The Identification of molecular chaperone associated proteins potentially involved in the regulation of Sperm-Oocyte interactions.

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This thesis includes one original paper published in a peer reviewed journal (Chapter 6). The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

Signed……..

Date……..

ACKNOWLEDGEMENTS

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SUMMARY

The union of the mammalian spermatozoon and oocyte represents the culmination of a series of complex interactions between male and female gametes. Yet despite the importance of this highly regulated and species specific event, relatively little is known about the molecular basis of the interaction.

Mammalian testicular spermatozoa, are incapable of fertilisation. Before fertilisation can occur, spermatozoa must undergo both epididymal maturation in the male reproductive tract and capacitation in the female tract. Only sperm that have traversed the epididymis attain the functional endpoints of capacitation, the ability to acrosome react and fertilise an egg. Capacitation is correlated with an increase in the level of tyrosine phosphorylation of a number of proteins, several of which become exposed on the cell surface.

Previous analysis of the surface phosphoproteome of capacitated sperm demonstrated that the molecular chaperone HSPD1 is exposed on the plasma membrane overlying the acrosome, an ideal position for interaction with the zona pellucida. Although HSPD1 is not directly involved in zona binding, it has been proposed that during capacitation, intracellular tyrosine phosphorylation activates HSPD1 inside the cell, orchestrating the assembly of a zona binding complex and its subsequent exposure on the outside of sperm.

To investigate the role of HSPD1 in fertilization, a proteomics-based approach was employed to identify chaperone associated proteins in capacitated sperm. HSPD1 was immunoprecipitated from capacitated sperm and associated proteins identified by liquid chromatography tandem mass spectrometry LC-MS/MS. analysis. Protein interactions were confirmed by reciprocal co-immunoprecipitation followed by Western blotting. The expression and localisation of the identified proteins during sperm maturation and fertilisation related events were investigated using indirect immunofluorescence and flow cytometry.

To this end we have identified a number of proteins including an aldose reductase, another chaperone HSPE1, citron kinase and the proacrosin binding protein. These proteins co-localise with HSPD1 to the acrosomal region. However, this expression pattern is lost once sperm have undergone calcium ionophore A23187 induced acrosome reaction, as would be expected of molecules potentially involved in sperm-egg interactions.

Based on these data we hypothesize that during epididymal transit, proteins important for fertilisation are deposited on sperm. During capacitation these proteins are assembled into functional protein complexes by chaperones including HSPD1, and chaperoned to sites including the cell surface where they affect the functional competence of sperm. The characterization of these HSPD1-interacting proteins is the subject of several chapters in this thesis.

In addition, a proteomic analysis of the surface proteome of functionally mature mouse sperm was performed. This work was designed to complement and reinforce the work identifying chaperone-associated proteins in sperm, and to establish a reference proteome for ongoing and future comparative proteome studies. Protocols were developed and optimized to label sperm surface proteins with a number of different biotinylation reagents. Isolated proteins were subsequently separated by SDS-PAGE and proteins identified by LC-MS/MS.

The broad goals of the work were to investigate the molecular basis of sperm-egg recognition with a view to identifying the key proteins that orchestrate the process.

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List of Abbreviations

2D-PAGE	two-dimensional polyacrylamide electrophoresis
AC	adenylyl cyclase
ACN	acetonitrile
AKAP	A-kinase anchor protein
AKR	aldo-keto reductase
AKR1B7	aldo-keto reductase family 1, member B7
AMP	adenosine monophosphate
amu	atomic mass unit
ATP	adenosine triphosphate
BN-PAGE	blue native polyacrylamide gel electrophoresis
BP	biological process
BSA	bovine serum albumin
BWW	Biggers, Whitten and Whittingham
cAMP	cyclic adenosine monophoshate
CAN	acetonitrile
CASA	computer-assisted sperm analysis
CBB	coomassie brilliant blue R-250
CD	cluster of differentiation
cDNA	complementary DNA
Cit-k	citron kinase
Co-Ip	co-immunoprecipitation
CRIK	citron kinase
Da	dalton
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DSRSB	double strength reducing sample buffer
DTSSP	3,3´-dithiobis (sulfosuccinimidylpropionate)
DTT	dithiothreitol
EGF	epidermal growth factor

ELISA	enzyme linked immunosorbant assay
EPF	early pregnancy factor
ESI	electrospay ionisation
EST	expressed sequence tag
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FITC-PNA	fluorescein isothiocyanate-conjugated peanut agglutinin
FL	fluorecsence
GABA	gamma-aminobutyric acid
GalTase	β-1,4-galactosyltransferase
GlcNAc	N-acetylglucosamine
GO	gene ontology
H342	Hoechst-3342
HCO3-	bicarbonate ion
HNE	4-hydroxynonenal
HPLC	high performance liquid chromatography
HSPD1	heat shock 60kDa protein 1
HUGO	human genome organisation
IAA	iodoacetamide
IEF	isoelectric focusing
IIF	indirect immunofluorescence
IP	immunoprecipitation
IPI	international protein index
kDa	kilodalton
KLH	keyhole limpet hemocyanin
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LPC	lyphosphatidylcholine
MALDI-TOF	matrix-assisted laser desorption/ionisation-time of flight
MF	molecular function
min	minutes
mM	millimolar

MMC	Monash Medical Centre
Mr	molecular mass
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MVDP	mouse vas deferens protein
NAD^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NHMRC	national health and medical research council
nm	nanometre
OAM	outer acrosomal membrane
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate-buffered Saline with Tween 20
pI	isoelectric point
PI	propidium iodide
PKA	protein kinase A
PKC	protein kinase C
PM	plasma membrane
PMP	plasma membrane proteome
PNA	peanut agglutinin
PR	progesterone receptor
PTM	posttranslational modification
ptx	pentoxifylline
PVA	polyvinyl alcohol
PVDF	polyvinylidene fluoride
ROCK	rho-associated kinase
ROS	reactive oxygen species
sAC	soluble adenylyl cyclase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
SR	sypro ruby
TCA	trichloroacetic acid

TCEP	tris(2-carboxyethyl) phosphine
TFA	trifluoroacetic acid
V	volts
v/v	volume to volume
w/v	weight to volume
Xcorr	cross-correlation score
ZP	zona pellucida
ZP1	zona pellucida glycoprotein 1
ZP2	zona pellucida glycoprotein 2
ZP3	zona pellucida glycoprotein 3
ZP4	zona pellucida glycoprotein 4
ZRK	zona receptor kinase
ΔCn	delta-correlation score
λ	wavelength
µl	microlitre
µM	micromolar

Published Papers

The following paper was accepted for publicationduring PhD candidature

Walsh A[1], Whelan D[1], Bielanowicz A, Skinner B, Aitken RJ, O'Bryan MK, Nixon B. (2008). Identification of the molecular chaperone, heat shock protein 1 (chaperonin 10), in the reproductive tract and in capacitating spermatozoa in the male mouse. Biol Reprod, 78 (6), 983-93.

[1] These authors contributed equally to this work.

1. Introduction and Review of Literature

1.1 Overview of mammalian post-testicular sperm maturation and fertilisation

Mammalian fertilisation is a multi-phasic event whereby male and female haploid gametes unite to form a diploid zygote (Fig.1.1). It begins with the ejaculation of sperm into the female reproductive tract, where they undergo a final maturation event regulated by the microenvironment in which they are immersed. This process termed capacitation confers on spermatozoa the ability to recognise and bind to the extracellular matrix surrounding the oocyte, the zona pellucida. Capacitation encompasses changes in motility patterns, alterations in membrane lipid architecture and protein distribution, and the initiation of complex signaling pathways.

Following adhesion to the zona pellucida, sperm undergo the acrosome reaction, an exocytotic process that facilitates penetration of the zona pellucida and entry of sperm into the perivitelline space (Fig. 1.1). The spermatozoon then binds to, and fuses with, the oocyte plasma membrane, triggering a rapid increase in the level of free intracellular calcium within the oocyte and stimulating signaling events that lead to egg activation and zygote formation.

The following review focuses on our current understanding of the sperm maturation events, both in the male (epididymal maturation) and female (capacitation) reproductive tracts that confer upon spermatozoa the competence to fertilise the ovum. Further, our present appreciation of the biochemical and molecular basis of the interaction between the male gamete and the zona pellucida is discussed. Emphasis is placed on murine reproduction due to its relevance to the work presented in this thesis.

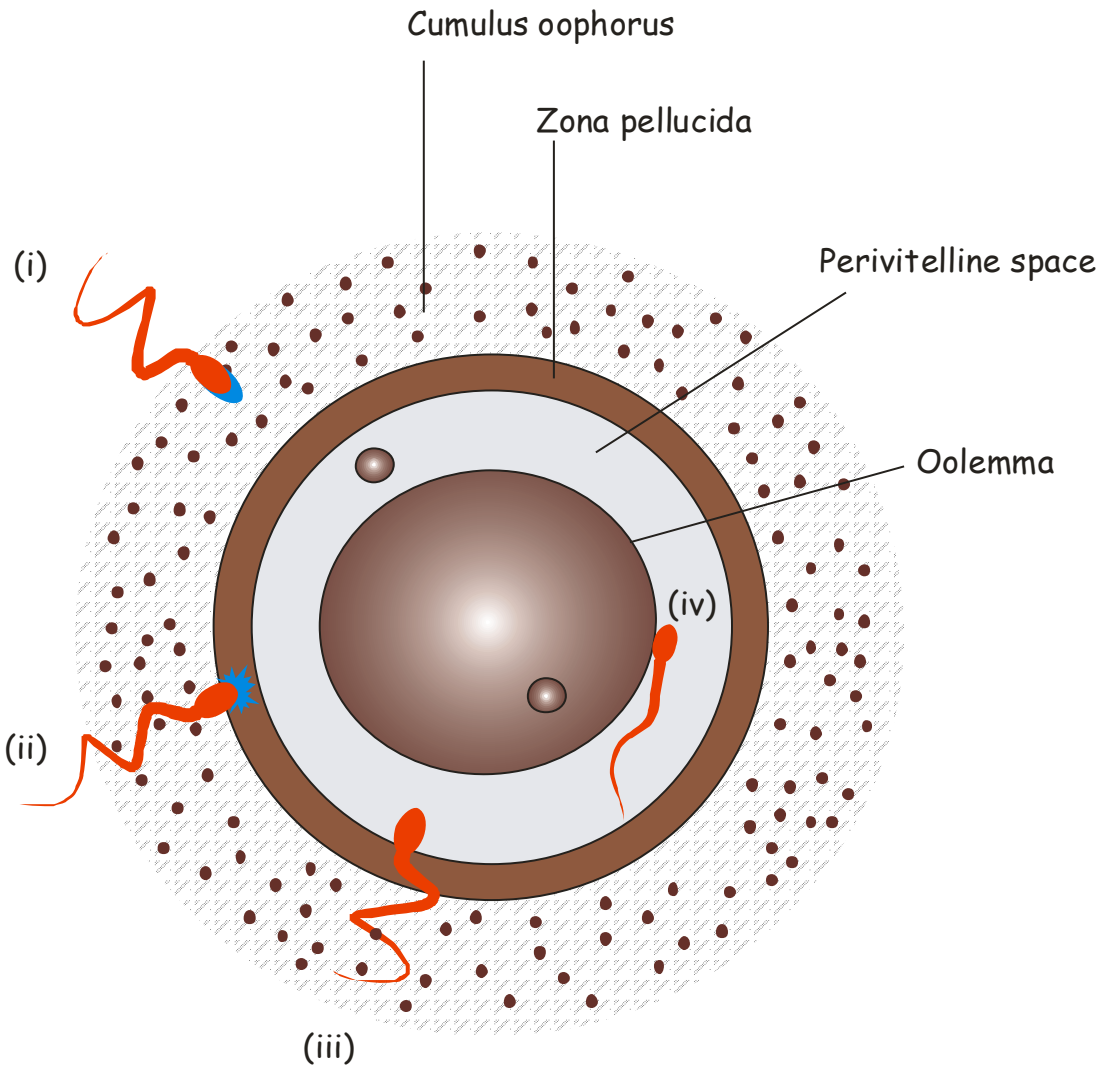


Figure 1.1. Overview of Mammalian Fertilization. Capacitated sperm penetrate the cumulus oophorus with the assistance of cell surface hyaluronidase (i), and bind to the zona pellucida inducing the acrosome reaction (ii). Hydrolytic enzymes facilitate passage through the zona into the perivitelline space (iii) and fusion with the oolemma (iv).

1.2 Epididymal Maturation of Spermatozoa

1.2.1 Overview

Testicular spermatozoa while morphogenically differentiated are neither fully motile nor capable of recognizing and fertilising an egg (Yanagimachi, 1994; Ecroyd *et al*., 2004; Gatti et al., 2004). These abilities are developed during passage through the epididymis in a process termed "epididymal maturation" (Yanagimachi, 1994; Robaire et al., 2006; Hinton and Cooper, 2010; Guyonnet et al., 2011). Spermatozoa leave the testis and pass through the epididymis acquiring the potential for coordinated motility and fertilization during their transit.

The function of the epididymis has been controversial. Historically, it was thought to have a role in the accumulation, storage and preservation of spermatozoa. However, the first indications that the organ played a more influential role in the process of fertilisation came in 1913 when Tournad found that sperm taken from the proximal regions of the organ were immotile when diluted in saline, whereas sperm from the distal epididymis exhibited full motility (Tournade, 1913). Then, in a series of papers published between 1929 and 1931, Young and Toothill demonstrated that in addition to acquiring motility, sperm become fertile while resident in the epididymis (Young, 1929; Young, 1929b; Young, 1931; Toothill and Young, 1931). It was concluded however, that time was the critical factor and that the changes taking place in spermatozoa were not influenced by the secretions of the epididymal epithelium. Thirty years later, in the mid to late 1960s, Bedford and Orgebin-Crist independently provided the first clear evidence for the role of the epididymis in the maturation of sperm. They showed that it was indeed the exposure of sperm to the *milieu* of the epididymal lumen that was the critical feature of the maturation process, and not simply the passage of time as proposed by Young (Bedford, 1967; Orgebin-Crist, 1967). It was demonstrated that sperm retained in the testis by epididymal ligation failed to mature functionally and did not develop the capacity for coordinated motility and fertilisation (Cooper and Orgebin-Crist, 1975; Cooper and Orgebin-Crist, 1977).

While the precise nature of the molecular events underpinning the functional maturation of sperm in the epididymis remains unclear, it is now accepted that sperm, and in particular the plasma membrane and surface proteome, are modified and

remodeled during migration through the epididymis (reviewed by Cornwall, 2009). In addition to acquiring new proteins, existing proteins may be post-translationally processed or removed altogether during passage through the epididymis (reviewed by Cuasnicu, et al., 2002).

Sperm are essentially transcriptionally silent, and therefore unable to create new proteins by ribosomal protein synthesis (reviewed by Guy and Breitbart, 2008). It is their sequential exposure to the *milieu* of the different microenvironments created along the epididymal lumen that is believed to facilitate the changes that ultimately render them competent for fertilization through their ability to move, capacitate and participate in sperm-zona interaction. The maturation process therefore relies on the successive intra-luminal microenvironments created by the secretory and absorptive functions of the epididymal epithelium. Maintaining the specificity of the environment is also critical. The tight junctions between the principal epithelial cells limit exchanges between the luminal compartment of the epididymis and the blood plasma (reviewed in Dacheux et al, 2003).

1.2.2 The Epididymis

Terminally differentiated sperm leave the testis and enter the epididymis where their post-testicular maturation begins. By the time they leave the epididymis, they will have undergone all the changes required to allow their continued maturation in the female reproductive tract; they will have gained the potential for coordinated motility and for fertilisation. This journey starts in the seminiferous tubules of the testis which converge to form the rete testis, a network of ducts that eventually converge to form a single, highly coiled duct, the epididymis (Fig. 1.2).

The epididymis sits adjacent to the testis and interconnects with the seminiferous tubules via the rete testes; it is extremely convoluted and ranges in length from one metre in mice (Takano et al., 1981) to up to six metres in humans (Von and Neuhauser, 1964).

The epididymis is usually divided into three gross anatomical regions: the caput, most proximal to the testis; the corpus; and cauda (Hoffer and Karnovsky, 1981; Hermo, 1995). The distal end of the cauda epididymis gradually becomes less convoluted and its diameter increases. After this point the epididymal duct becomes the ductus (vas) deferens. The vas deferens which is surrounded by a thick muscular layer of cells connects with the urethra. Functionally, the vas deferens stores sperm and conveys them toward the urethra during emission by peristaltic contraction of the muscular coat (reviewed by Lohiya et al., 2001).

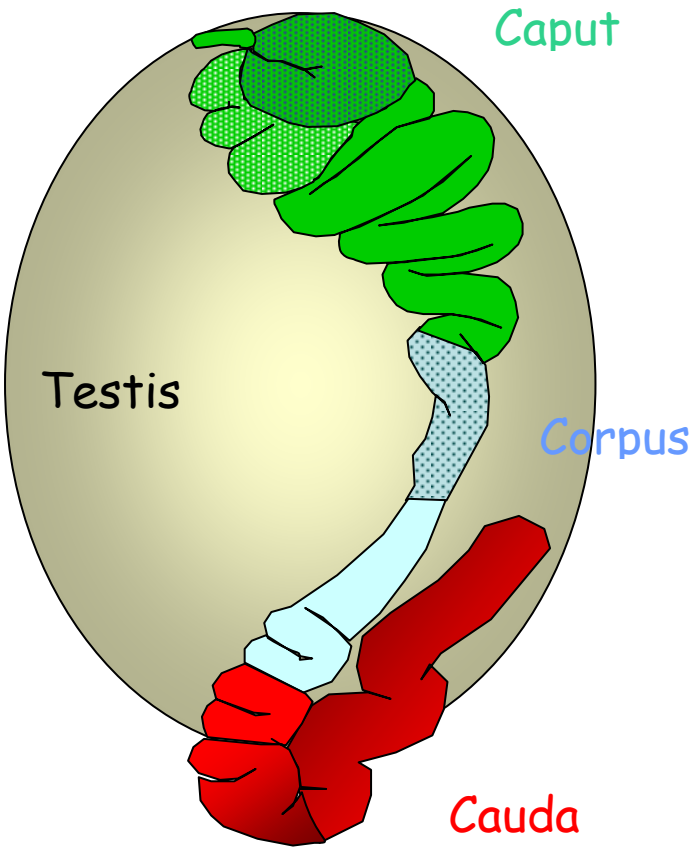


Figure 1.2. Schematic representation of the mammalian testis and epididymis. The major anatomical regions of the mammalian epididymis are marked: caput in green, corpus in blue, and the cauda in red. Sperm released from the testis enter the caput region and mature as they migrate to the distal caudal region, gaining the potential for fertilization. The epididymis is continuous with the efferent ducts of the rete testis and terminates at the vas deferens (not shown).

The predominant cell type present in the epididymal epithelium is the principal cell, which accounts for between 65-80% of the total epithelial cell population depending, on the region (Trasler et al., 1988). Although continuous throughout the organ, the structure and function of principal cells varies in different segments of the epididymis. Differences are primarily reflected in the organisation of the endocytotic (coated pits, endosomes and lysosomes) and secretory (endoplasmic reticulum and Golgi apparatus) machinery (Hamilton et al., 1977; Hermo et al., 1994). Functionally, these cells play a central role in maintaining the luminal environment through which sperm migrate (reviewed in Robaire and Viger, 1995; Robaire *et al*., 2007). Moreover the principal cells have been shown to synthesize numerous proteins, many of which are secreted into the lumen of the epididymis (Hermo et al., 1994; Holland and Orgebin-Crist, 1988).

Apical cells, found only in the epithelium of the initial segments of the caput (Sun and Flickinger, 1980), have an apically located nucleus, and exhibit a different expression profile compared to neighboring principal cells (Adamali and Hermo, 1996). Although their specific function remains controversial, they have been shown to endocytose material from the epididymal lumen and to contain numerous proteolytic enzymes (Adamali and Hermo, 1996).

Narrow cells are only found in the initial segments of the mouse and rat caput epididymis (Sun and Flickinger, 1980). Similar cells have been reported in the same region of epididymides from a number of species including, human (Palacios, *et al*., 1991), bovine (Goyal, 1985), and hamster (Flickinger *et al*., 1978). These cells are narrower than principal cells and differ from apical cells in their distinct morphology and protein production profiles (Adamali and Hermo, 1996).

Clear cells are found throughout the epididymis and play a role in the endocytosis of material from the epididymal lumen. Both Hermo *et al*., (1988) and Moore and Bedford (1979) demonstrated the endocytotic function of clear cells by showing the uptake of a tracer injected into the lumen of the epididymis. These cells are found in many species including humans (Vendrely, 1981), and have been shown to take up cytoplasmic droplets shed by sperm as they migrate through the duct (Hermo *et al*.,

1988). In addition, it has been reported that clear cells are involved in region-specific endocytosis of different proteins (Hermo *et al*., 1992; ; Vierula *et al*., 1995).

Halo cells are characterised by a narrow rim of clear cytoplasm and are found throughout the epididymis (Robaire and Viger 1995). They have been postulated to be lymphocytes or monocytes and are believed to play a role in the immunological barrier of the male reproductive duct (Wang and Holstein, 1983; Flickinger *et al*., 1997; Serre and Robaire, 1999)

Basal cells have been documented in all species studied to date (Robaire and Hermo, 1988; Robaire and Viger, 1995) and are so named because of their adherence to the basement membrane. Although their exact function remains unclear, it has been proposed that basal cells are involved in the regulation of principal cell function (Robaire *et al.*, 2006).

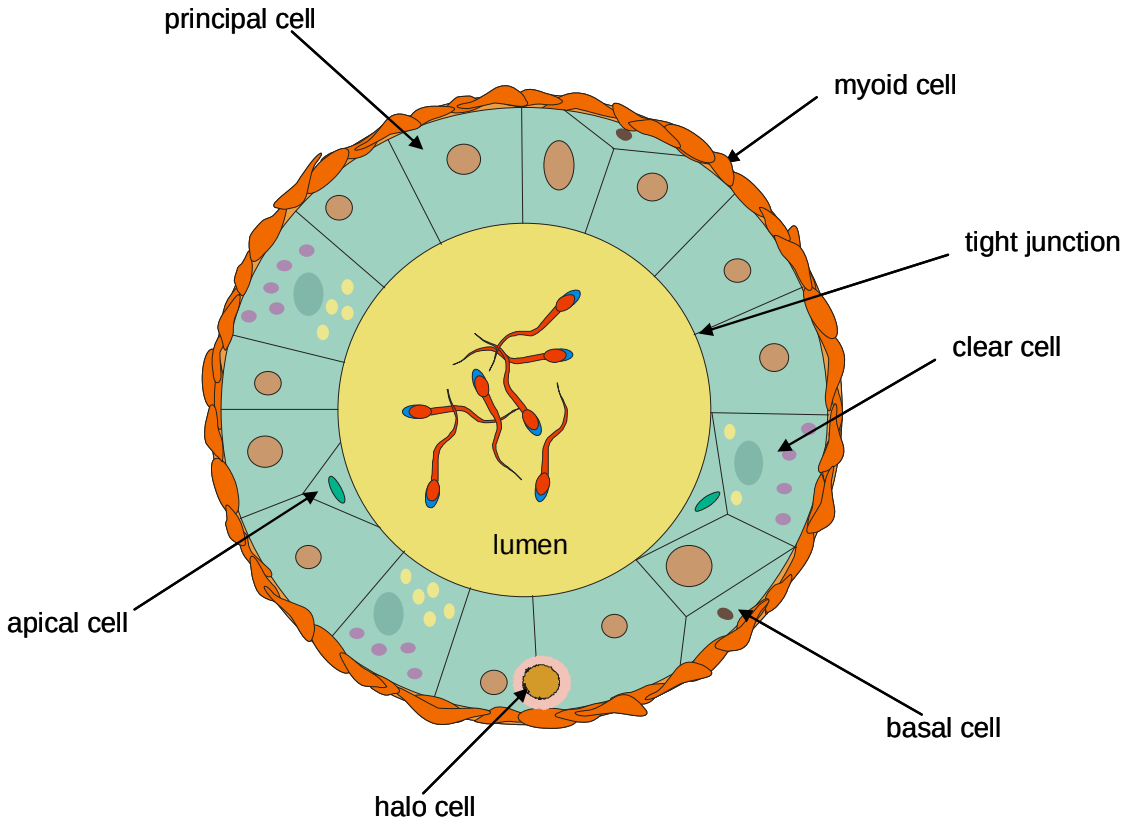


Figure 1.3. Schematic organisation of the major cell types in the epididymal lumen. The relative position and distribution of each of the main cell types are illustrated.

Since sperm are produced in the immune privileged testis and contain highly immunogenic proteins (Dym and Fawcett, 1970; Hinton, 1995; Cheng and Mruk, 2002), the continuation of a physical barrier between sperm and the immune system beyond the testis to include the epididymis is critical for sperm survival and male fertility. The epithelial cells of the epididymis are tightly joined to form a closed functional unit. Thus, protecting sperm from the immune system as they migrate through the epididymis (Hinton and Palladino, 1995).

The points of contact between adjacent epithelial cells, the cell junctions, are comprised of apically located gap, adherens, and tight junctions. Adherens junctions are made of proteins that mechanically hold neighboring cells together by connecting to microfilaments of the cytoskeleton (Gumbiner, 1996), while gap junctions mediate communication between adjacent cells by allowing the passage of small molecules (Soranzo et al., 1982). However, the fidelity of the blood-epididymis barrier relies on the integrity of the tight junctions at the luminal surface between adjacent principal cells (Cyr *et al.*, 1995). The tight junctions seal the spaces between cells, physiologically separating the luminal compartment from the intercellular space (Agarwal and Hoffer, 1989).

The blood-epididymis barrier also facilitates the creation and preservation of specific luminal microenvironments which are thought to be critical to the functional maturation of sperm as they travel down the epididymis (Hoffer and Hinton, 1984). This is achieved by restricting the exchange of ions, solutes and other macromolecules between the luminal compartment and the blood plasma (Hinton and Palladino, 1995).

1.2.3 Epididymal Function

1.2.3.1 Sperm Transport

Several studies have determined the time required for sperm to migrate through the epididymis. Rowley and colleagues using a thymine labelling approach determined the mean transit time in humans to be 12 days (Rowley et al., 1970). However, others have observed shorter transit times in the order of 3-4 days by measurement of the ratio of extra-testicular sperm reserves to daily testicular sperm production (Amann and Howard, 1980). Turner and Reich proposed that the discrepancy may be attributed to

the paucity or supply of sperm (Turner and Reich, 1985). This view is supported by work estimating faster transit times for men with large daily sperm production (Johnson and Varner, 1988). Consistent with this view is the decreased transit times documented in chimpanzees with high sperm production rates (Smithwick *et al*., 1996). Studies in other species, including the mouse, bull, boar, rabbit, ram, rat, hamster, rhesus monkey, and wallaby, have determined that the minimal time required for sperm to traverse the length of the epididymis is approximately ten days (Amann *et al*., 1976; Ortavant, 1954; Meistrich *et al*., 1975 Orgebin-Crist, 1965; Setchell and Carrick, 1973; Hikim and Hoffer, 1988; Sujarit and Pholpramool, 1985; Swierstra, 1968).

1.2.3.2 Functional Maturation

By the time sperm reach the cauda epidiymis, they will have undergone all the physiological changes required for them to recognise, interact with, and fertilize an egg after capacitation in the female reproductive tract (Robaire and Hermo, 1988; Guyonnet et al., 2011). While resident in the epididymis, spermatozoa are held in an immotile state and only become motile after being deposited in the female reproductive tract or an equivalent physiological medium *in vitro*. Testicular sperm and sperm taken from the proximal regions of the epididymis are not progressively motile when diluted into media, whereas sperm taken from the cauda region exhibit the ability to swim forward in a coordinated fashion. Progressive motility is essential for sperm to ascend the female genital tract (reviewed by Suarez and Pacey, 2006)

Tournade, in 1913 was the first to recognise the importance of the epididymis in the maturation of sperm potential for motility (Tournade, 1913). Tournade's observations have subsequently been confirmed by many studies in numerous species: mouse (Soler *et al*., 1994), rat (Hinton *et al*., 1979), hamster (Kann and Serres, 1980), rabbit (Pérez-Sánchez *et al*., 1996), boar (Dacheux *et al*., 1979), goat (Jaiswal and Majumder, 1996), ram (Chevrier and Dacheux, 1992), bull (Acott *et al*., 1983), monkey (Mahony *et al*., 1993), and human (Dacheux *et al*., 1980; Mooney *et al*., 1972; Yeung *et al*., 1993). In addition to an increase in the percentage of motile sperm, the maturation of the potential for motility involves a change in the pattern of motility. When released into media, testicular sperm remain largely immotile, caput sperm swim in circles, but

sperm taken from the cauda epididymis move progressively forward (Pérez-Sánchez *et al*., 1996).

The changes taking place in the epididymis that facilitate the development of the potential for motility involve the maturation of signal transduction mechanisms linking the plasma membrane to the flagellar machinery. After removal of the plasma membrane, the tails of testicular and caput sperm beat with frequencies approaching those observed with mature sperm, in media containing adenosine triphosphate (ATP) and cyclic adenosine monophosphate (cAMP) (Ishijima and Witman, 1987).

Although the nature of the signaling pathways involved remains unclear, increased phosphorylation is observed on proteins in the axoneme, the outer dense fibres (ODFs) and the fibrous sheath (Tash and Bracho, 1998; Yeung and Cooper, 2003). However, the tyrosine phosphorylation of proteins localized to the plasma membrane (Devi et al., 1997) and the inhibition of motility in intact but not in demembranated sperm by tyrosine kinase inhibitors (Uma Devi et al., 2000), suggests that tyrosine phosphorylation regulates motility via signal transduction at the level of the plasma membrane rather than by direct modification of flagellar structures (Yeung and Cooper, 2003).

Concomitant with the development of the capacity for forward motion is the ability to recognise an outer vestment of the oocyte, the zona pellucida. Following a period of maturation in the female reproductive tract, the fertilising ability of maturing sperm can be measured by artificial insemination or *in vitro* binding assays (Gatti et al., 2004). In all mammalian species studied to date, a gradient of increasing fertilising ability is observed as sperm move along the epididymis. Down this fertility gradient, the number of sperm capable of binding the zona pellucida increases in parallel to the number of sperm showing progressive motility (Gatti et al., 2004). However, the development of motility potential has been shown to be a separate phenomenon to the maturation of zona binding ability. Studies have shown that sperm taken from successive regions of the epididymis display an increasing ability to bind to the zona pellucida at 4°C when motility is significantly reduced (Burkin and Miller, 2000; Gatti *et al*., 2004; Asquith et al.,2005; reviewed by Aitken et al., 2007). Also, the retention of the zona binding

ability of immobilized caudal sperm suggests that the two physiological properties are unrelated (Saling, 1982).

Although there is some species variation as to the exact location in the epididymis where sperm acquire the ability to interact with the zona (Orgebin-Crist and Olson, 1984), the number of sperm capable of binding the zona increases strongly in the proximal corpus and reaches a maximum in the cauda (Burkin and Miller, 2000). *In vitro* maturation experiments have confirmed the role of the epididymis in the maturation of fertilising ability. In numerous studies, the co-incubation of caput sperm with epididymal epithelial cell cultures, not only increases sperm motility, but also their capacity to bind the zona pellucida (Moore *et al*., 1992). Since sperm are transcriptionally inactive, it is interpreted that epididymal proteins are involved, either directly or indirectly, in sperm gaining the ability to interact with the oocyte.

The sperm plasma membrane undergoes intense remodeling in response to the luminal environment encountered in the epididymis (Marengo, 2008; Belleannee et al., 2011; reviewed by Dacheux et al., 2003). In addition to the acquisition of new proteins, it is well documented that existing surface protein are modified or processed during epididymal transit (Gatti *et al*., 2004). Several studies have implicated proteolytic mechanisms in the maturation of the sperm plasma membrane in the epididymis (Myles *et al*.,1987; Lum and Blobel, 1997; Petruszak *et al*., 1991). Proteolysis may induce a redistribution of the protein within the plasma membrane (reviewed by Evans, 1999), or its release from the sperm surface into the epididymal lumen (Métayer et al., 2002). Fertilin (PH30), a member of the ADAM family of metalloproteases reported to have a role in gamete interactions (Primakoff *et al*., 1987), is present over the entire sperm head in the testis. However, successive cleavage of the protein in the caput results in its redistribution to the posterior head (Frayne *et al.*, 1998; Phelps *et al*., 1990; Blobel, 2000). Other proteins processed in a similar fashion include hyaluronidase PH20 (Hou *et al*., 1996; Seaton *et al*., 2000), alpha-D-mannosidase (Tulsiani et al., 1995), SP10 (Foster *et a*l., 1994), and β-galactosyltransferase (Shur and Hall, 1982a). An example of a protein removed from sperm altogether in the epididymis is angiotensin converting enzyme (ACE). ACE exists as two different isoforms in the male, a somatic form (sACE) and a testis-specific, germinal form (gACE) (Gatti *et al.*, 1999; Bernstein, 1998). gACE is present on the surface of the mid- and principal-piece of the flagellum

in testicular sperm, but is completely removed by proteolytic cleavage during transit through the caput (Gatti *et al*., 1999; Métayer *et al*., 2002).

In conjunction with the processing and removal of existing surface proteins, many studies report that proteins secreted by the epididymal epithelium are incorporated into the plasma membrane of the maturing spermatozoon (Hall *et al*., 1996). Many of these proteins are thought to be simply adsorbed to the sperm surface by virtue of their high extra-cellular concentration, while others like rat epididymal HIS50 establish ionic interactions (Rifkin and Olsen, 1985). Alternatively, proteins like glycoprotein HE5 (CD52), which is inserted into human sperm, are anchored via glycosylphosphatidylinositol (GPI) linkages during epididymal transit (Kirchhoff *et al*., 1998; Yeung et al., 1997). The precise mechanisms by which secreted proteins end up GPI-anchored to the sperm surface remain unclear. However, the process is thought to involve direct exchange between sperm and either the apical plasma membrane of epithelial cells or vesicles released from the epithelial cells (Moore *et al*., 1989; Kirchhoff, 1996; Frenette and Sullivan, 2001).

Different parts of the male reproductive tract are known to secrete membranous vesicles via an apocrine mode of secretion (Sullivan et al., 2005). Collectively, these particles have been termed exosomes and some studies have indicated that they may be involved in the transfer of new proteins to sperm. It is well documented that exosomes derived from the prostate are constituents of seminal plasma (Saez et al., 2003) where they are called prostasomes. Prostasomes have been the subject of many investigations since their discovery in 1978 (Ronquist et al., 1978). Despite no definite function being attributed to them, prostasomes have been implicated in a number of different processes and functions: antibacterial (Carlsson et al., 2000), immunomodulation (Rooney et al., 1993; Rooney et al., 1996), capacitation (Cross, 1996a; Cross, 1996b), sperm motility (Carlsson et al., 1997), and antioxidant production (Saez et al., 1998; Saez et al., 2000). A rich source of proteins, proteomic investigations indicate prostasomes contain as many as 139 proteins, 34% being enzymes (Utleg et al., 2003). A complement-inhibitory protein, CD59, is GPI-anchored to prostasomes but is capable of being transferred to the surface of sperm *in vitro* (Rooney et al., 1993). Although the mechanism of transfer remains unclear, Arienti et al. (1997) demonstrated the ability of prostasomes to fuse with sperm in slightly acidic conditions.

In the epididymis, similar exosome-type vesicles have been observed in a number of species including the rat (Fornes et al., 1995; Eickhoff et al., 2001), bull (Frenette and Sullivan, 2001; Frenette et al., 2003), mouse (Rejraji et al., 2002), and the hamster (Légaré et al., 1999; Yanagimachi et al., 1985). Termed epididymosomes, these vesicles range in diameter from 50 - 500 nanometres (Sullivan et al., 2005), and have been proposed to mediate the transfer of specific epididymal proteins to the surface of sperm (Saez et al., 2003). Believed to be the product of apocrine secretion from the epididymal epithelium (Hermo and Jacks, 2002; Aumuller et al., 1999), epididymosomes contain a unique protein complement. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) showed that epididymosomes isolated from the epididymal lumen have a protein complement distinct from the soluble protein content from the same epididymal region (Sullivan et al., 2005). In addition, the protein composition of epididymosomes is different from exosome type vesicles isolated from ejaculated semen (Frenette et al., 2003). A growing list of proteins of epididymosomal origin have now been conclusively identified. They include a unique glutathione peroxidase, lacking the selenocysteine residue characteristic of other GPXs, termed GPX5; this protein is secreted by the caput epididymis in a number of mammalian species (Ghyselinck and Dufaure, 1990). In the mouse, the protein has been shown to be associated to epididymosomes (Rejraji et al., 2002) and to be transferred to the sperm surface covering the acrosome where it is believed to have a protective function preventing premature acrosomal exocytosis (Vernet et al., 1997). Other proteins include ADAM7, which is transferred to the sperm surface during epididymal transit (Oh et al., 2009). Sullivan and colleages analysed the protein composition of human epididymosomes and identified a total of 146 different proteins (Thimon et al., 2008)

Further, macrophage inhibitory factor (MIF), which was originally identified as a T cell cytokine (Bloom and Bennett, 1966), has been shown to be associated with epididymosomes in the bull (Frenette et al., 2003) and the rat (Eickhoff et al., 2001). The MIF containing epididymosomes were shown to be in close association with sperm and are thought to orchestrate the transfer of the protein to structures in the sperm flagellum (ODF) (Eickhoff et al., 2001). Based on the thiol-protein oxidoreductase catalytic property of MIF, it has been suggested that MIF has a role in the acquisition of sperm motility during maturation by modulating the thiol status of sperm proteins

(Eickhoff et al., 2004). However, the true function of the protein in sperm remains to be established. The essential nature of this cytokine is however in doubt as MIF knockout (KO) male mice have normal fertility (Honma et al., 2000).

Together with ubiquitin, which may have a role in eliminating defective sperm in the epididymis (Sutovsky et al., 2001), bovine epididymosomes also contain two enzymes involved in the polyol pathway (Frenette et al., 2004): an aldose reductase that reduces glucose to sorbitol and sorbitol dehydrogenase which subsequently oxidizes the sorbitol to fructose (Kobayashi et al., 2002). Both enzymes have been shown to be associated with bovine epididymosomes and epididymal sperm, supporting the notion that epididymosomes are involved in the polyol pathway during sperm transit through the epididymis.

Interestingly, proteins proposed to have a role in binding the zona pellucida are constituents of epididymosomes and have been shown to be transferred to sperm during maturation in the epididymis (Frenette and Sullivan, 2001). Sullivan and colleagues demonstrated that the hamster protein, P26h is associated to epididymosomes and is GPI-anchored to the sperm surface during epididymal transit and P26h exhibits a species-specific affinity for the zona pellucida glycoproteins (Legaré et al., 1999). Orthologs of P26h have been identified in other mammalian species including P25b in bulls (Parent et al., 1999), and P34H in humans (Boué et al., 1994; Boué et al., 1996).

The precise mechanism by which epididymosomes mediate protein transfer to sperm in the epididymis remains to be elucidated. However, the fact that the major proteins present on these vesicles are not detected on caudal or ejaculated sperm indicates that it is not simply a membrane fusion process.

In addition to epididymosomes, Asquith and colleagues reported the presence of 'dense bodies' in the epididymal lumen (Asquith et al., 2005). Dense bodies first appear in the proximal corpus and persist throughout the cauda and the vas deferens, these structures averaged 900 nm in diameter and were distinguished from epididymosomes by their lack of membrane. Dense bodies do not physically associate with sperm, but are suspended in the epididymal fluid between the maturing gametes. They were found to contain the molecular chaperones heat shock protein 1 (HSPD1, formally known as

HSP60) and heat shock protein 90 beta (HSP90B1, formally known as endoplasmin). Interestingly, both chaperones which are closely associated with the mitochondria of spermatogonia and primary spermatocytes in the testis, disappear from the male germ line during spermatogenesis and are undetectable in testicular sperm. However, both proteins re-appear in epididymal sperm and localise to the head of spermatozoa taken from all regions of the murine epididymis. Whether the proteins are truly lost from sperm in the testis or the relevant epitopes are simply masked, then subsequently unmasked, in the epididymis remains to be determined.

Although the origins of HSPD1 and HSP90B1 expressed by epididymal sperm has not been conclusively linked to the dense bodies, the fact that the dense bodies appear in the epididymal region where sperm gain the ability to recognise and bind the zona; suggests they may be involved in the transfer of key proteins to sperm required for fertilisation.

1.2.3.3 Sperm Protection

In addition to providing the correct luminal microenvironment for the functional maturation of sperm, the epididymis plays an important role in protecting sperm from a range of potential threats which include the immune system, oxidative stress, and xenobiotics (Robaire *et al.*, 2006). Since sperm contain surface proteins that are immunogenic, they must be shielded from the host immune system (Robaire and Hermo, 1988). The blood-epididymis barrier does this by acting as an extension of the blood-testis barrier (reviewed by Mital et al., 2011; Cyr *et al*., 1995). However, relatively little is known about the exact mechanisms by which sperm are protected from the immune system in the epididymis (Li et al., 2012).

In biological systems, oxidative stress results from imbalances between reactive oxygen species (ROS) generating processes and the enzymatic or non-enzymatic scavengers which remove ROS (Chen et al., 2013; Auten, 2009; Aitken and Baker, 2002; Baker et al., 2003b). In normal sperm physiology, ROS have been implicated in the regulation of events including hyperactivation, capacitation, acrosome reaction, zona pellucida binding and oocyte penetration (Chen et al., 2013; de Lamirande et al., 1997b). Mammalian sperm possess a high membrane content of polyunsaturated fatty acids (Aitken and Clarkson, 1987; Vernet et al., 2004). This fact, coupled with

increasing sperm concentrations and metabolic rates during epididymal transit, means that sperm are highly susceptible to oxidative damage in the epididymis (Vernet et al., 2004). Lipid peroxidation of the sperm membrane affects the cells capacity for fertilisation (reviewed by Vernet et al., 2004); and has been correlated with reduced intracellular ATP levels (Alvarez and Storey, 1982; de Lamirande and Gagnon, 1992), and defects in the midpiece (Rao et al., 1989) and axoneme (de Lamirande and Gagnon, 1992).

While in residence of the epididymis, sperm are protected from oxidative attack by a variety of antioxidant enzymes and molecules (Vernet et al., 2004; Drevet, 2006; Aitken and Roman, 2008). However, different levels of protection are required in different regions of the epididymis reflecting the varying susceptibility of sperm at different stages of maturation, and the different metabolic rates of the epithelial cells in each region. The major antioxidant enzymes present in the epididymis include superoxide dismutase (SOD) (Perry et al., 1993), γ-glutamyl transpeptidase (Hinton et al., 1991; Palladino et al., 1994), glutathione transferases (Veri et al., 1993; Montiel et al., 2003), catalase (Vernet et al., 2004), indolamine dioxygenase (Yoshida et al., 1980) and members of the glutathione peroxidase (GPX) family (Perry et al.,1992; Rejraji et al., 2002). All of these enzymes, found to varying degrees in different regions of the epididymis, play a role in controlling the fine balance of ROS production and scavenging that is crucial to sperm maturation and function (de Lamirande *et al*., 1997b).

1.3 Capacitation

1.3.1 Overview

While resident in the epididymis, morphologically differentiated but functionally incompetent male gametes undergo maturational changes that confer the potential for fertilisation on them. However, the expression of this functional potential gained in the epididymis is suppressed until spermatozoa enter the female reproductive tract. In the

female, sperm undergo a final series of biochemical and physiological changes, collectively termed capacitation, that facilitate oocyte binding and penetration (Visconti et al., 1998; Gadella et al., 2008; Baker et al., 2009).

This requirement for additional maturation outside the male reproductive tract was first recognised more than 50 years ago following *in vivo* studies investigating the fertilization of freshly ovulated eggs in rats and rabbits. Austin and Chang independently demonstrated that ejaculated mammalian sperm were unable to fertilise eggs *in vivo* until they had resided for a period of time in the female reproductive tract (Austin, 1951; Chang, 1951). Sperm that have completed this process are referred to as capacitated. Following these initial investigations fertilisation was defined as the endpoint of the capacitation process. However, it is understood today that while capacitation permits sperm to bind to, and undergo the acrosome reaction at the surface of the zona, it does not include the acrosome reaction itself (Ward and Storey, 1984; Tulsiani and Abou-Haila, 2004).

Although the site of capacitation for most mammalian species studied is the female reproductive tract, under appropriate conditions it can be induced *in vitro* (Yanagimachi, 1994). One of the major breakthroughs in reproductive research, which facilitates much of todays work, came in 1967 with the development of techniques for the *in vitro* capacitation of mammalian sperm.

The process of capacitation involves a series of biochemical and biophysical modifications that render sperm competent for fertilisation of the oocyte. One of the functional consequences of capacitation is the development of a distinct motility pattern called hyperactivation. Although hyperactivation has been considered part of the capacitation process because sperm have been observed to hyperactivate while undergoing capacitation *in vitro*, hyperactivation can be stimulated to occur independently of cAMP-regulated capacitation (Marquez and Suarez, 2004). Other changes correlated with capacitation include the loss of decapacitation factors (Nixon et al., 2006) and additional plasma membrane remodeling (Nixon et al., 2011), the activation of signaling cascades that result in the downstream induction of tyrosine kinases and phosphorylation, and the ability to recognise the zona pellucida and undergo the acrosome reaction (Platt et al., 2009; Baker et al., 2009; Mitchell et al.,

2008). The exact relationships between these correlates is not yet completely understood and although the conditions for *in vitro* capacitation have been established for a number of mammalian species, the molecular events that control and regulate this critical event are not fully understood.

1.3.2 In vivo capacitation and transport in the female reproductive tract

Considering the diversity of events that have been described as occurring during capacitation (section 1.3.4), it is not surprising that *in vivo*, the process is not an "all or nothing" affair, but rather a process of sequential steps or events. The advantage of the female reproductive tract being able to regulate the rate of capacitation is the staggered delivery of capacitated sperm to the ovulated cumulus-oocyte complex, regardless of the time of mating (De Lamirande et al., 1997a). Capacitation *in vivo* can be considered a sperm priming process that prepares sperm to interact with the oocyte and undergo the acrosome reaction on making contact with the zona pellucida. It has been reported that in humans, *in vivo* capacitation can maintain sperm in a responsive state for several days while they wait for the oocyte to be ovulated (Aitken, 1997). Although little is known about the mechanisms controlling the kinetics of capacitation *in vivo*, factors in the female reproductive tract provide signals that regulate the process in such a way that by the time sperm arrive at the site of fertilization they are primed for a fast response to the zona pellucida.

The site at which the process begins in the female reproductive tract varies among mammalian species reflecting the initial site of sperm deposition upon ejaculation (Yanagimachi, 1994). Ejaculation facilitates the dilution of sperm into the female reproductive tract secretions. Within minutes of ejaculation, human sperm begin to leave the seminal fluid and swim into the cervical canal (Sobrero and MacLeod, 1962). In contrast, rodent sperm are swept completely out of the vagina and into the uterus along with seminal plasma (Bedford and Yanagimachi, 1992; Carballada and Esponda, 1997). With mice, some semen remains in the vagina where it coagulates and forms a copulatory plug. The plug forms a cervical cap that promotes sperm transport into the

uterus (Matthews and Adler, 1978; Carballada and Esponda, 1992). Other species, like pigs, bypass the vagina altogether. In these cases semen is deposited directly into the uterine cavity, where sperm may quickly gain access to the oviduct (Hunter, 1981). Although the site at which the process begins may vary among species, capacitation is completed in the oviduct (Bedford, 2004).

For sperm, the ascension of the female reproductive tract to the site of fertilization in the Fallopian tube is one fraught with danger. The anti-microbial defenses of the vagina, low pH and immunological responses, can damage sperm as well as the infectious agents for which it has evolved (Boskey et al., 2001). Once transported through the cervix, which like the vagina can also mount an immune response, sperm enter the uterus (Pandya and Cohen, 1985). By the time sperm navigate the uterus, they may have lost much of their protective seminal plasma coating, rendering them increasingly susceptible to immunological attack (Suarez and Pacey, 2006).

The uterotubual junction, in addition to presenting an anatomical barrier, presents a physiological barrier to sperm (Suarez and Pacey, 2006). Experiments with knockout mice indicate that certain proteins must be available and exposed on the surface of sperm to interact with the uterotubal junction, promoting sperm passage. Male mice that are null mutants for the genes encoding fertilin B (Cho et al., 1998), calmegin (Yamagata et al., 2002), and testis-specific ACE (Krege et al., 1995) are infertile as their sperm fail to pass through the uterotubal junction or bind to the zona pellucida.

In contrast to the vagina, cervix and uterus, the Fallopian tube provides a relative haven for sperm while they await the oocyte (Rodriguez-Martinez et al., 1990). There is evidence in a number of species including the hamster (Smith et al., 1987), mouse (Suarez, 1987), rabbit (Harper, 1973), bovine (Hunter and Wilmut, 1984), pigs (Hunter, 1981) and sheep (Hunter and Nichol, 1983) that upon entering the oviduct, sperm are held in a reservoir that facilitates their survival. This tubal reservoir is thought to be formed when sperm bind to glycans on the epithelial cell lining of the oviduct. Fertilin and its terminal sugar, sialic acid, competitively inhibit the binding of inseminated hamster spermatozoa to the oviductal epithelium (Demott et al., 1995). The binding of stallion sperm was inhibited by asialofetuin and its terminal sugar, galactose, (Dobrinski et al., 1996), while binding of boar sperm was blocked by

mannose (Wagner et al., 2002). Bovine sperm were prevented from binding by fucose (Lefebvre et al., 1997). The physiological relevance or function of such sperm reservoirs is thought to be related to the preservation of fertility and viability (Suarez and Pacey, 2006).

Sperm from a number of mammalian species, including humans, remain viable for extended periods *in vitro* when incubated in media supplemented with oviductal epithelium (Kervancioglu et al., 1994; Pollard et al., 1991; Ellington et al., 1993; Kawakami et al., 2001; Quintero et al., 2005). Incubation of sperm with vesicles prepared from the apical membranes of the endosalpinx produced the same extension of viability (Murray and Smith, 1997; Dobrinski et al., 1996; Smith and Nothnick, 1997), indicating that the effect can be mediated by direct contact rather than by secretions.

Although the mechanism by which epithelial binding improves the viability of sperm *in vivo* is unclear, it has been reported that epithelial-bound equine spermatozoa maintain a lower cytoplasmic Ca^{2+} concentration compared to unbound cells (Dobrinskii et al., 1997). In addition, the rate of *in vitro* capacitation is reduced when oviductal epithelial membrane vesicles are added to the capacitation media (Murray and Smith, 1997). It is believed that bound sperm may be preserved by the stalling of capacitation and the associated rises in intracellular calcium levels (Suarez and Pacey, 2006).

The suppression of capacitation and the slowing of sperm progress in the Fallopian tube would prolong viability, ensuring a continual delivery of functionally competent cells to the site of fertilisation. Additionally, the chances of polyspermic fertilisation are minimized by ensuring that only a low number of cells reach the site of fertilisation at any one time.

Despite having not been observed in humans, the creation of a sperm reservoir within the oviduct cannot be dismissed. As with other species, the viability of human sperm *in vitro* is maintained by incubation with oviductal epithelium (Murray and Smith, 1997). Moreover, sperm do bind the endosalpingeal epithelium intermittently *in vitro*

(Pacey et al., 1995). This may facilitate the creation of a functional reservoir by detaining sperm in the tubal isthmus.

Sperm remain in the discrete microenvironments within the oviductal isthmus until ovulation occurs (Hunter et al., 1987) and signals arising from the ovulated cumulus-oocyte complex stimulate the continuation of the capacitation process (Topper et al., 1999; Hunter and Rodriguez-Martinez, 2004). Release of sperm from the tubal epithelium is not regulated by a reduction in the number of sperm binding sites; but rather by changes in sperm cells themselves (Hunter and Rodriguez-Martinez, 2004). Changes associated with the process of capacitation facilitate the release of sperm from the epithelium to continue their journey toward the oocyte. The capacitation associated changes within the plasma membrane, including the shedding of proteins and cholesterol which prepare the cells for interaction with the oocyte (reviewed by De Jonge, 2005) are thought to perturb the cells interaction with the epithelium(Suarez and Pacey, 2006). In addition, the capacitation-associated onset of hyperactivated motility, provides the necessary force required to overcome the attraction between sperm and epithelium (Suarez and Ho, 2003). Although hyperactivation has been considered to be part of the capacitation process, there is now evidence that it is regulated by a different or divergent pathway from that controlling acrosomal responsiveness (Marquez and Suarez, 2004).

1.3.3 In vitro capacitation

It is now more than half a century since Austin and Chang independently reported their observations on what we now refer to as sperm capacitation. However, even in this era of sophisticated molecular genomic and proteomic technologies we know relatively little about the process of capacitation *in vivo*. Much of our understanding of the process comes from the multitude of *in vitro* experiments conducted in numerous species. Sperm capacitation can be readily accomplished *in vitro* provided that the culture conditions facilitate and support membrane remodeling and activation of signaling pathways similar to those occurring *in vivo* (De Jonge, 2005).

While different species may have specific requirements, several critical media constituents must be present for capacitation to proceed. Generally the media requires a sterol acceptor molecule, such as albumin; and energy substrates such as glucose, pyruvate and lactate (Bavister, 1969; Bavister, 1973; Yanagimachi, 1994). The media must also contain an ionic composition that is conducive to sperm homeostasis and facilitative of signal transduction processes. Ions such as Ca^{2+} and HCO^{3-} have been identified as critical to the capacitation process (Boatman and Robbins, 1991; Baldi et al., 1991). Temperature is also an important consideration, 37°C being optimal for most species studied (Yanagimachi, 1994). However, there has been wide variation in the length of incubation times reported in experiments investigating the process of capacitation (De Jonge, 2005), as well as considerable heterogeneity in the different media preparations that have been used. As such, conclusions about molecules and processes involved in capacitation drawn from *in vitro* studies may not be physiologically relevant and should only be considered in the context of the experiment until they can be verified *in vivo* (De Jonge, 2005). With such a diverse range of media preparations and conditions reported to stimulate capacitation *in vitro*; it raises the question, which set of *in vitro* conditions are more closely reflective of those occurring during capacitation *in vivo*?

The definition of capacitation *in vitro* is also important because the assays used to determine whether capacitation has occurred are based on it. *In vitro* fertilisation (IVF) assays may be used to assess the level of capacitation of a sperm sample. However, only a small proportion of the sperm population, those that penetrate eggs, are truly assayed. Moreover, a population of sperm is heterogeneous with respect to their ability to respond to a capacitative environment. Thus, results of IVF-based assays for capacitation may not be reflective of the entire population. Because of the technical difficulties associated with IVF-based assays, many investigators now assay an event that is correlated with capacitation.

Correlates of capacitation include, but are not limited to, acrosomal exocytosis (Visconti et al. 1998; Cross, 2000), hyperactivation (Burkman, 1984; Mahony and Gwathmey, 1999), the ability to bind the zona pellucida (Shur and Hall, 1982a;

Breitbart and Naor, 1999), and an increase in global tyrosine phosphorylation (Naaby-Hansen et al., 2002; Kerr et al., 2002; Hellsten et al., 2001).

1.3.4 Correlates of capacitation

Capacitation regulates the ability of sperm to reach the site of fertilisation and interact with the egg. Attainment of the capacitated state has been correlated with a number of physiological and biochemical changes in sperm. Reviewed in detail by Yanagimachi (Yanagimachi, 1994), these changes encompass extensive remodeling of the plasma membrane, changes in metabolism, intracellular pH, and changes in the tyrosine phosphorylation status of a number of proteins (Visconti et al., 1995a).

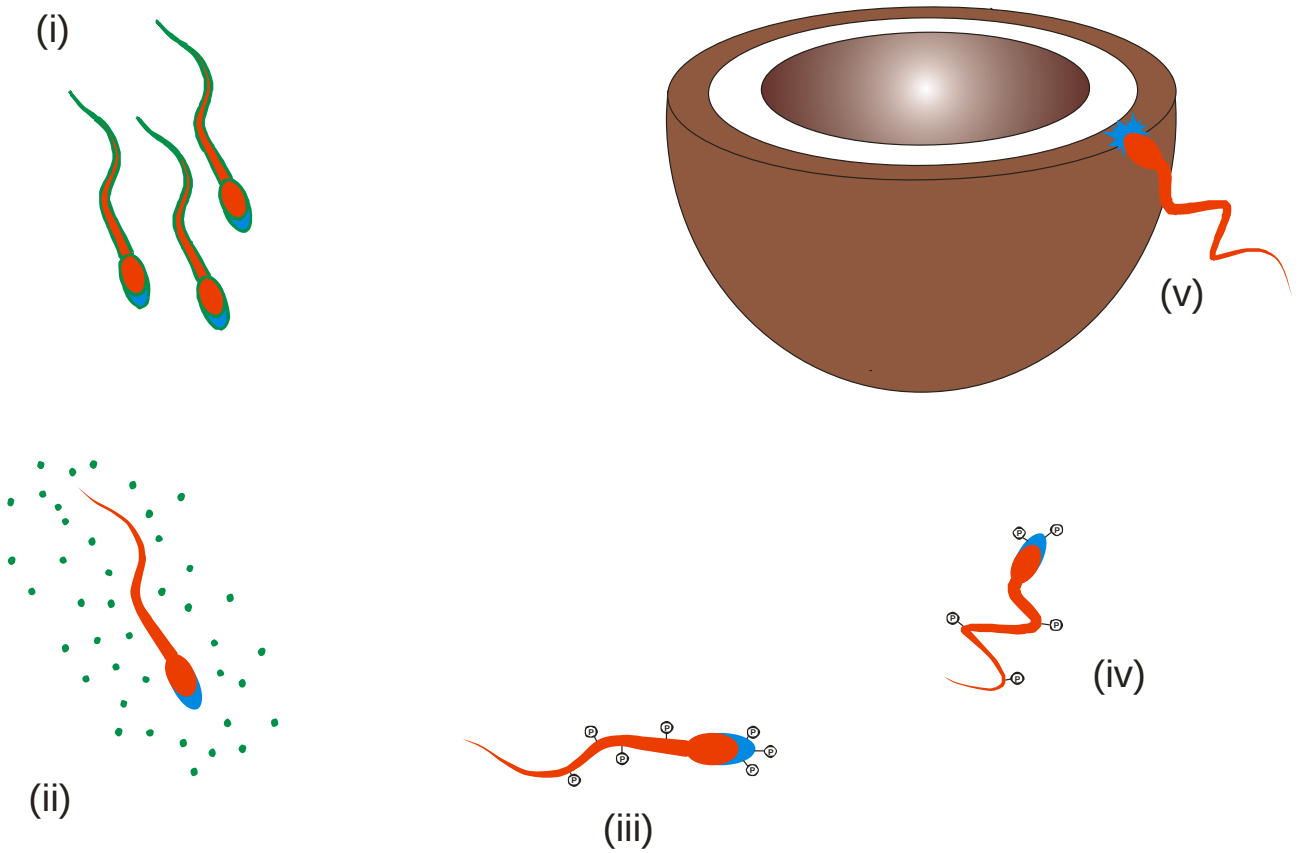


Figure 1.4. Correlates of Capacitation. A number of physiological and biochemical changes in sperm are correlated with the process of capacitation. These correlates include, but are not limited to, the loss of decapacitation factors (ii), extensive remodeling of the plasma membrane, the induction of a unique cAMP mediated tyrosine phosphorylation signal transduction cascade (iii), the onset of hyperactivated motility (iv), the ability to recognise and bind the zona pellucida, and undergo the acrosome reaction (v).

1.3.4.1 Loss of decapacitation factors

The initial stages of the capacitation process involve the reversal of some of the changes that took place in the epididymis. During transit through the epididymis proteins, and other macromolecules from the epididymal *milieu*, adsorb to the surface of sperm (Tezon et al., 1985; Ross et al., 1990; Moore et al., 1992; Boue et al., 1994; Nixon et al., 2005a). In addition, constituents of seminal plasma from the male accessory organs are found to adsorb to the surface of sperm (Audhya et al., 1987; Topfer-Petersen et al., 1998). The adsorbed materials are thought to have an inhibitory effect, holding sperm in a quiescent and immotile state, in preparation for storage in the cauda epididymis. It has been proposed that the environment of the female reproductive tract may be particularly efficient in driving the removal of these factors (Bedford, 1970; Oliphant and Brackett, 1973). The loss, or desorption, of these inhibitory factors from non-capacitated sperm correlates temporally with the acquisition of fertilising ability (Fraser, 1984). Furthermore, consistent with the notion that capacitation is a reversible process; the addition of decapacitation factors back to capacitated cells reduces their fertilizing ability (Fraser et al., 1990, Nixon et al., 2006). Additional support for the loss of coating material from the sperm surface comes from the observation that many sperm surface proteins become accessible to antibodies during capacitation (Margalioth et al., 1992).

The protein lactoferrin coats human sperm (Hekman and Rümke, 1969) but is completely removed by movement through cervical mucus (Rosselli et al., 1990). In addition, Nixon and co-workers identified four putative decapacitation factors; plasma membrane fatty acid binding protein, cysteine-rich secretory protein 1 (CRISP1), phosphatidylethanolamine binding protein 1 (PEBP1), and an unnamed protein product they termed decapacitation factor 10 (DF10); released from the surface of murine sperm when incubated in media supportive of capacitation (Nixon et al., 2006). The addition of the proteins back to a suspension of sperm suppressed several correlates of the capacitation process. However, although some decapacitation factors lost by sperm have been identified in *in vitro* studies, it is rarely established whether the same factors are lost *in vivo*.

While proteinaceous decapacitation factors appear to exhibit some degree of species specificity (Topfer-Petersen et al., 1998), cholesterol has been identified as a decapacitation factor in numerous species, including the rat (Davis, 1976; Davis et al., 1979), humans (Langlais et al., 1988), rabbit (Davis, 1982), ram (Darin-Bennett and White, 1977), guinea pig (Jha et al., 2008) and goat (Iborra et al., 2000). Cholesterol, a major constituent of seminal plasma, is thought to stabilize the phospholipid bilayer (Go and Wolf, 1985), and is believed to play an important role in preventing the premature capacitation of spermatozoa (Begley and Quinn, 1982). The loss of cholesterol in the early stages of capacitation leads to a reduction in the cholesterol/phospholipid ratio. This is believed to increase the fluidity of the plasma membrane and facilitate the completion of the capacitation process (Davis et al., 1981). The slowing and inhibition of capacitation *in vitro* when cholesterol is added to the incubation medium supports the notion that cholesterol efflux plays a part in the process of capacitation (Visconti *et al.,* 1998). Moreover, the rate of capacitation is increased when a sterol acceptor is added to the culture media (Harrison and Gadella, 2005; Visconti *et al.,* 1999a; Visconti *et al.,* 1999b).

Although the precise role of cholesterol abstraction in capacitation is not yet clear, cholesterol efflux has been proposed as the primary signal/event activating the signaling pathways which culminate in increased protein tyrosine phosphorylation, a hallmark of the capacitated state. Moreover, it is now accepted that cholesterol is enriched in membrane microdomains, or lipid rafts, which have been shown to concentrate a variety of signal transduction proteins (Nixon et al., 2010). Several studies have also shown that these raft domains are modified by the abstraction of cholesterol (Golub et al., 2004; Edinin, 2001). It has been proposed that modulation of lipid raft domains in the plasma membrane of mammalian sperm may account for several aspects of capacitation (Osheroff et al., 1999; Travis et al., 2001).

1.3.4.2 Plasma membrane remodeling

The plasma membrane of mammalian sperm exhibits a pronounced domain organisation. Arguably, one of the most important changes during capacitation is

the reorganization of the plasma membrane, particularly over the head region (reviewed by Gadella et al., 2008). Biophysical studies have revealed that the lipids and proteins are organized into lateral regions of the sperm head surface. The capacitation associated reorientation and modification of these surface molecules permits the sperm cell to bind to the zona pellucida (Flesch and Gadella, 2000). Capacitation associated changes to the plasma membrane include the removal of inhibitory decapacitation factors which unmasks components of the glycocalyx (Saxena et al., 1986), adsorption of new components from the female genital fluids and enzymatic modification of glycocalyx components (Mahmoud and Parrish, 1996; Revah et al., 2000). In addition, a lateral reorganization of membrane proteins takes place (Aguas and Pinto-Da-Silva, 1989).

Capacitation induces changes in the distribution and composition of the plasma membrane lipids and phospholipids. Visconti and co-workers furthered our understanding of the consequences of cholesterol abstraction demonstrating a link between the loss of cholesterol and the activation of signaling pathways that culminate in increased protein tyrosine phosphorylation, and ultimately the attainment of fertilization competence (Visconti et al., 1999a; Visconti et al., 1999b).

In addition to cholesterol, sperm from a number of different species have been shown to contain other sterols which may be active participants in the capacitation process (Travis and Kopf, 2002). Desmosterol has been shown to undergo efflux from the plasma membrane of sperm from a number of species including rodents and primates during capacitation (Visconti et al., 1999a; Lin et al., 1993). Sperm of different species also contain varying amounts of sterol sulfates (Cross, 1998); and Langlais and co-workers propose that sterol sulfotransferases, which have been reported to exist within the female reproductive tract, may help render the sperm membrane more fluid as part of the capacitation process (Langlais et al., 1981).

Another class of membrane lipid that has been implicated in the control of sperm function are the ceramides (Flesch and Gadella, 2000). Cross (2000) reported

that increasing sperm ceramide levels enhanced capacitation by increasing the rate of cholesterol and desmosterol efflux from the plasma membrane. Ceramides, in addition to effecting lipid packing and membrane fluidity, can exert signaling effects by directly influencing the activity of protein phosphatases and kinases (Kolesnick, 2002).

Recently, the cAMP/PKA signaling pathway has been implicated as a key mediator in the lipid remodeling or 'scrambling' process (Gadella and Harrison, 2002; Harrison and Miller, 2000). Working on boar sperm and using flow cytometry, Gadella showed that the pathway was initiated by bicarbonate stimulation of soluble adenylyl cyclase (sAC), and appeared to culminate in the activation of a sperm specific phospholipid scramblase (Gadella and Harrison, 2000; Harrison and Miller, 2000). Evidence of bicarbonate induced lipid scrambling during capacitation has subsequently been found in sperm from humans (De Vries et al., 2003), the stallion (Rathi et al., 2001), and the mouse (Wang et al., 2003).

Increased membrane fluidity facilitates the lateral redistribution of proteins within the plasma membrane. It has been proposed that the capacitation-associated redistribution of membrane components facilitates the reorganisation and preparation of molecules involved in intercellular gamete interactions (Flesch and Gadella, 2000). Working with human sperm, Focarelli and co-workers identified a change in the plasma membrane distribution of the sialoglycoprotein GP20, coincident with capacitation (Focarelli et al., 1998). This protein, which is homologous to the leukocyte antigen CD52, can be localized over the entire sperm surface of freshly ejaculated cells; but is restricted to the equatorial region of the sperm head in capacitated cells (Focarelli et al., 1998). Similar findings of altered protein distribution as a consequence of capacitation have been reported for other proteins and other species including the boar (Saxena et al., 1986), guinea pig (Myles and Primakoff, 1984), mouse (Lopez and Shur, 1987), and rat (Jones et al., 1990).

1.3.4.3 Tyrosine phosphorylation

It is widely accepted that the reversible protein phosphorylation of serine, threonine and tyrosine residues can control a diverse range of biological functions and activities in eukaryotic cells (Koch et al., 1991; Hunter, 1994). Cell cycle progression (Nasmyth, 2001), energy metabolism (MacKintosh, 1998), differentiation (Rane and Reddy, 2002), transduction of extracellular signals (Manning et al., 2002), and gene expression (Dever, 2002) are just a few of the many processes controlled by this reversible post-translational modification. Phosphorylation and dephosphorylation, catalyzed by protein kinases and protein phosphatases, can modify the function of a protein in almost any conceivable way; for example by increasing its stability, altering cellular localization, marking it for destruction, and initiating or disrupting interactions with other molecules (Köcher et al., 2003). The reversibility of phosphorylation, coupled with the ready availability of ATP as the phosphoryl donor, explains its selection as the most general regulatory mechanism adopted by eukaryotic cells.

The correlation of serine and threonine phosphorylation of sperm proteins with the ability to undergo the acrosome reaction has been recognised for more than thirty years (Garbers and Kopf, 1980). More recently, the influence of the capacitation-associated increases in the level of tyrosine phosphorylation on sperm fertilizing ability has been appreciated (Visconti et al., 1995a; Visconti et al., 1995b). In 1995 Visconti and colleagues reported the correlation between the *in vitro* capacitation of murine caudal sperm and the tyrosine phosphorylation of a number of sperm proteins ranging in molecular weight from 40 to 120kDa. It was proposed that tyrosine phosphorylation represented an important regulatory pathway modulating events associated with capacitation (Visconti et al., 1995a). In addition, the process was found to be dependent on PKA (Visconti et al., 1995b). These observations have subsequently been extended to other mammalian species (Visconti, 2002). The tyrosine phosphorylation of proteins during the capacitation process correlates temporally with the ability of sperm to undergo a zona-induced acrosome reaction (Visconti, 2002).

Until recently, with the exception of two A-kinase anchor protein (AKAP) family proteins (Carrera et al., 1996) and CABYR (Naaby-Hansen, 2002), few proteins

phosphorylated on tyrosine residues during capacitation had been identified. However, proteomic investigations are beginning to uncover the repertoire of proteins phosphorylated during capacitation in mammalian spermatozoa. The usual approach taken by investigators, characterise the changes in tyrosine phosphorylation using 2D-PAGE followed by immunoblot analysis with anti-phosphotyrosine antibodies. Proteins determined to have an altered phosphotyrosine profile are excised from a duplicate gel and identified by peptide mass fingerprinting (PMF) or tandem mass spectrometry (MS/MS). Working with human sperm, Ficarro and colleagues used a similar approach to identify eighteen proteins tyrosine phosphorylated as a consequence of capacitation (Ficarro et al., 2003). Other investigation have looked at the phosphoproteome of capacitated sperm in other species (Geussova, 2002; Ecroyd et al., 2003; Asquith et al., 2004; Mitra and Shivaji, 2004; Luconi, 1998; Naaby-Hansen, 2002; Dubé et al., 2005; Arcelay et al., 2008; Baker et al., 2010).

Protein	Species	Reference
Voltage-dependent anion select channel 2	human	Ficarro et all., 2003
Keratins	human	Ficarro et all., 2003
Phospholipid hydroperoxide glutathione peroxidase	human	Ficarro et all., 2003
Ubiquinol cytochrome c, Reductase 1	human	Ficarro et all., 2003
Glutamate ammonia ligase	human	Ficarro et all., 2003
Pyruvate dehydrogenase B	human	Ficarro et all., 2003
F-actin capping protein B	human	Ficarro et all., 2003
A kinase anchor protein 3	human	Ficarro et all., 2003
	hamster	Jha, 2002
A kinase anchor protein 4	human	Ficarro et all., 2003; Mandal, 1999
TRAP-1 (tumor necrosis factor type 1 receptor associated protein)	human	Ficarro et all., 2003
N-Acyl-aminoacylpeptide hydrolase	human	Ficarro et all., 2003
Valosin-containing protein (VCP or p97)	human	Ficarro et all., 2003
	boar	Geussova, 2002
HSP70	human	Ficarro et all., 2003
HSP90	human	Ficarro et all., 2003
	mouse	Ecroyd et al. 2003
Proacrosin binding protein	human	Ficarro et all., 2003
	boar	Dubé et al., 2005
Glutathione s-transferase M3	human	Ficarro et all., 2003
Outer dense fiber 1 (ODF 1)	human	Ficarro et all., 2003
α-Tubulin		Ficarro et all., 2003
HSP60	mouse	Asquith et al., 2004
GRP94	mouse	Asquith et al., 2004
dihydrolipoamide dehydrogenase	hamster	Mitra, 2004
extracellular signal related kinases ERK01 and ERK-2	human	Luconi,1998
calcium-binding tyrosine phosphorylation regulated protein (CABYR)	human	Naaby-Hansen, 2002
$p52^{SHC}$	human	Morte, 1998

Table 1.1. Proteins that have been shown to be phosphorylated on tyrosine residues during *in vitro* capacitation of sperm from different mammalian species.

Caution must be shown when interpreting these data as while the protein identification may be of high confidence, more often than not, actual sequence data confirming the relevant post-tranlational modification (phosphorylation) is very hard to obtain. As such, although a result may strongly suggest that a given protein is phosphorylated; unequivocal evidence requires confirmation with an alternative method like anti-pY immunoprecipitation, mutagenesis analysis, or direct sequencing.

In order to establish a connection between the different phosphorylated proteins and a specific sperm function, the localization of each phosphoprotein within the capacitated cell is critical. With the exception of the boar (Petrunkina et al., 2001) it appears that in most species, proteins of the flagellar structures are major targets of tyrosine phosphorylation during capacitation (Naz and Rajesh, 2004). Tyrosine phosphorylated proteins have been localized to the sperm tail in humans (Naz et al., 1991a; Carrera et al., 1996; Leclerc et al., 1997), monkey (Mahony and Gwathmey, 1999), hamster (Fujinoki et al., 2003), rat (Lewis and Aitken, 2001), and mouse (Urner et al., 2001). This supports a role for phosphorylation in the changes to motility patterns that accompany capacitation. Tyrosine phosphorylation of flagellar proteins has been linked to the onset of hyperactive motility, which is a prerequisite for penetration of the oocyte vestments.

In humans, AKAP3 and AKAP4 are the most well-characterized of the proteins shown to be tyrosine phosphorylated during *in vitro* capacitation. Localized primarily to the fibrous sheath, the AKAPs are essential scaffolding proteins that bind protein kinase A (PKA) providing spatial regulation of downstream signaling (Eddy et al., 2003; Tasken and Aandahl, 2004). AKAP proteins have been linked to the onset of hyperactivated motility during capacitation (Vijayaraghavan et al., 1997); and recently, Luconi and co-workers reported that AKAP3 phosphorylation in human sperm is connected to soluble adenylyl cyclase (sAC) activity and coincided with an increase in motility (Luconi et al., 2005). Although the AKAPs have been identified as targets of capacitation-associated tyrosine phosphorylation in humans and the hamster, they are not tyrosine phosphorylated in the mouse and rat. Rather the AKAPs present in the tails of rodent sperm undergo phosphorylation on serine/threonine residues (Platt et al., 2009; Johnson et al., 1997).

Although the phosphorylation of sperm proteins is a key feature of capacitation, it is not clear how, or indeed if, tyrosine phosphorylation is involved in zona binding and the acrosome reaction (Naz and Rajesh, 2004). The increased tyrosine phosphorylation occurring during capacitation however, has been linked with the enhanced ability of sperm to recognise and bind to the zona pellucida (Asquith *et al*., 2004). Immunocytochemical analysis of live sperm, facilitated the localization of tyrosine phosphorylated proteins to the plasma membrane overlying the acrosome in capacitated cells (Asquith *et al*., 2004), an ideal spot for interacting with the zona pellucida. Interestingly, this pattern of labeling was absent from uncapacitated cells. Further analysis identified two molecular chaperones, heat shock protein 1 (HSPD1) and heat shock protein 90 beta (HSP90B1) as tyrosine phosphorylated proteins localizing to the plasma membrane of the sperm head (Asquith et al., 2005). It has been proposed that the phosphorylation of these two chaperones during capacitation induces a conformational change facilitating the assembly or exposure of a functional receptor for the zona pellucida (Asquith et al., 2004). The tyrosine phosphorylation of another molecular chaperone, heat shock protein 90 (HSP90) during capacitation has also been observed in murine sperm (Ecroyd et al., 2003b).

In boar spermatozoa, capacitation has been shown to induce the tyrosine phosphorylation of plasma membrane proteins, which are thought to facilitate zona binding and induction of the acrosome reaction (Flesch et al., 1999). For example, Valosin-containing protein/p97 (VCP) has been shown to be phosphorylated in the boar (Geussova et al., 2002). Although the function of this protein remains to be elucidated, it is a homolog of the SNARE-interacting protein NSF (Ficarro et al., 2003) and has been proposed to have a role linking capacitation and the acrosome reaction (Ficarro et al., 2003).

Dubé and co-workers reported that the proacrosin binding protein (ACRBP), previously identified as a target of tyrosine phosphorylation during capacitation of human sperm (Ficarro et al., 2003), is tyrosine phosphorylated during capacitation of boar sperm (Dubé et al., 2005). In the boar, the tyrosine phosphorylation of ACRBP appears to result in its translocation or redistribution to the plasma membrane (Dubé et al., 2005). Little is known about the function of ACRBP. However, it has been implicated in the activation and localization of proacrosin and acrosin within the

acrosomal matrix (Baba et al., 1994a). Acrosin is a multifunctional acrosomal enzyme reported to have a role in retaining the binding of acrosome reacted sperm to the zona pellucida by association with ZP2 (Howes et al., 2001).

1.3.4.4 Hyperactivated Motility

Concomitant with the later stages of capacitation, sperm exhibit a distinctive pattern of motility termed hyperactivation (Suarez and Ho, 2003). Hyperactivated motility refers to the changes in the pattern of flagellar beat observed in mammalian sperm during capacitation. Immediately following deposition in the female reproductive tract, or upon release into *in vitro* media, mammalian sperm exhibit linear and progressive motility as a result of relatively symmetrical and low amplitude flagellar bends (Yanagimachi, 1994). In contrast, sperm flushed from the oviduct near the time of fertilization, and sperm that have been incubated in media conducive to capacitation, exhibit hyperactivated motility. This altered pattern is characterized by increased velocity, deeper and more asymmetric flagellar bends, and increased lateral head displacement (Yanagimachi, 1994). Consequently, progressively forward swimming in viscous and viscoelastic substances is enhanced (Yanagimachi, 1970; Yanagimachi, 1994). Hyperactivation has been observed in numerous mammalian species including the rat (Shalgi ad Phillips, 1988), human (Burkman, 1984), mouse (Suarez and Osman, 1987), rabbit (Suarez *et al*., 1983), hamster (Yanagimachi, 1970; White and Aitken, 1989) and monkey (Boatman and Bavister, 1984). Crucial for fertility *in vivo*, several roles for hyperactivated motility have been proposed.

Mucus fills the uterotubal junction and extends into the isthmus in humans (Jansen, 1980), rabbits (Jansen, 1978), pigs (Suarez *et al*., 1991), and cattle (Suarez *et al*., 1997). Suarez showed that hyperactivated spermatozoa enter viscous substances with greater efficiency (Suarez, 1996). This would assist sperm in penetrating mucus secretions in the oviduct. Hyperactivation also gives sperm the improved flexibility required for turning around in pockets of mucosa (Suarez et al., 1983). Additionally, hyperactivation would facilitate penetration of the intercellular matrix in the cumulus oophorus and the zona pellucida (Suarez and Pacey, 2006). When hyperactivation was blocked in capacitation, acrosome-reacted hamster sperm bound to the zona pellucida were unable to penetrate it (Stauss *et al*., 1995). Others have suggested that hyperactivated motility may facilitate ascension of the female reproductive tract by

helping sperm to dissociate from transient adhesion to the oviductal epithelium (Yanagimachi, 1994; DeMott and Suarez, 1992; Suarez, 1996).

In contrast to the acrosome reaction, hyperactivation is a reversible phenomenon; and there is little consensus about the identity of *in vivo* factors that regulate its onset. However, some insight into the intracellular signaling processes has been provided by the recognition that bicarbonate (HCO_3^-) is required for the onset of hyperactivation *in vitro* (Boatman and Robbins, 1991). Hyperactivation is also characterized by an increase in intracellular calcium via Catsper ion channels (Kirichok *et al*., 2006). Other potential regulators include progesterone and cAMP (reviewed by Suaez and Ho, 2003). The targets of HCO_3^- are unknown but may include the modulation of Ca^{2+} entry mechanisms (Wennemuth et al., 2003), or stimulation of soluble adenylyl cyclase (sAC) activity with subsequent activation of cAMP-dependent protein kinase (Chen et al., 2000; Wennemuth et al., 2003).

Knockout studies have implicated both sAC and PKA in the signaling pathways controlling motility. Null mutant mice for sAC (Esposito et al., 2004) and for the sperm-specific PKA catalytic subunit (Nolan et al., 2004) are both infertile due to a lack of motility and deficiencies in $HCO3^-$ -induced capacitation responses. In the case of the sAC knockout, motility was restored by the addition of membrane permeable cAMP analogs to the incubation medium. Other knockout studies have implicated the sodium-hydrogen exchangers NHE1 and NHE5 in the regulation of sperm motility (Wang et al., 2003). Sperm from mice in which these genes have been silenced are immotile and infertile. However, addition of cAMP agonists to media restores motility and fertility through the direct activation of PKA.

1.3.4.5 Zona binding and the acrosome reaction

The culmination of capacitation, or what many investigators consider to be the end point of the process, is the ability of spermatozoa to bind to the zona pellucida and undergo the acrosome reaction (reviewed by Cross, 1998 and Tulsiani et al., 2007). The acrosome reaction is an exocytotic process whereby the release of hydrolytic enzymes from the acrosomal region facilitates the penetration of the spermatozoon through the zona pellucida (reviewed by Baldi et al., 2000; Breitbart, 2003; Abou-

haila and Tulsiani, 2009). Acrosomal exocytosis can be quantified by microscopic analysis following staining with a number of reagents including fluorescently tagged monoclonal antibodies (Carver-Ward et al., 1994), lectins (Aitken and Brindle, 1993), soybean trypsin inhibitor (Arts et al., 1994) or chlortetracycline (Saling and Storey, 1979). These visualization techniques are generally combined with a cell viability assay such as ethidium monoazide (Henley et al., 1994), Hoescht 33258 (Tao et al., 1993) or the hyperosmotic swelling test (Aitken and Brindle, 1993). Different approaches for the determination of acrosomal status have been reviewed by Cross and Meizel (Cross and Meizel, 1989).

The archetypal physiological agonist for inducing the acrosome reaction is the zona pellucida (Bleil and Wassarman, 1983). However, the difficulty in obtaining large quantities of native material has led to the use of alternative inducers such as progesterone (Barros et al., 1972; Parinaud et al., 1992), lyphosphatidylcholine (LPC) (Yanagimachi, 1977) and the calcium ionophore A23187 (Jamil and White, 1981). Both LPC and A23187 are potentially cytotoxic and the dose applied must represent a compromise between efficacy and cell viability (de Lamirande et al., 1998a). In addition, these agents may bypass traditional capacitation pathways at high concentrations and so caution must be used when interpreting such data.

1.3.5 Signaling Pathways

The precise mechanisms that control the process of capacitation and prepare sperm for interaction with the ovulated oocyte are not completely clear. However, there is evidence signaling events leading to mammalian sperm capacitation rely on activation/deactivation of proteins by phosphorylation. This cascade includes soluble adenylyl cyclase, an atypical bicarbonate-stimulated adenylyl cyclase, and is mediated by protein kinase A and the subsequent stimulation of protein tyrosine phosphorylation (Visconti et al., 1997; Aitken et al., 1998a; Visconti and Kopf, 1998).

The process is also mediated by pathways involving PKC (Furuya et al., 1993) and protein tyrosine kinases (Leclerc et al., 1996; Leclerc et al., 1997).

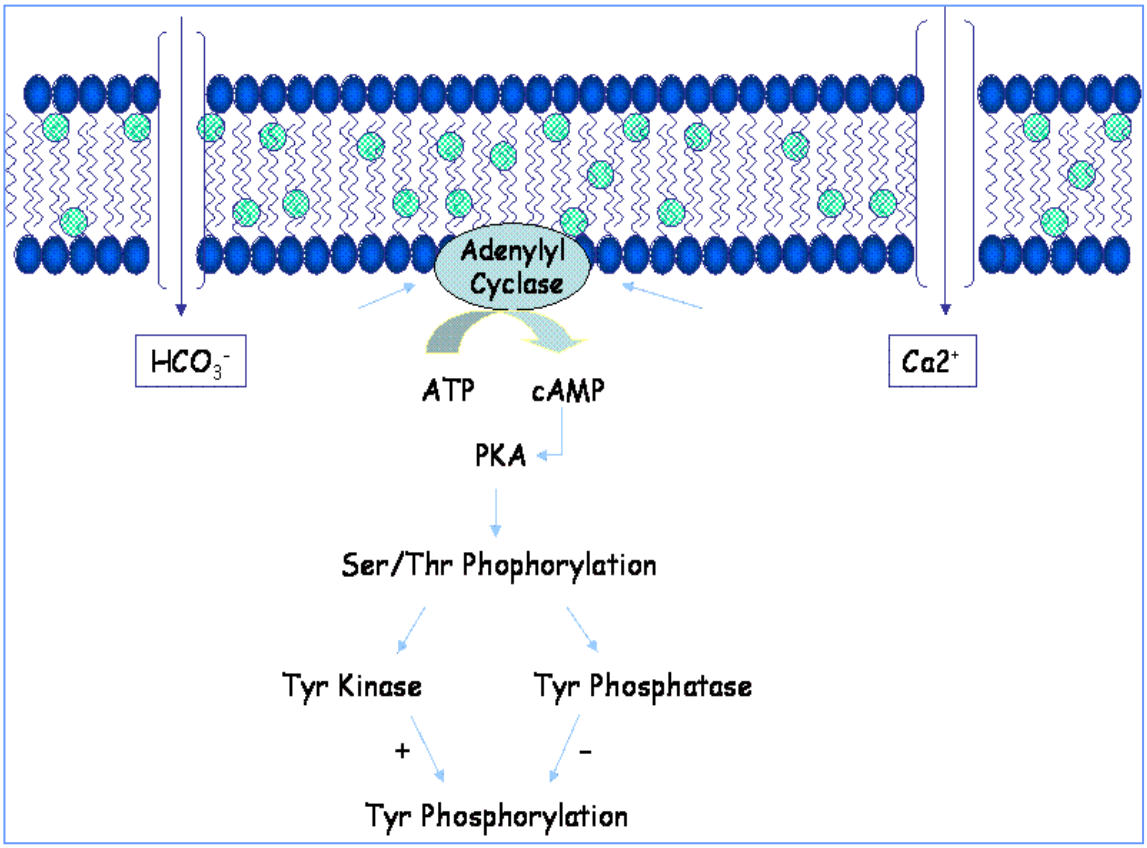


Figure 1.5. Signalling pathways in capacitation

In mammals, various sperm proteins are tyrosine phosphorylated during capacitation (Table1.1). Although there is consensus that a unique cAMP/PKA-dependent signal transduction cascade is associated with protein tyrosine phosphorylation (Fig. 1.5) (Leclerc et al., 1997; Aitken et al., 1998a), the precise mechanisms leading to increased tyrosine phosphorylation during capacitation are yet to be determined.

Elevated levels of cAMP during capacitation and the observation that membrane permeable analogues of cAMP accelerate some aspects of capacitation have led to the

general conclusion that capacitation is dependant on adenylyl cyclase activity and cAMP production.

Mammalian spermatozoa contain two adenylyl cyclase enzymes for the production of cAMP; a G-protein regulated transmembrane enzyme (tmAC), and a soluble isoform (sAC) (Harrison, 2003; Wuttke et al., 2001). All nine members of the tmAC family have been detected in mammalian sperm (Spehr et al., 2004), and they localize to different regions of the cell indicating their potential involvement in compartmentalized signaling pathways. Class VIII tmACs are prominent in the flagellum while class III enzymes localize primarily to the sperm head (Spehr et al., 2004). Although some evidence has linked tmAC to the initiation of the acrosome reaction, there is no compelling data supporting their role in capacitation (Fraser et al., 2003; Leclerc et al., 1996; Liguori et al., 2004; Mehlmann et al., 1998).

Activity of the soluble isoform, sAC, was first described in rat testis homogenates in 1975 (Braun and Dods, 1975). It is present in a range of tissues, but highly enriched in male germ cells (Sinclair et al., 2000), sAC differs from tmAC in its insensitivity to G-protein modulation (Buck et al., 1999). Rather, sAC has been shown to be regulated by HCO_3^- and Ca^{2+} (Chen et al., 2000; Litvin et al., 2003; Jaiswal and Conti, 2003). Bicarbonate is essential for capacitation *in vitro* and is believed to exert its effects, in part, by modulating sAC activity (Harrison, 1996). Consistent with the notion of an adenylyl cyclase signaling cascade in sperm is the restoration of motility and fertility in sAC knockout mice following the addition of membrane permeable cAMP analogs to incubation media (Esposito et al., 2004).

Further support for the existence of the AC-PKA pathway in sperm comes from the numerous reports documenting the suppression of capacitation by inhibitors of PKA (Aitken et al., 1998a; Galantino-Homer et al., 1997; Visconti et al., 1995b). Moreover, the process of capacitation could be induced by addition of biologically active cAMP agonists (Visconti et al., 1995b).

The cAMP-dependent protein kinase, PKA, is a target of elevated cAMP during capacitation. Both regulatory and catalytic subunits of PKA have been identified in sperm, including a novel sperm-specific splice variant of the catalytic subunit

designated C_s or $C_{\alpha 2}$ (Nolan et al., 2004; San Agustin et al., 1998). The role of PKA in normal sperm function is reinforced by the observation that mice lacking the unique C_s of PKA are infertile and fail to exhibit the usual increase in tyrosine phosphorylation associated with capacitation (Nolan et al., 2004). The increase in cAMP concentration in spermatozoa is dependent on the presence of HCO_3^- and Ca^{2+} in the extracellular medium (Carlson et al., 2007). Bicarbonate presumably binds to sAC and thereby activates the enzyme to produce increased levels of cAMP (Okamura and Sugita, 1983; Okamura et al., 1985; Garty and Salomon, 1987). Increased cAMP levels activate cAMP dependent protein kinases (PKA), and the activated PKA indirectly induces tyrosine phosphorylation.

The kinetics of cAMP activation through sAC are variable. Porcine sperm respond rapidly to the addition of HCO_3^- in the external media, generating a peak cAMP response within sixty seconds (Harrison and Miller, 2000); and PKA-dependent protein phosphorylation peak within 90 seconds of addition (Harrison, 2004). It has been proposed that this initial rapid response is associated with the control of motility during capacitation (Nolan et al., 2004; Wennemuth et al., 2003). Other cAMP/PKA responses like membrane lipid reorganization and protein tyrosine phosphorylation are more protracted.

Any model of capacitation needs to take into account cAMP involvement in both early and late capacitation-associated events. One model that is consistent with observations in a number of species, proposes that initial HCO_3^- transport increases the concentration of cAMP and consequently the activity of PKA locally, promoting lipid scrambling. Scrambling is facilitated by the activity of several phospholipid transferases, including the bidirectional carrier scramblase (Flesch and Gadella, 2000). Once scrambling occurs, cholesterol acceptors are able to facilitate cholesterol efflux which is proposed to have a positive feed back effect on HCO_3^- transport with consequent activation of sAC and cAMP synthesis. This second round of cAMP synthesis is more sustained and thought to be responsible for the later capacitation associated processes, hyperactivation and protein tyrosine phosphorylation. The spatial regulation of PKA signaling is facilitated through the enzymes interactions with relevant scaffolding proteins in specific subcellular compartments. This permits

local domains of HCO_3^-/Ca^{2+}-cAMP signaling and has significant implications for sperm function (Zippin et al., 2003).

As PKA is a serine/threonine kinase and therefore not able to directly phosphorylate proteins on tyrosine residues, an intermediate tyrosine kinase (TK) must be involved in the capacitation process. PKA may influence tyrosine phosphorylation in a number of ways. Firstly, it may directly or indirectly stimulate a TK; or it may inhibit tyrosine phosphatases. Alternatively, the phosphorylation of proteins on serine and threonine residues may prime these proteins for subsequent tyrosine phosphorylation by a TK. Further support for the suggestion that tyrosine phosphorylation is regulated by the serine/threonine phosphorylation of intermediary proteins comes from the observation that the inhibition of serine/threonine phosphatases increases tyrosine phosphorylation during capacitation (Leclerc et al., 1996).

Tyrosine kinases are classified as either receptor tyrosine kinases (RTKs) or non-receptor protein tyrosine kinases (PTKs). RTKs are transmembrane proteins containing an extracellular ligand binding domain and an intracellular tyrosine kinase domain (Cadena and Gill, 1992). RTKs are activated by extracellular ligand binding resulting in either autophosphorylation or the phosphorylation of other proteins (reviewed by Fantl et al., 1993). Consistent with the suggestion of RTK mediated signaling in mammalian sperm is the localization of phospholipase Cγ (PLCγ) on the plasma membrane of murine sperm and its tyrosine phosphorylation-dependent activation (Tomes et al., 1996). Offering further support for RTK mediated signaling is the documentation of phosphoinositide 3 kinase (PI3 kinase) activity operating downstream of tyrosine phosphorylation in human sperm (Fisher et al., 1998).

The presence of various tyrosine kinases has been demonstrated in the spermatozoa of several mammalian species. These include cAbl in human sperm (Naz, 1998), c-ras in human sperm (Naz et al., 1992b), p190 c-met tyrosine kinase in human sperm cells (Herness and Naz, 1999), TK32 in the pig (Tardif et al., 2003), EGF receptor tyrosine kinase in human, mouse, rabbit and rat sperm (Naz and Ahmad, 1992a) and bovine sperm (Lax et al., 1994), and fibroblast growth factor receptor (FGFR) in mouse and rat sperm (Cotton et al., 2006).

A bioinformatics search of kinases known to be potentially regulated by PKA led to the hypothesis that SRC is involved in capacitation. The addition of SRC inhibitors prevented the appearance of tyrosine phosphorylated proteins in human sperm induced by wheat germ agglutinin binding to platelet cell adhesion molecule 1 (PECAM-1); (Nixon et al., 2005b). Subsequent studies further support the notion that SRC is present in human (Lawson et al. 2008; Mitchell et al., 2008) and murine (Baker et al., 2006; Krapf et al., 2010) sperm.

Baker and colleagues proposed that the capacitation-associated increase in tyrosine phosphorylation is governed by SRC tyrosine kinase activity (Baker et al., 2006). This conclusion was based primarily on the observation that SRC is present in sperm and that the Src kinase family inhibitor SU6656 blocked the capacitation-associated increase in tyrosine phosphorylation. A model was proposed where PKA plays a dual role. First, it phosphorylates SRC at serine residues, activating the enzyme and promoting tyrosine phosphorylation. Also, PKA phosphorylates CSK, inhibiting its activity. This facilitates optimal SRC activity and the global increase in tyrosine phosphorylation

Although Krapf and colleagues reported observations supporting this model proposed by Baker, they concluded that c-Src is not directly involved in the increase in tyrosine phosphorylation that occurs during sperm capacitation in the mouse (Krapf et al., 2010). This conclusion is based on results using sperm from *Src*-null mice. Curiously, sperm from both Src-null and wild-type mice display similar levels of capacitation-associated tyrosine phosphorylation. However, it may be possible that SRC deletion can be compensated for by other tyrosine kinases or pathways. Considering the complexity of the subcellular localization and substrates of the capacitation-associated tyrosine phosphorylation events, it is possible that multiple PKA-activated tyrosine kinases may participate, possibly in a species specific manner.

The *src*-related PTK, c-*yes* (cellular-yamaguchi sarcoma viral oncogene) has also been localized primarily to the acrosomal region and to a lesser extent, the midpiece of human sperm (Leclerc and Goupil, 2002). Furthermore, it has been demonstrated that the activity of c-*yes* is activated by cAMP and inhibited by extracellular calcium (Leclerc and Goupil, 2002); reinforcing the notion that tyrosine phosphorylation of

proteins in the male gamete is a result of cross-talk between the cAMP/PKA pathway and tyrosine kinase/s (Naz and Rajesh, 2004).
However, YES1, which is a member of the Src family, does not have a cAMP-dependent phosphorylation site, so the mechanism by which cAMP stimulates this kinase is unclear. Moreover, it is negatively regulated by calcium, which is inconsistent with the calcium-dependency of capacitation-associated protein tyrosine phosphorylation.

The non-receptor tyrosine kinase c-Abl which localizes to the head and flagellum of mouse sperm and whose activity is up-regulated by PKA, may be involved. It was demonstrated that Inhibition of c-Abl partly decreased sperm protein tyrosine phosphorylation induced by exogenous cAMP analogues (Baker et al., 2009). These observations are consistant with the kinase having a role in sperm capacitation. Other receptor PTKs have been reported in mature sperm of several species, reviewed by (Naz and Rajesh, 2004).

Originally generated to study flagellar function, transgenic mice were developed to express a dominant-negative variant of FGFR-1 in male haploid germ cells. These transgenic mice revealed subfertility in part due to poor sperm production; however, those sperm that were produced had a severely compromised ability to undergo capacitation. Wild-type mice had functional FGFR-1 that suppressed downstream mitogen activated protein kinase (MAPK) signalling and protein tyrosine phosphorylation; FGFR-1 activation thus negatively regulates capacitation (Cotton et al., 2006). These findings indirectly support the hypothesis that the MAPK signaling cascade promotes capacitation-associated tyrosine phosphorylation.

The process of capacitation in mammalian sperm is therefore regulated by the activation of intracellular signaling pathways involving various molecules such as cAMP, PKA, RTKs, and PTKs. Several studies have documented the importance of the capacitation-associated tyrosine phosphorylation of sperm proteins to hyperactivated motility, zona pellucida binding and the acrosome reaction. The presence of three major pathways involving cAMP/PKA, RTKs, and PTKs has been demonstrated. In reality, it is unlikely that these pathways are mutually exclusive, but rather, operate synergistically with cross-talk among several molecules.

The extracellular signal-regulated kinase (ERK) module of the MAPK pathway plays a role in generating tyrosine phosphorylated sperm proteins during capacitation. Whereas a significant effect of PKA-mediated PTK signaling during capacitation is on sperm hyperactivation, ERK signaling seems to be particularly directed to modifications in the sperm head.

Luconi showed that an ERK is active in human sperm during capacitiation (Luconi et al. 1998). Further, modulation of the ERK cascade with specific inhibitors indicated that it plays a role upstream of protein tyrosine phosphorlation (de Lamarinde and Gagnon 2002). ERK1/2 activation was evident by enhanced phosphorylation during capacitation, and ERK1/2 inhibitors blocked both protein tyrosine phosphorylation and the ability of the sperm to acrosome-react. Later, O'Flaherty demonstrated that typical stimulators of human sperm capacitation and protein tyrosine phosphorylation, stimulated the phosphorylation of three MEK-like proteins (O'Flaherty et al., 2005). MEK is a dual specificity kinase that phosphorylates the tyrosine and threonine residues on ERK1/2 required for activation.

In the mouse, Nixon and colleagues demonstrated that sperm surface phosphotyrosine expression is strongly driven by the ERK module of the MAPK pathway (Nixon et al., 2010). Immunolabeling revealed core elements of the MAPK signaling pathway including SHC1, GRB2, RAS, RAF1, MEK, and ERK1/2, to be present primarily in the periacrosomal region. Phospho-specific antibodies revealed a dramatic increase in phosphorylation when sperm were incubated in capacitating conditions, reflecting cascade activation. Surface phosphotyrosine expression of capacitated sperm was suppressed in the presence of specific inhibitors of the ERK cascade, although levels never fell to those observed in non-capacitated sperm. It was suggested that since the inhibitors used were not perfectly specific for the ERK participants, it was unlikely that tyrosine phosphorylation would be completely eliminated. Similarly, inhibition of the ERK cascade reduced the ability of the sperm to bind homologous ZP. Collectively, these data highlight a previously unappreciated role of the ERK module in the modification of the sperm surface during capacitation to render these cells functionally competent to engage in the process of fertilization (Nixon et al., 2010).

1.3.6 Factors that regulate tyrosine phosphorylation and capacitation

Capacitation is a process that prepares spermatozoa for interaction with the cumulus-oocyte complex. It ensures that sperm arrive at the site of fertilization at the appropriate time and rapidly undergo the acrosome reaction on contacting the zona pellucida.

1.3.6.1 Cholesterol Efflux

The plasma membrane of mature sperm cells differs from that of somatic cells in its relatively high cholesterol content (Marttinez and Morros, 1996). Cholesterol efflux from the plasma membrane has been linked to the activation of membrane signal transduction pathways related to the process of capacitation (Visconti et al., 1999).

It has been demonstrated that the abstraction of cholesterol during capacitation increases the fluidity and permeability of the plasma membrane, facilitating the entry of calcium and bicarbonate ions through specific membrane ion channels (Summers et al., 1976; Visconti et al., 1999a). This in turn activates a unique cAMP/PKA-dependent signaling pathway associated with protein tyrosine phosphorylation (Leclerc et al., 1997; Aitken et al., 1998). Visconti and co-workers showed that cholesterol efflux during capacitation leads to increased tyrosine phosphorylation of sperm proteins through the cAMP/PKA pathway (Visconti et al., 1999).

The important role of cholesterol removal in the initial stages of capacitation is reinforced by the findings of a number of studies. Firstly, in the absence of a suitable cholesterol acceptor molecule in the external media, analogues of cAMP restore the normal pattern tyrosine phosphorylation associated with capacitation (Visconti et al.,1999b). Furthermore, the addition of cAMP overcame the inhibitory effect of exogenous cholesterol sulphate in media containing BSA (Visconti et al.,1999b), suggesting that the cholesterol effect is upstream of cAMP metabolism. Consistent with cholesterol efflux having a regulatory role is the observation that inhibitors of PKA activity limit the degree of tyrosine phosphorylation induced by BSA and β-

cyclodextrins (Osheroff et al., 1999). The use of alternative cholesterol binding proteins, such as the β-cyclodextrins, confirms that the effect is mediated by cholesterol removal rather that albumin itself.

Taken together, these studies strongly indicate that cholesterol efflux induces changes in the sperm plasma membrane facilitating the influx of calcium and bicarbonate. This in turn affects the cAMP-dependent signaling pathway leading to tyrosine phosphorylation.

1.3.6.2 Bicarbonate and intracellular pH

The requirement for bicarbonate in sperm capacitation has been well documented for a number of mammalian species (Neill and Olds-Clarke, 1987; Boatman and Robbins, 1991). Although, the precise mechanism by which the influx of HCO_3^- regulates capacitation remains unclear, it has been correlated with an increase in intracellular pH, regulation of cAMP levels, hyperpolarization of the plasma membrane, increased tyrosine phosphorylation, and changes in the distribution membrane lipids.

The influx of HCO_3^- into sperm during capacitation is thought to be facilitated by specific ion channels within the plasma membrane. The involvement of a Na^+/ HCO_3^- co-transporter in the capacitation of murine sperm has been demonstrated (Demarco et al., 2003). Alternatively, intracellular bicarbonate levels may be raised from the conversion of diffused carbon dioxide to bicarbonate by carbonic anhydrases present in the head of sperm (Wandernoth et al., 2010; Parkkila et al., 1991). Although the increase in intracellular pH observed during capacitation has been attributed to the influx of bicarbonate (Vredenburgh-Wilberg and Parrish, 1995), the role of pH remains uncertain as increased pH fails to induce tyrosine phosphorylation and capacitation (Fraser, 1995).

Rather than the modulation of pH, bicarbonate may exert its effect by stimulation of the pathways leading to protein tyrosine phosphorylation during capacitation. Murine sperm incubated in bicarbonate deficient media fail to exhibit the increased level of tyrosine phosphorylation normally associate with capacitation . Furthermore, the

normal level of capacitation induced phosphorylation is restored by the addition of cAMP agonists, indicating that bicarbonate exerts its effects upstream of cAMP.

Harrison (2004) and Da Ross et al. (2004) demonstrated the role of bicarbonate in protein tyrosine phosphorylation in sperm during capacitation and fertilization. In addition to triggering changes in the plasma membrane lipid architecture of boar sperm (Gadella and Harrison, 2000; Gadella and Harrison, 2002; Harrison and Miller, 2000), bicarbonate induces rapid changes in motility via a cAMP/PKA-dependent pathway (Harrison, 2004).

Luconi et al. recently demonstrated the effect of *in vitro* addition of bicarbonate on intracellular cAMP production and tyrosine phosphorylation (Luconi et al., 2004). The addition of HCO_3^- resulted in increased sperm motility and hyperactivation, mediated by increased cAMP production and tyrosine phosphorylation of AKAP3. The stimulatory effects of bicarbonate were nullified by the inclusion of a bicarbonate transport inhibitor to the media (Luconi et al., 2004). It is interpreted that bicarbonate stimulates sperm motility and hyperactivation through activation of sAC and tyrosine phosphorylation of AKAP3, resulting in increased recruitment of PKA to AKAP3.

The current consensus is that bicarbonate exerts its influence by stimulating sAC activity; thereby mediating downstream signaling events culminating in the tyrosine phosphorylation of numerous sperm proteins. However, there is some evidence suggesting that bicarbonate may not directly regulate sAC activity in some species. Contrary to observations in the mouse (Visconti et al., 1995b), Aitken and Baker have demonstrated that rat sperm undergo tyrosine phosphorylation in the absence of bicarbonate following adjustment of intracellular pH (Aitken et al., 1998b; Baker, et al., 2003b). It is thought that some species may contain a pH-dependent isoform of the AC enzyme (Peterson et al., 1980); and that the increase in intracellular pH accompanying bicarbonate influx underlies the stimulatory effects of bicarbonate, facilitating cAMP synthesis and downstream signaling.

In addition to influencing the cAMP/PKA pathway, studies in the mouse suggest that bicarbonate may have another mode of action, regulating the potential of the plasma membrane through the $Na^+/HCO3^-$ co-transporter (Demarco et al., 2003).

Interestingly, the bicarbonate levels within the epididymal lumen are relatively low in comparison to those experienced by sperm in seminal plasma and the oviduct (Brooks, 1983). This differential may play an important role in the suppression and promotion of capacitation in the epididymis and the female reproductive tract respectively.

1.3.6.3 Calcium

Numerous studies have demonstrated the importance of calcium to fertilization related events. There is a considerable amount of evidence supporting the requirement of calcium for capacitation (Fraser, 1987; DasGupta et al., 1993) and the acrosome reaction (Florman et al., 1992). However, the role of calcium in the capacitation associated increase of protein tyrosine phosphorylation remains controversial.

It has been demonstrated in several mammalian species that there is an increase in the intracellular Ca^{2+} concentration during the process of capacitation (Zhou et al., 1990; White and Aitken, 1989; Coronel and Lardy, 1987; Fraser and McDermott. 1992; Okamura et al., 1993; Suarez et al., 1993). It has also been observed that there is considerable variation in the requirement for calcium between species during capacitation. Murine sperm require micromolar (μM) concentrations of extracellular Ca^{2+} for capacitation (Fraser, 1987), whereas human sperm need millimolar (mM) levels (DasGupta et al., 1993; Stock and Fraser, 1989).

Although there is consensus on the requirement of calcium for capacitation and other fertilization related events, there are contrasting reports on the impact of extracellular calcium on tyrosine phosphorylation during capacitation. Considering that calcium can simulate both the production of cAMP by adenylyl cyclase (Gross et al., 1987) and its degradation by cAMP nucleotide phosphodiesterase (Wasco and Orr, 1984), it is not surprising that the cation can have both a negative and positive effect on capacitation associated signaling pathways.

Recently, in an attempt to reconcile these data and elucidate the mechanisms by which this cation exerts its regulatory effects on the signaling cascades that culminate in tyrosine phosphorylation, Baker and co-workers investigated the impact of

extracellular calcium on the tyrosine phosphorylation of human and mouse sperm. Results demonstrated that homeostatic regulation of $[Ca^{2+}]i$ in Ca^{2+}-supplemented media is an energy dependent process requiring the consumption of intracellular ATP (Baker et al., 2004). It was concluded that Ca^{2+} suppresses tyrosine phosphorylation by limiting the availability of intracellular ATP, and not by activating tyrosine phosphatases or inhibiting tyrosine kinases as previously suggested (Luconi et al., 1996).

Calcium has a number of roles in the regulation of signaling pathways involved in preparing sperm for fertilisation and there is evidence indicating that high concentrations of extracellular calcium may provide a means of regulating sperm function *in vivo*. The calcium concentration of human seminal plasma is 170 μM (Arver and Sjoberg, 1983), more than sufficient to limit the availability of internal ATP and prevent premature tyrosine phosphorylation and capacitation. In the female reproductive tract, sperm bind to the oviductal epithelium (discussed in section x.x). Compared to free swimming cells, bound sperm exhibit a reduced intracellular calcium concentration (Petrunkina et al., 2001). It has been proposed (Baker et al., 2004) that this facilitates a build up of ATP within sperm. The increased availability of ATP may then lead to the stimulation of signaling culminating in hyperactivation. The onset of hyperactivated motility facilitates the release of sperm from the epithelium to continue their journey toward the oocyte.

1.3.6.4 Reactive Oxygen species (ROS)

The first indication that reactive oxygen species (ROS) may be involved in capacitation came in 1993 with the observation that superoxide dismutase (SOD) prevents, and the addition of exogenous superoxide anion promotes, capacitation in human sperm (de Lamirande and Gagnon, 1993). Since then, despite the documented pathological effects of ROS, their capacity to induce oxidative damage to DNA, lipids and proteins; a number of studies have implicated ROS such as hydrogen peroxide (H_2O_2) and superoxide anion in the regulation of human sperm capacitation and protein tyrosine phosphorylation (Leclerc et al., 1997; Aitken et al., 1996).

The enzymes responsible for tyrosine phosphorylation and dephosphorylation of sperm proteins may be targets for ROS. Consistent with this proposal is the

observation that the tyrosine phosphorylation of proteins during capacitation is enhanced under oxidizing conditions and inhibited by reducing conditions (Aitken et al., 1995). Furthermore, the biological response of human sperm to progesterone and recombinant ZP3 is enhanced by stimulation of ROS generation and inhibited by catalase, a ROS scavenger (Aitken et al., 1996).

ROS is thought to influence capacitation associated tyrosine phosphorylation at the level of cAMP production. The generation of ROS is believed to stimulate tyrosine phosphorylation by mediating the intracellular concentration of cAMP. A link between ROS and the synthesis of cAMP and tyrosine phosphorylation has been made for sperm from different species including the human (Aitken et al., 1995, 1998), mouse (Ecroyd et al., 2003), rat (Lewis and Aitken, 2001), bovine (Rivlin et al., 2004), and equine (Baumber et al., 2003). The mechanism is believed to involve the stimulation of adenylyl cyclase to produce cAMP, leading to PKA-dependent tyrosine phosphorylation (Aitken et al., 1998; Lewis and Aitken, 2001; Rivlin et al., 2004) and possibly the suppression of tyrosine phosphatase activity (Hecht and Zick, 1992).

Although the identity of the ROS involved in the process is yet to be confirmed, data from a number of independent investigations suggests that H_2O_2 plays a role. The exogenous addition of H_2O_2 by the combination of glucose and glucose oxidase, or the direct addition of this ROS to incubation media, leads to the stimulation of tyrosine phosphorylation and capacitation in hamster (Bize et al., 1991) and human sperm (de Lamirande and Gagnon, 1995; De Lamirande et al., 1997; Griveau et al., 1994; Leclerc et al., 1997; O'Flaherty et al., 2005). Moreover, the observation that the addition of catalase reverses these effects is consistent with the involvement of H_2O_2 (Aitken et al., 1995 2017-25; Baumber et al., 2003; Bize et al., 1991; Rivlin et al., 2004).

Although controlled low concentrations of ROS are beneficial for sperm capacitation and tyrosine phosphorylation, elevated concentrations lead to impaired function, immobilization and cell death. It is the fine balance between the amount of ROS produced and scavenged at any particular time that determines whether normal sperm function will be promoted or hindered.

1.3.6.5 Other Regulators

The interaction of progesterone with spermatozoa has been known for several decades. In 1989 and 1990, Thomas and Blackmore independently documented the rapid, and now characteristic, influx of calcium in human spermatozoa (Thomas et al., 1989; Blackmore et al., 1990)

Progesterone has been reported to affect capacitation and the acrosome reaction in mammalian sperm (Therien and Manjunath, 2003). Parinaud and Milhet showed that in human sperm, progesterone stimulates tyrosine phosphorylation resulting in hyperactivation (Calogero et al., 1996) and increased synthesis of cAMP (Parinaud and Milhet, 1996). Progesterone has also been shown to enhance the fluidity of the plasma membrane in human sperm which facilitates capacitation (Revelli et al.,1998).

Progesterone has been shown to exert its influence on sperm through a progesterone receptor (PR) or binding sites on the sperm plasma membrane (Shah et al., 2003). A number of studies have described the presence of different progesterone receptors in the plasma membrane. These are: plasma membrane Ca^{2+} channel (PR1), a membrane associated protein tyrosine kinase (PTK; PR2), and a plasma membrane chloride channel (PR3) (Revelli et al., 1998).

Although a deluge of research papers have been published, the identity of the receptor binding progesterone and the channel responsible for the influx has remained elusive (Publicover et al., 2007). Recently, two groups using patch clamping techniques on human sperm demonstrated that the universal characteristic effect of progesterone on sperm, a rapid influx of calcium, is *via* the sperm-specific channel CatSper. Lishko and Strunker independently provided direct evidence of stimulation of CatSper with progesterone (Lishko et al., 2011; Strünker et al., 2011)

Gamma-aminobutyric acid (GABA) may stimulate the capacitation of sperm via a signaling pathway involving Ca^{2+}, cAMP and protein tyrosine phosphorylation. In cells, three types of membrane receptors (A, B and C) mediate the inhibitory effects of GABA, the most widely distributed neurotransmitter in the central nervous systems of vertebrates. The GABA-A receptor has been identified in human spermatozoa (Naz and Rajesh, 2004). The addition of GABA to incubation media

results in a dose dependent increase in the percentage of capacitated sperm (Ritta et al., 2004). Moreover, increased levels of intracellular Ca^{2+} and cAMP were induced by GABA, and these effects were abrogated by the addition of GABA antagonists (Ritta et al., 2004).

Cytokines are a family of peptide hormones shown to have both positive and negative affects in a variety of cell types and tissues. Although they are produced primarily by cells of the immune system in response to various stimuli, non-immune cells have been reported to secrete cytokines. Cytokines have been identified in genital tract secretions of both men and women (Temma et al., 2004; Fichorova et al., 2004), and several studies have identified cytokine receptors in sperm. Naz and co-workers demonstrated the presence of receptors for interferon alpha (IFNα) and interferon gamma (IFNγ) in mouse, rabbit, pig and human sperm (Naz et al., 2000). Others have detected interleukin 2 alpha (IL2α) (Fierro et al., 2002), interleukin 2 beta (IL2β) (Fierro et al., 2002), and insulin like growth factor 1 (IGF1) (Naz and Padman, 1999; Henricks et al., 1998) receptors in human sperm.

Cytokines have been shown to influence sperm functions including motility, capacitation, acrosome reaction, and zona binding in both a stimulatory and inhibitory manner (Naz et al., 1994). The mechanism by which cytokines exert their influence on sperm function is not clear. However, it has been reported that certain cytokines namely, interferon alpha (IFNα), interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), interfere with sperm motility (Naz et al., 1994); while interleukin 6 (IL6) enhances capacitation and the acrosome reaction (Naz and Kaplan, 1994). The impact cytokines have on protein tyrosine phosphorylation is unclear. However, the fact that many of them affect capacitation, cytokines may have a role modulating the pathways controlling protein tyrosine phosphorylation.

The number and diversity of factors reported to influence tyrosine phosphorylation and other correlates of capacitation, emphasizes the complexity of the signaling pathways involved in the regulation of the process. It must also be considered that much of the data pertaining to the regulation of the capacitation process have been garnered from *in vitro* studies and that the culture conditions used represent a

dramatic simplification of the environment sperm encounter within the female reproductive tract.

1.4 Sperm-Zona Pellucida Interaction

1.4.1 Overview

Sperm-zona interaction is a complex capacitation-dependent phenomenon comprising three stages: primary binding of acrosome intact sperm to the zona pellucida, secondary interaction of acrosome reacted sperm with the zona pellucida, and penetration of acrosome reacted sperm through the zona pellucida into the perivitelline space (Florman and Storey, 1982; Saling et al., 1979). Primary zona pellucida binding is mediated by interaction between ZP carbohydrates and lectin-like proteins on the surface of the sperm head (Gwatkin and Williams, 1977; reviewed by Töpfer-Petersen, 1999 and Clark, 2010). Early studies suggested that primary zona binding encompasses a non-specific, reversible 'loose' attachment, followed by a species-specific, irreversible 'tight binding' (Bleil and Wassarman, 1983; Hartmann et al., 1972). Loose attachments are defined as weak bonds that can be easily disrupted by serial rinsing of oocytes through narrow bore pipettes, while tight binding cannot be disrupted by physical manipulation (Hartmann et al., 1972). Studies in the mouse have shown that capacitated sperm rapidly attach to the zona but require a period of at least ten minutes before tight binding is established (Schmell and Gulyas, 1980). Tight binding appears to be a prerequisite for induction of the acrosome reaction (Tollner et al., 2003).

1.4.2 Structure and biochemical composition of the zona pellucida

In order to reach, bind to, and fuse with the oocyte plasma membrane, capacitated sperm must first bind and penetrate the zona pellucida, a protective glycoprotein matrix surrounding the mammalian oocyte (Wassarman, 1988). Composed of up to four glycoproteins designated ZP1, ZP2, ZP3 and ZP4, the zona pellucida mediates

taxon specific sperm binding at fertilization (Wassarman et al., 2001), and has a protective role during early embryonic development (Hoodbhoy and Dean, 2004). Although all mammalian eggs are surrounded by the zona pellucida, there are differences between species with respect to composition and structure (Wassarman, 2005). It is generally accepted that the zona of most mammalian species is comprised of three or four proteins ZP1, ZP2, ZP3 and ZP4 (Lefievre et al., 2004; Conner et al., 2005; Hughes and Barratt, 1999).

In the mouse, the ZP1 polypeptide consists of 623 amino acid residues, while ZP2 comprises 713 amino acids, and ZP3 424 residues. The three proteins have relative molecular masses (Mr) of 200, 120 and 83 kDa respectively (Bleil and Wassarman, 1980). Synthesized and secreted by the growing oocyte (Wassarman et al., 2001), each zona protein is extensively glycosylated at serine/threonine (O-linked) and asparagine (N-linked) residues (Wassarman, 1988; Greve et al., 1982; Florman and Wassarman, 1985; Hoodbhoy et al., 2005). Since all three proteins are sulfated and heavily glycosylated, the masses of the protein cores are considerably lower than the overall molecular masses, oligosaccharides account for roughly half of the mass of each protein (Easton et al., 2000).

The three proteins form a thick extracellular coat or matrix that completely surrounds the plasma membrane of the oocyte. Several lines of evidence suggest that a domain common to each of the zona proteins, the ZP domain (Bork and Sander, 1992), is important for protein-protein interactions and has a role in the assembly of the zona filaments during oocyte growth (Jovine et al., 2005). The current model of the murine zona pellucida is based on that first proposed by Wassarman in 1988 (Figure 1.6). Supported by extensive biochemical data, the model dictates filaments are constructed of repeating ZP2-ZP3 units held together by non-covalent interactions. Filaments are cross-linked at intervals by the ZP1 protein or a combination of ZP1 and ZP4 in rats and humans. The recognition that ZP1 is a homodimer, composed of 120 kDa subunits linked by a disulfide bond is consistent with the proposed role for the protein as an interfilament crosslinker (Bleil and Wassarman, 1980b; Greve and Wassarman, 1985).

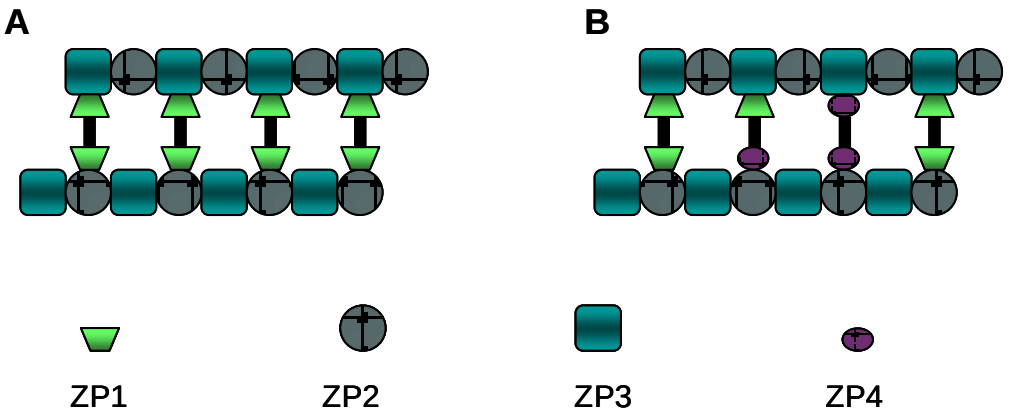


FIGURE 1.6. Structural organisation of the zona pellucida according to the Wassarman model. **A**: Model of the mouse zona comprising ZP1-3. ZP2/ZP3 filaments are crosslinked by ZP1 homodimers. **B**: Hypothetical model of a zona containing all four zona proteins. This model assumes a conserved function between ZP1 and ZP4. (Adapted from Wassarman et al., 2005.)

The binding of mouse sperm to the murine zona can be inhibited by preincubation of the sperm with zona glycoproteins. Bleil and Wassarman determined that the active competitor in the inhibition assay was the 83kDa ZP3 glycoprotein; ZP1 and ZP2 failed to compete for sperm binding (Bleil and Wassarman, 1980a; 1986; 1988). There is evidence that the sperm binding activity of the zona pellucida resides on the O-linked oligosaccharides of ZP3 (Florman and Wassarman, 1985). Studies have demonstrated that sperm-zona binding can be blocked by O-linked oligosaccharides of ZP3, present on Ser^{334} near the C-terminus of the protein (Chen et al., 1998; Kinloch et al., 1995). Although these investigations suggest that sperm-zona binding is a carbohydrate mediated event, the molecular composition of the sperm-binding oligosaccharide remains obscure. Different studies have implicated no less than four different monosaccharide residues as being crucial for initial sperm binding : α-galactose (Bleil and Wassarman, 1988), β-N-acetylglucosamine (Miller et al., 1992b), fucose (Johnston et al., 1998), and mannose (Tulsiani et al., 1992). The discrepancy between these studies is not surprising considering the heterogeneity in the distribution of carbohydrate in the murine zona (Avilés et al., 1997). Avilés and colleagues demonstrated that certain sugars are restricted to the inner regions of the zona while others were more evenly distributed throughout the structure (Avilés et al., 2000).

In addition to providing the site for initial adhesion of capacitated sperm cells, ZP3 stimulation triggers the acrosome reaction in sperm bound to the zona pellucida (Florman and Storey, 1981; Bleil and Wassarman, 1983). However, whilst they account for adhesion, ZP3 carbohydrate chains are not sufficient to drive acrosomal exocytosis (Florman et al., 1984). Moreover, glycopeptides produced from the enzymatic digestion of native zona and purified ZP3 retain the ability to bind sperm but are unable to induce the acrosome reaction (Florman et al., 1984). Two models have been proposed to account for these observations. The first proposes that ZP3 may be polyvalent with regard to the adhesion carbohydrates and that the crosslinking of the complementary ligand on the sperm surface may be the catalyst for exocytosis (Leyton and Saling, 1989; Macek et al., 1991). The second proposes that discrete domains of ZP3 are responsible for sperm binding and the induction of the acrosome

reaction (Endo et al., 1987). Conclusive evidence for either model is yet to be obtained.

In addition to the involvement of carbohydrate moieties, studies have implicated the ZP protein backbone in sperm-zona interaction. ZP peptides have been shown to function in both high affinity interaction between the sperm and ZP carbohydrate side chains (Yonezawa et al., 1995) and induction of the acrosome reaction (Chapman et al., 1998; Florman et al., 1984). It is possible that the initial attachment between the sperm and zona pellucida is mediated by carbohydrate residues on ZP3 and subsequent tight binding is a function of the peptide core (Prasad et al., 2000).

An alternative model dictates that initial sperm-egg binding depends on the interaction of a sperm surface protein with a supramolecular complex of the three mouse zona pellucida glycoproteins, the role of carbohydrate recognition in this paradigm is thought to be minimal (Gahlay et al., 2010).

Fusion of the gametes triggers the cortical reaction, a secretory discharge from the oocyte that acts as a block to polyspermic fertilization. Enzymes released from cortical granules lying just below the plasma membrane diffuse across the perivitelline space and either modify or mask sperm receptors in the zona pellucida; thereby preventing the binding and induction of the acrosome reaction on the outer surface of the zona (Florman and Wassarman, 1985).

These two models were tested by replacing endogenous protein with mutant ZP2 that could not be cleaved or with ZP3 lacking implicated O glycans. Sperm bound to two-cell Zp2 mutant embryos despite fertilization and cortical granule exocytosis. Contrary to prediction, sperm fertilized *Zp3 mutant* eggs. Sperm at the surface of the zona pellucida remained acrosome-intact for more than 2 hours and were displaced by additional sperm (Gahlay et al., 2010). These data indicate that sperm-egg recognition depends on the cleavage status of ZP2 and that binding at the surface of the zona is not sufficient to induce sperm acrosome exocytosis.

Historically, the characterization of substances released from cortical granules has been impeded by the small amount of material available for analysis. However,

proteomic studies using artificially activated mouse eggs identified six major proteins in the 20-70kDa mass range (Moller and Wassarman, 1989; Pierce et al., 1990; Gross et al., 2000). Consistent with the models of the zona block to polyspermy is the observation that one of the proteins exhibits proteinase activity facilitating cleavage of the ZP2 protein (Moller and Wassarman, 1989).

Based on the cleavage site of ZP2, ovastacin was selected as a candidate protease. Ovastacin is an oocyte-specific member of the astacin family of metalloendoproteases. Using specific antiserum, ovastacin was detected in cortical granules before, but not after, fertilization. Recombinant ovastacin cleaved ZP2 in native zonae pellucidae, documenting that ZP2 was a direct substrate of this metalloendoprotease (Burkart et al.,2012)

1.4.3 Sperm binding proteins/receptors for the zona pellucida

Mammalian fertilisation has been the subject of intensive research for many years. Yet, despite the fundamental importance of this highly regulated and species-specific event, the molecular basis of how the gametes recognise and interact with each other remains unclear. One of the key issues remaining contentious is the identity of the proteins on the surface of sperm that recognize and bind the zona pellucida. No less than twenty different sperm proteins have been implicated in species-specific binding of sperm to eggs.

Putative ZP receptors have been identified through the use of a range of techniques including analysis of mutations influencing fertility, development of inhibitory monoclonal antibodies, analysis of sperm autoantigens, ZP affinity columns, photoaffinity crosslinking and binding of radiolabelled ZP to sperm lysates (reviewed by McLeskey et al., 1998). The controversy in this field of research may also be partially attributed to species differences in gamete interaction proteins, and to the fact that many of the *in vitro* assays used to identify and assess such molecules do not necessarily mirror *in vivo* events. One of the most common assays for ZP receptor activity is to block or impede sperm-zona binding with a specific antibody against a

protein of interest. However, the interpretation of such data can be difficult due to the likelihood of non-specific steric hindrance caused by relatively large antibody molecules coating the surface of the cell. In addition, the assumption that gamete adhesion and downstream signaling are mediated by a single receptor-ligand interaction has confounded identification of the ZP receptor. A number of studies have suggested that the ZP receptor is a multimeric protein complex comprising both low and high-affinity binding partners, which may impart some measure of redundancy to the process. The following sections review some of the molecules proposed to mediate sperm-zona interactions in mammalian species. The list of candidates is by no means exhaustive and a fuller treatment of zona receptor candidates can be found in the following publications (McLeskey et al., 1998; Topfer-Petersen et al., 2000; Tulsiani and Abou-Halia, 2001; Wassarman, 1999a; Ensslin et al., 2007; Tulsiani and Abou-Halia, 2011).

1.4.3.1 β-1,4-Galactosyltransferase (GalTase)

A carbohydrate dependent model of interaction suggests that a specific β-1,4-galactosyltransferase (GalTase) expressed on the head of mouse sperm mediates initial zona binding by interacting with N-acetylglucosamine (GlcNAc) residues at the terminal ends of O-linked ZP3 sugars (Shur and Hall, 1982a; Lopez et al., 1985). This is consistent with the findings of Florman and Wassarman (1985) who demonstrated that O-linked sugars in mouse ZP3 are specifically recognised by murine spermatozoa.

Consistent with a role for GalTase in sperm-zona interaction is the dose-dependent inhibition of sperm-zona binding *in vitro* by GalTase inhibitors, purified GalTase, and Fab fragments of anti-GalTase antibodies (Shur and Hall, 1982a; Shur and Hall, 1982b; Lopez et al., 1985). A potential role for GalTase in the induction of the acrosome reaction has been proposed (Miller et al., 1992b). In this model, multivalent binding between ZP3 and several GalTase moieties leads to receptor aggregation and the activation of heteromeric G-protein signaling complexes and the acrosome reaction.

Transgenic mouse studies have however raised questions about the importance of GalTase in the fertilization process. While sperm from mice over-expressing a surface GalTase transgene are able to bind more solubilized ZP3 compared to wild-type animals, they show a decreased capacity to bind intact zonae (Youakim et al., 1994). It is interpreted that the over-expression of GalTase impairs the release of decapacitation factors, previously shown to be necessary for the exposure of the GalTase binding site for ZP (Shur and Hall, 1982). Also surprising is the fact that, GalTase-null knockout mice are fertile even though sperm from these animals are unable to undergo the ZP3-induced acrosome reaction (Lu and Shur, 1997). The authors suggest that GalTase is essential for the induction of the acrosome reaction *in vivo*, and that fertility was retained by spontaneous acrosomal exocytosis.

While GalTase has not been detected in human sperm, it has been localized to the plasma membrane of porcine sperm. It appears however, that it is not required for sperm-zona binding to take place in this species (Rebeiz and Miller, 1999).

1.4.3.2 SED1

Currently one of the preferred candidates for the mouse sperm zona receptor is SED1, a homolog of the boar sperm surface protein p47 (Ensslin and Shur, 2003). Boar p47 was identified as a zona binding protein through affinity chromatography using immobilized zona proteins and found to be homologous to mammary epithelial proteins shown to have roles in cell-cell interactions (Ensslin et al., 1998). This protein was localized to the acrosomal cap of boar and homologous cDNA was cloned from bovine, mouse and human testis. The murine ortholog, subsequently named SED1, contains Notch-like EGF repeats and discoidin/F5/8 type C domains that may contribute to the affinity of this for the zona pellucida (Ensslin and Shur, 2003). Localization of SED1 to the plasma membrane overlying the acrosome and surface exposure in live, capacitated mouse sperm are consistent with the argument for SED1 involvement in zona binding. Further, recombinant SED1 and anti-SED1 antibodies impair *in vitro* zona pellucida interaction (Ensslin and Shur, 2003). However, targeted disruption of the *Sed1* gene does not result in infertility. *Sed1* null mice are fertile and their sperm bind to the zona pellucida *in vitro*, although litter sizes and sperm-egg binding levels are reduced (Ensslin and Shur, 2003). Additional characterization of SED1 is required for elucidation of the role and mechanism by which this protein

influences murine fertilization. SED1 has also been localized to the surface of acrosome-intact human sperm (Copland et al., 2009).

1.4.3.3 Protein tyrosine kinase (ZRK)

One of the more controversial zona receptor candidates is the 95kDa zona receptor kinase (ZRK). In 1989, Leyton and Saling observed that a 95kDa tyrosine phosphorylated protein on the mouse sperm head with intrinsic tyrosine kinase activity showed affinity for the zona pellucida (Leyton, 1989). The level of phosphorylation of ZRK was reported to increase during capacitation and in response to exposure to solubilized zona pellucida (Leyton et al., 1992). This group subsequently published the sequence of the human homolog of ZRK, designated Hu9 (Burks et al., 1995). Like the murine version, this protein was tyrosine phosphorylated, demonstrated tyrosine kinase activity, and had an affinity for the zona pellucida. Further, synthetic peptides corresponding to the predicted extracellular domain of the Hu9 inhibited sperm-zona binding *in vitro*.

A number of reports disputing these results however were subsequently published. Firstly, Kalab and colleagues proposed that the 95kDa tyrosine phosphoprotein in mouse sperm was not a zona receptor kinase, but rather a tyrosine phosphorylated form of hexokinase with a possible role in the compartmentalisation of glycolytic pathways in the sperm flagellum (Kalab et al.,1994; Travis et al., 2001a). Despite a subsequent report from Saling and co-workers challenging these findings (Leyton et al., 1995), the role of ZRK in zona pellucida interaction is presently unclear. Soon after this, the identification of HU9 as a sperm-specific zona receptor was challenged. Several independent researches found that the HU9 sequence contained a number of errors and that rather than a novel sperm-specific protein, it showed 100% sequence identity with a proto-oncogene c-mer, which is expressed in a number of cell types (Bork, 1996; Tsai and Silver, 1996). While a more recent report maintains the importance of a 95kDa tyrosine phosphoprotein in human sperm function (Brewis et al., 1998), the classification of this protein as a zona receptor candidate remains controversial.

1.4.3.4 SP56

Another candidate for the zona receptor in the mouse is the lectin-like 56kDa zona oligosaccharide-interacting protein, sperm protein-56 (SP56) (Bliel and Wassarman, 1990). First identified twenty years ago as a direct binding partner for mZP3 by photoaffinity cross-linking studies (Bliel and Wassarman, 1990), Sp56 localizes to the plasma membrane in the head of acrosome intact, but not acrosome reacted sperm (Cheng et al., 1994). Sequence analysis of the cDNA encoding sp56 indicates that the protein is a member of the C3/C4 superfamily of binding proteins that includes the alpha subunits of complement 4B-binding protein (Bookbinder et al., 1995). Consistent with sp56 having a role in mediating sperm interaction with the zona pellucida are the results of inhibition assays where purified sp56 (Bookbinder et al., 1995), and anti-sp56 antibodies inhibit sperm-zona binding *in vitro* (Cohen and Wassarman, 2001).

Collectively, these observations suggest that sp56 may be the sperm protein responsible for sperm-egg recognition in the mouse. However, questions have been raised about the localization of the protein to the outer leaflet of the plasma membrane. Working with guinea-pig sperm, Foster and colleagues identified an acrosomal matrix protein, AM67, closely related to the murine sp56 protein (Foster et al., 1997). Prompted by the discrepancy between the localization of the protein in the guinea pig and the mouse, the authors re-evaluated sp56 in the mouse and found it to be a part of the acrosomal matrix (Foster et al., 1997). The results, which are in conflict to those previously published by Bleil and colleagues have since been independently confirmed (Kim et al., 2001). For sperm with an intact acrosome, dogma dictates that acrosomal matrix proteins should be unavailable for binding to mZP3. Recently however, acrosomal molecules have been implicated in sperm-egg adhesion prior to the acrosome reaction (Tardif and Cormier, 2011). Further, it has been recognised that some acrosomal matrix (AM) proteins, including sp56, are released to the sperm surface during capacitation (reviewed by Wassarman, 2009). This may explain why uncapacitated mammalian sperm are unable to bind to the unfertilized egg ZP.

1.4.4 Unresolved issues in mammalian fertilisation

While significant advances have been made in the characterization of capacitation and zona pellucida interaction in mammalian spermatozoa, many issues remain unresolved. The association between capacitation-associated signal transduction events, particularly tyrosine phosphorylation, and gamete interaction is at present unclear. Additionally, the nature of the ZP receptor on the surface of sperm is uncertain. While current evidence suggests that the ZP receptor may be a multimeric protein complex comprising zona binding components of both low and high affinities, the identity of such molecules and the mechanisms for downstream signaling following zona adhesion are open to question. The findings discussed in this review highlight the biological complexity of sperm-egg interaction and emphasizes the importance of integrating observations made *in vitro* with our knowledge of fertilization *in vivo* to elucidate the physiological basis of how the gametes recognize and interact with each other.

1.5 Aims and Hypotheses

Mammalian testicular spermatozoa are incapable of fertilisation. Before fertilisation can occur, spermatozoa must undergo both epididymal maturation in the male reproductive tract and capacitation in the female tract. Only sperm that have traversed the epididymis attain the functional endpoints of capacitation, the ability to acrosome react and fertilise an egg. Capacitation is correlated with an increase in the level of tyrosine phosphorylation of a number of proteins, several of which become exposed on the cell surface.

Recent work from a collaborators laboratory demonstrated a close correlation between capacitation associated, surface phosphotyrosine expression and the ability of mouse spermatozoa to recognize the oocyte and engage in sperm-zona pellucida interaction. Furthermore, an analysis of the surface phosphoproteome of capacitated sperm identified two molecular chaperones, heat shock protein 1 (HSPD1) and heat shock protein 90 beta (HSP90B1). Both proteins have well characterised roles in protein

folding and the assembly of multimeric protein complexes. In addition, both chaperones show a restricted localisation in the apical head of mouse spermatozoa, an ideal position from which to direct sperm-zona pellucida interaction.

We hypothesize that the activation of HSPD1 and HSP90B1, by the tyrosine phosphorylation events associated with sperm capacitation triggers these proteins to assemble and present a functional zona pellucida recognition complex on the cell surface prior to fertilization. The studies presented in this thesis were targeted towards elucidation of the molecular basis of the initial recognition and adhesion events between the mammalian spermatozoon and oocyte. The aims of the project were to identify and characterise chaperone-associated proteins on the surface of capacitated mouse spermatozoa.

The research presented in chapter four investigated the identities of other members of this putative receptor complex of chaperone-associated proteins, with a particular interest in ascertaining the specific molecule responsible for zona adhesion. A proteomics-based approach utilizing co-immunoprecipitation in concert with tandem mass spectrometry was employed to identify chaperone-associated proteins in lysates derived from capacitated mouse sperm. HSPD1 was immunoprecipitated from capacitated sperm and associated proteins identified by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Several proteins were putatively identified as binding partners of HSPD1 on the surface of capacitated mouse spermatozoa: HSPE1 (formally known as hsp10), another molecular chaperone; citron kinase; and an aldose reductase and the proacrosin binding protein.

As an initial step to characterising the function of the identified HSPD1-associated proteins in the maturation of spermatozoa and fertilization-related events, we sought to determine the expression and localisation of each candidate protein during epididymal maturation, capacitation and the acrosome reaction. The characterization of each candidate protein forms the basis of chapters five, six, seven and eight.

The investigation of chaperones and chaperone-associated proteins in spermatozoa may shed new light on the potential importance of these molecules in the assembly and expression of receptor complexes on the surface of a wide variety of different cell

types, not just spermatozoa. Furthermore, the characterization of the identified HSPD1-associated proteins in spermatozoa will inform future studies on sperm-zona interaction and may have implications in the treatment of male factor infertility and the development of novel methods of male-oriented fertility control.

Proteins at the cell surface play a key role orchestrating and regulating the sequential interactions that occur between the male and female gametes. The surface proteome is likely to contain many functionally important proteins, the identification of which will be critical to understanding the molecular basis of how the gametes recognise and interact with each other.

Complementry to identifying chaperone-associated proteins as a means to characterizing important proteins in the male gem line, experiments were performed to catalogue the repertoire of proteins localized to the plasma membrane of murine sperm. The objective of the research detailed in chapter three was to profile the surface proteome of functionally mature murine sperm taken from the cauda epididymis.

In order to understanding initial gamete recognition events at fertilization (capacitation, zona binding and acrosomal exocytosis) it is imperative to study the sperm surface proteome by using purified plasma membrane fractions. Although this task is challenging there are now strategies at our disposal to achieve comprehensive coverage of the proteins at the sperm surface. For this purpose, a dedicated protocol based on specific purification of cell surface proteins labeled with membrane impermeable biotinylation reagents was developed. Appropriate gel electrophoresis separation and purification methods combined with mass spectrometry were then used to identify surface membrane proteins.

In addition, such a resource will represent an invaluable reference tool for future comparative type proteome studies. Such studies comparing sperm at different stages of epididymal maturation, or capacitated versus non-capacitated cells will provide a means of identifying surface proteins that are altered during epididymal maturation and/or capacitation, proteins likely to be key players in the molecular events that underpin the process of fertilisation.

CHAPTER 2

MATERIALS & METHODS

2. Materials and Methods

2.1 Reagents and Solutions

Further information regarding reagents and solutions employed in these studies appears in the appendices as follows:
Appendix A: Reagents, materials and kits.
Appendix B: Buffers and solutions.

2.2 Animals

All experiments using mice described in these studies were performed in accordance with the National Health and Medical Research Council's (NHMRC) guidelines on ethics in animal experimentation and were approved by the Monash Medical Centre (MMC) standing committee on Ethics in Animal Experimentation (Ethics approval number MMCA 2003/56). Male Swiss mice (8-12 weeks old) were obtained from the central animal house at Monash University and housed in the animal holding facilities at MMC. The animals were exposed to a twelve hour light cycle at a temperature of 22°C and had free access to food and water. Euthanasia was by carbon dioxide asphyxiation and cervical dislocation.

2.3 Sperm and Testicular Cell Preparation

2.3.1 Media and incubation conditions

Sperm were routinely prepared in HEPES-buffered Biggers-Whitten-Whittingham media (BWW, Appendix B) (Biggers et al., 1971). In some experiments BWW media was prepared without $NaHCO_3$ (BWW-HCO_3^-), without $CaCl_2$ (BWW-Ca^{2+}) or

without $CaCl_2$ but with 1.7mM $SrCl_2$ (BWW-Ca^{2+} + Sr^{2+}). BWW-HCO_3^- and BWW-Ca^{2+} were supplemented with additional NaCl to maintain an osmolarity of 300 mos^{-1}. BWW was sometimes prepared with bovine serum albumin (BSA) substituted with polyvinyl alcohol (PVA) to a final concentration of 1mg/mL. Some samples were treated with 1mM pentoxifylline (ptx) and 1mM dibutyryl cyclic adenosine monophoshate (cAMP).

2.3.2 Testicular cell preparation

Total testicular cell preparations were obtained by macerating the testis tissue in incomplete BWW. Cells were then collected by centrifugation at 500xg for 3min. The preparation was washed three times with incomplete BWW media prior to protein extraction.

2.3.3 Caput and corpus epididymal spermatozoa

The epididymis was removed from the euthanased animal, excess fat and connective tissue trimmed away and the organ blotted on filter paper to remove any excess blood. The caput or corpus region was isolated and placed in a droplet of media under water-saturated mineral oil that had been pre-warmed to 37°C. A 25 gauge needle was used to puncture the epididymis several times and gentle pressure was applied to release spermatozoa into the surrounding media. After removing the epididymis, the preparation was subjected to a density gradient centrifugation to enrich spermatozoa and remove contaminating red blood cells. The preparation was gently layered over 5mL of 30% (v/v) percoll in PBS and centrifuged at 500xg at 37°C for 15min.

2.3.4 Cauda epididymal spermatozoa

The cauda epididymis and vas deferens was dissected from the animal, excess fat and connective tissue removed and the organ blotted free of excess blood on filter paper.

Sperm were isolated by epididymal back-flushing using the method of Ecroyd et al., 2003a. A cannula of ethylene tubing was inserted into the lumen of the vas deferens and secured with surgical suture. A small nick was carefully made in the proximal region of the cauda. With the aid of a syringe, water-saturated paraffin oil with a small amount of sudan black was forced through the cannular, vas deferens and the duct network of the cauda. Epididymal fluid was collected, at the site of the fine nick in the proximal cauda, into a 5µl microcappillary. The microcapillary was then stored temporarily under water-saturated paraffin oil at 37°C. Spermatozoa were permitted to disperse in the incomplete BWW for 5-10 min before a cell count was performed to determine the concentration of spermatozoa.

2.3.5 Preparation of capacitated sperm

Capacitated sperm were prepared, by diluting cells to a concentration of $6x10^6$ cells/mL in complete BWW media and incubation at 37°C in an atmosphere of 5% CO_2 for 90 min unless otherwise stated. All media and glassware were pre-warmed to 37°C. Incubation was carried out in open, flat-bottomed tubes.

2.3.6 Preparation of acrosome reacted sperm

The acrosome reaction was induced using the calcium ionophore A23187. Following capacitation, caudal epididymal sperm were treated with 1.25µM A23187 at 37°C for 15 min.

2.4 Protein Extraction and Purification

2.4.1 Total cellular protein extraction

Spermatozoa were centrifuged at 500g for 3 min and washed three times with incomplete BWW media. The pellet was then solubilised in sperm lysis buffer

containing 1mM sodium orthovanadate and heated to 95°C for 5 min. Insoluble material was removed by centrifugation (20,000g, 10min) and an aliquot taken for the determination of protein concentration. The remainder of the sample was then either stored at -20°C or diluted 1:1 with double strength reducing SDS-PAGE sample buffer (DSRSB) for SDS-PAGE analysis.

2.4.2 Protein extraction for co-immunoprecipitation

For co-immunoprecipitation (Co-IP) experiments, sperm were capacitated for 90 min in BWW supplemented with 1mM pentoxifylline (ptx) and 1mM dibutyryl cyclic adenosine monophoshate (cAMP). After washing three times with incomplete BWW to remove BSA, cells were lysed in cold co-immunoprecipitation buffer containing protease inhibitors and 1mM sodium orthovanadate for 60 min at 4°C with constant gentle agitation. Insoluble material and cell debris were then removed by centrifugation (20,000g for 10 min at 4°C) and the clarified lysates removed to a new tube. In some experiments surface proteins were treated with the chemical crosslinker 3,3´-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) after capacitation but before lysis. After capacitation, sperm were washed and incubated in PBS containing 1mM DTSSP for 30 min at room temperature. Cells were then washed and lysed.

2.4.3 Plasma membrane protein extraction

Sperm were isolated from the cauda epididymis by back-flushing and diluted to 10 mL with incomplete BWW media. Cells were then centrifuged for 90 sec at 40g. The supernatant was removed to a new tube and centrifuged for an additional 10 min at 280g. The sperm pellet was then resuspended in 1 mL of incomplete BWW. Surface proteins were labeled by the addition of sulfo-NHS-SS-Biotin (a thiol-cleavable amine-reactive biotinylation reagent that contains an extended spacer arm to reduce steric hindrances associated with avidin binding) to a final concentration 0.1 mg/mL and incubation for 15 min at room temperature. Unreacted biotin label was quenched by the addition of Tris-HCl to 1 mM and incubation at room temperature for 5 min.

Cells were then pelleted at 300g for 5 min, resuspended in lysis buffer supplemented with protease inhibitors and sodium orthovanadate for 60 min at 4°C with constant agitation. Insoluble material was removed by centrifugation (20,000g for 10 min at 4°C). Biotinylated proteins were recovered from the clarified lysate by incubation with 500 µL of immobilised NeutrAvidin™ agarose resin (Pierce Cell Surface Protein Isolation Kit, cat.# 89881), prepared according to the manufacturer's instructions, for 60 min at room temperature with constant agitation. Non-specifically bound proteins were removed by washing with three changes of lysis buffer followed by three PBS washes. Proteins were then eluted by boiling in SDS-PAGE sample buffer for 5 min.

2.4.4 Protein precipitation

The volume of protein extracts was reduced to below 500 µL by centrifugal lyophilysation. A -20°C solution comprising an equal amount (w/v) of trichloroacetic acid (TCA) and eight volumes of acetone was added, and the mixture vortexed immediately. Samples were then left to precipitate overnight at -20°C. Extracts were then centrifuged (20,000g, 20 min, 4°C) and washed by three changes of cold acetone before being allowed to air dry. Proteins were then resuspended in the appropriate buffer.

2.4.5 Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) experiments were performed using either the ProFound™ Mammalian Co-Immunoprecipitation Kit (Pierce, product number: 23600) or Dynabeads® M-280 tosyl-activated superparamagnetic polystyrene beads from Dynal Biotech (Dynal Biotech product number: 142.03). Both kits were used according to the manufacturer's instructions.

ProFound™ Mammalian Co-Immunoprecipitation Kit: 50 µL of the supplied antibody coupling gel slurry (AminoLink® Plus gel) was washed three times with

binding/wash buffer (PBS) followed by conjugation with 50 µg of primary antibody. The primary antibody was covalently linked to the coupling gel by the addition of sodium cyanoborohydride. Following a four hour incubation at room temperature with constant agitation, unreacted sites on the coupling gel were quenched by the addition of 1M Tris-HCl, pH 7.4. The antibody conjugated gel was then washed twice with 1M NaCl followed by two washes with PBS pH 7.4. The antibody-gel was then stored at 4°C in PBS containing 0.02% w/v sodium azide between experiments.

Dynabeads® M-280 tosyl-activated superparamagnetic polystyrene beads: the beads were washed three times with 0.1M Na-phosphate buffer pH 7.4 for 5 min prior to being resuspended in the same buffer containing the antibody to be immobilised. After incubation for 24 hours at 37°C with constant gentle agitation, the beads were washed twice with PBS pH 7.4 containing 0.1% (w/v) BSA for 5 min at 4°C. Beads were then blocked by incubation with 0.2 M Tris pH 8.5 containing 0.1% (w/v) BSA for 24 hours at room temperature with constant gentle agitation. Finally beads were washed twice with PBS pH 7.4 containing 0.1% (w/v) BSA for 5 min at 4°C.

For Co-IP experiments, lysates were diluted 1:1 with PBS containing protease inhibitors and sodium orthovanadate and added to the prepared antibody-coupled gel. After incubation overnight at 4°C with constant agitation unbound proteins were removed by centrifugation at 4000g for 1 min. The Co-IP gel was washed five times with PBS before eluting the captured Co-IP complexes in ImmunoPure® elution buffer at room temperature for 5 min. Eluates were then precipitated and stored at -20°C until digestion with trypsin.

2.4.6 Protein quantitation

Estimations of protein concentrations were made using the detergent compatible (DC) protein assay kit™ from BioRad following the manufacturer's instructions in

accordance with the Lowry method (Lowry, 1951). BSA protein standards were prepared with the relevant lysis buffer for each experiment.

2.5 Protein Separation

2.5.1 Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve proteins according to molecular weight. Protein extracts were disrupted by diluting 1:1 with double strength Laemmli sample buffer (Laemmli, 1970) containing 10% (v/v) β-mercaptoethanol, and incubation for 2 hrs at room temperature. Proteins were separated on 1.5 mm thick 10% acrylamide gels with a 4.0% stacking gel and a discontinuous buffer system (Laemmli, 1970). Electrophoresis was performed at 110V constant voltage and terminated when the bromophenol blue tracking dye reached 1 cm from the bottom of the gel. Details of individual reagents can be found in appendix B.

2.6 Protein detection and localisation

Proteins within polyacrylamide gels were visualised by staining gels with either Coomassie brilliant blue R-250 (CBB), sypro ruby (SR) or silver staining. Alternatively, some gels were not stained but transferred to membranes for detection of proteins by western blotting.

2.6.1 Coomassie Brilliant Blue R-250 staining

Coomassie brilliant blue R-250 (CBB) staining of SDS-PAGE gels employed overnight exposure to 0.03% (w/v) CBB in a solution of 50% (v/v) methanol in water containing 8.75% (v/v) acetic acid, prior to destaining for one hour in the same

solvent, excluding the dye. Gels were then rinsed by several changed of distilled water.

2.6.2 SYPRO® Ruby staining

In case of sypro ruby (SR) staining, gels were exposed to a solution of stain as supplied by BioRad. Gel resolved proteins were stained overnight with SR prior to destaining for one hour with deionised water. Sypro stained gels were visualized with the aid of an ultra violet transilluminator (λ=254nm).

2.6.3 Silver nitrate staining

Silver staining of protein gels was carried out using a modification of Blum's method (Blum, 1986). Briefly, gels were fixed in a solution of 50% (v/v) methanol, 12% (v/v) acetic acid for 30 min followed by two 5 min washes in 10% (v/v) ethanol, 5% (v/v) acetic acid. Acid was removed by washing overnight in a minimum of four changes of 10% (v/v) ethanol. Gels were placed in 1.6 mM sodium thiosulfate for 1 min, washed four times with distilled water and stained in 0.2% (w/v) silver nitrate in the dark for 6 min. Following three washes in distilled water, the gels were developed in 90 μM sodium thiosulfate, 0.09% (v/v) formaldehyde and 283 mM sodium carbonate solution until bands appeared. The reaction was stopped by the addition of 1% (v/v) acetic acid. Gels were then washed in distilled water.

2.6.4 Western blotting

Following SDS-PAGE, proteins were transferred to polyvinylidene fluoride (PVDF) membrane, either at 200V constant voltage for 60 min or at 30V constant voltage for 16 hours. Prior to transfer, the membrane was wet with methanol, washed for 2 min in distilled water followed by equilibration in western transfer buffer for 10 min. After transfer the membrane was blocked in Odyssey blocking buffer (LI-COR

Biosciences), diluted 1:3 with PBS pH 7.4, for 60 min at room temperature. Blocked membranes were probed with the primary antibody diluted 1:1000 (unless otherwise stated) in Odyssey blocking buffer diluted 1:3 with PBS pH 7.4 containing 0.05% (v/v) Tween-20 (PBST) for 60 min at room temperature with constant gentle agitation. After washing the membrane with three changes of PBST (10 mL, 5 min) to remove unbound antibody, secondary antibody was added to the membrane diluted 1:5000 in PBST and incubated at room temperature with constant gentle agitation. After three more PBST washes and a single PBS wash, proteins were detected on blots using infrared (LI-COR Odyssey) imaging.

2.6.5 Immunolocalisation of proteins*:* Indirect immunofluorescence *(IIF)*

Sperm were plated onto glass slides coated with 0.1% (v/v) poly-L-lysine and air dried. Cells were fixed in ice cold methanol for 1 min, then washed in ethanol for 1 min before being air dried. All subsequent incubations were performed in a humidified chamber. Fixed cells were permeabilised with 0.2% (v/v) Triton X-100 for 30 min at room temperature. Following three 5 min PBS washes, slides were blocked with 10% (v/v) donkey serum in CAS block for 60 min. Slides were then incubated with primary antibody diluted 1:50 in blocking solution overnight at 4°C. After three 5 min PBS washes, cells were incubated with secondary antibody diluted 1:100 in blocking solution for 60 min at room temperature. Following three 5 min PBS washes, cells were counterstained with DAPI, washed again and mounted with Vectashield mounting media. Slides were viewed and images captured using fluorescence and phase contrast microscopy.

2.6.6 *Flow cytometry*

Following preparation, spermatozoa were diluted to $1x10^6$ cells/mL, and incubated in either non-capacitating or capacitating media. The sperm suspension was then incubated with primary antibody at 1:100 dilution for ten minutes at 37°C. Sperm

were subsequently washed twice with BWW and incubated with FITC-conjugated secondary antibody at a dilution of 1:200 for an additional ten minutes at 37°C. Following two additional BWW washes, the cells were incubated with propidium iodide (PI) (20 mg/mL) and analyzed using a fluorescence-activated cell sorting (FACS) Calibur FACS (Becton Dickinson, Franklin Lakes, NJ, USA) with an FL4 530/30 nm band-pass filter, allowing the collection of fluorescence data in logarithmic mode and light-scatter data in linear mode. Ten thousand cells were counted in each sample at a rate of 50–500 events per second. Data were analysed using the Cell Quest package (BD Biosciences, San Jose, CA).

2.7 Protein Identification

2.7.1 In-gel proteolytic digestion

Stained protein bands of interest were excised with a scalpel and placed into 0.6 mL polypropylene microfuge tubes. Unless otherwise stated all steps were performed at room temperature and all incubations were performed with agitation. CBB-stained gel pieces were destained by washing twice with 200µl of 50% acetonitrile in 200 mM NH_4HCO_3 for 45 min at 37°C. After drying the gel pieces *in vacuuo*, using a centrifugal concentrator, sufficient 2 mM Tris(2-carboxyethyl) phosphine (TCEP), in 25 mM NH_4HCO_3 (pH 8.0), was added to cover the gel pieces, and the proteins were reduced for 15min at 37°C. After cooling to room temperature, the TCEP solution was replaced with approximately the same volume of 20 mM iodoacetamide in 25 mM NH_4HCO_3 (pH 8.0). After a 30 min incubation at 37°C in the dark, the gel pieces were washed three times with 200 µL of 25 mM NH_4HCO_3 for 15 min at 37°C and dried *in vacuuo* with a centrifugal concentrator. The gel pieces were rehydrated in a volume 1.5 times that of the gel volume in a digestion buffer of 10% acetonitrile (ACN) in 40 mM NH_4HCO_3 containing 0.02 µg/µL sequencing grade modified trypsin and left to stand at room temperature for 60 min. An additional 50 µL of digestion buffer, without trypsin, was added to keep the gel pieces wet during subsequent incubation at 37°C for 18 hours.

After incubation, tryptic peptides were extracted and processed separately for mass spectrometry (MS). Digest supernatants were removed (extract 1) and peptides extracted by two subsequent incubations for 45 min at 37°C with separate 50 μL aliquots of 60% (v/v) methanol in 0.1% (v/v) TFA (extracts 2 and 3).

Combined extracts (1-3) were diluted with 0.1% (v/v) TFA to reduce the ACN concentration to 5% (v/v) and partially fractionated on C18 ZipTips that had been pre-wetted with methanol and washed with 5% (v/v) aqueous methanol containing 0.1 % (v/v) formic acid. 20μL aliquots of the diluted extracts were aspirated through the ZipTips and out into a waste tube until the entire sample had been transferred to the waste tube. The ZipTips were then washed 3 times with 20μL aliquots of 5% (v/v) aqueous methanol containing 0.1% (v/v) formic acid and the adsorbed peptides were subsequently eluted by repeatedly aspirating 10μl of 70% (v/v) aqueous methanol containing 0.1% (v/v) formic acid through the ZipTip.

2.7.2 In-solution proteolytic digestion

Protein extracts were resuspended in 80 μl of 50 mM NH_4HCO_3, 10% (v/v) acetonitrile. To reduce disulfide bonds, TCEP was added to a final concentration of 10 mM and samples heated to 95°C for 10 min. Reduced thiol groups were subsequently blocked by the addition of iodoacetamide to 15 mM and incubation in the dark for 45 min at room temperature. Unreacted iododacetamide was quenched by the addition of dithiothreitol (DTT) to 5 mM and incubation at room temperature for 10 min. One microgram of sequencing grade modified trypsin was added to samples prior to incubation at 37°C for 4hrs. An additional microgram of enzyme was added and digestion continued for 12 hrs at 37°C. The enzyme was inactivated by the acidification of samples to pH 3-4 with formic acid. Digests were then desalted with C18 ZipTips prior to MS analysis.

2.7.3 Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS)

Spectra were acquired with an Applied Biosystems 4700 Proteomics Analyzer. The MALDI matrix used was alpha-cyano-4-hydroxycinnamic acid. Collision energies were 1 keV and air was used as the collision gas. The acquired MS and MS/MS data were then submitted to a database for protein identification.

2.7.4 LC-MS/MS tandem mass spectrometry

Tryptic peptides were separated by nanoscale reversed phase high performance liquid chromatography (HPLC) and analysed by mass spectrometry (MS). A linear gradient of 0-60% buffer B over 50 min was employed. A 300 μm x 5 mm trap column was employed to desalt samples prior to separation on a 75 μm x 150 mm analytical C18 reversed phase column. MS analysis was performed on an LTQ linear ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). A nanospray ion source was used with a New Objectives electrospray (ESI) needle (30 μm tip). The needle voltage was 1.6kV in positive ion mode. The scan cycle consisted of a survey scan (mass range 500-2000 amu) followed by MS/MS of the six most intense signals in the spectrum.

2.7.5 Mass spectrometric data interpretation

All MS/MS spectra were searched against the International Protein Index (IPI) database (Version 3.32) using the SEQUEST algorithm. Parameters used for peptide identification were partial oxidation of methionine residues (+16 Da), fragment ion and peptide mass tolerance of ± 1.5 Da, and tryptic cleavage of peptides with up to 2 missed cleavages.

Identified peptide sequences were filtered in terms of cross-correlation value (Xcorr) and delta-correlation score (ΔCn, where Cn = normalized correlation). Only

conventional tryptic peptides displaying an Xcorr of at least 2.0 and a ΔCn of at least 0.08 were considered for protein identification.

2.8 Functional assays

2.8.1 Viability assay

The LIVE/DEAD® sperm viability kit from Molecular Probes (cat # L-7011) was used to assess the viability of different sperm preparations. Briefly, sperm were washed and resuspended in 1 mL of BWW media. SYBR 14 dye was added to a final concentration of 100 nM and the cells incubated for 10 min at 37°C. Propidium iodide was then added to a final concentration of 4.8 μM and cells incubated for an additional 10 min in the dark. Cells were then mounted using Vectashield and observed with a fluorescence microscope.

2.8.2 Assessment of acrosomal integrity

To assess the acrosomal integrity of sperm, cells were labeled with fluorescein isothiocyanate-cinjugated peanut agglutinin (FITC-PNA) (0.1 mg/mL in PBS). 5 μl of FITC-PNA was added to a 100 μl sperm suspension and incubated at 37°C for 15 min in the dark. Cells were then washed in PBS and mounted with Vectashield medium and observed with a fluorescence microscope.

2.9 Bioinformatics

2.9.1 Gene ontology analysis.

All identified proteins were mapped to PANTHER molecular function categories, and compared to a reference list to statistically determine over- or under-representation of PANTHER classification categories. Each list was compared to the reference list

using the binomial test (Cho & Campbell, 2000) for each molecular function. www.pantherdb.org/

2.9.2 Tissue Expression

WebGestalt, a web-based Gene Set Analysis Toolkit, maintained by Vanderbilt University (http://bioinfo.vanderbilt.edu/webgestalt/option.php), was used to determine the pattern of EST tissue distribution for protein identifications. For each of the identified proteins, a virtual, *in silico* generated Tissue Northern Blot was created. The normalised expression distribution of each protein across a variety of tissues was determined using EST abundance data from the Unigene database.

2.9.3 Cellular location.

In the absence documentation in the literature, gene ontology (GO) annotations were used to describe the subcellular localization of each of the identified proteins (http://www.geneontology.org).

2.10 Anti-peptide Antibody Production

2.10.1 Design and preparation of the peptide antigen

2.10.1.1 B-cell epitope prediction

Peptide immunogens were selected based on their predicted antigenicity. For this purpose a bioinformatic analysis was performed to predict likely B-cell epitopes. Three web-based servers were employed:

(1) Bcepred. The server was used to predict linear B-cell epitopes based on the physico-chemical properties of amino acids known to correlate with B-cell epitopes.

Physico-chemical properties used were hydophilicity (Parker et al., 1986), flexibility (Karplus and Schulz, 1985), accessibility (Emini et al., 1985), polarity (Ponnuswamy et al., 1980) and exposed surface (Janin and Wodak, 1978). The server is accessible at www.imtech.res.in/raghava/bcepred.

(2) BetaTPred2. The server was used to predict the location of beta turns in proteins. The server is accessible at www.imtech.res.in/raghava/betatpred2/

(3) ABCpred. Tne ABCpred server was used as the ultimate validation to confirm the pridictions based on physio-chemical properties (BCEpred server) and the location of ß-turns (BetaTPred2 server).The ABCpred server predicts linear B-cell epitopes in a protein sequence using a recurrent neural network. The server is accessible at www.imtech.res.in/raghava/abcpred/

2.10.1.2 Peptide synthesis and conjugatuon

Peptide synthesis and conjugation was performed by Mimotopes Pty Ltd (Clayton, VIC, Australia). Peptides were synthesised by solid phase peptide synthesis (SPPS) utilizing Fmoc chemistry and purified by HPLC to a minimum of 75% homogeneity as determined by HPLC and mass spectrometry. Purified peptides were then conjugated individually to diphtheria toxoid (DT) via an N-terminal cysteine residue using the heterobifunctional reagent, 6'-maleimido-caproyl n-hydroxy succinimide (MCS).

2.10.2 Antibody production

Anti-peptide antibodies were produced in goats by Antibodies Australia (Werribee, Victoria, Australia) using their standard method. Individual DT-conjugated peptides were used to immunize three goats. Pre-immune serum was collected before the initial immunization. Subsequent injections were at 6, 10, 15, 18, 22, 25 and 30 weeks post-initial immunisation. Serum was collected at 10, 22, 25 and 30 weeks.

CHAPTER 3

PROFILING THE SURFACE PROTEOME OF MURINE SPERMATOZOA

3. Profiling the surface proteome of murine spermatozoa

3.1 Introduction

All cellular functions rely on the separation of the internal and external environments. The plasma membrane provides a physical boundary between the cell and its environment. However, the plasma membrane and the proteins therein, the plasma membrane proteome (PMP), are more than just a structure defining the limits or boundary of the cell. The PMP is defined as the entire protein composition of a particular cell types plasma membrane, under specific conditions, at a specific time (Aebersold and Goodlett, 2001; Wilkins et al., 1996). Proteins of the plasma membrane participate in many fundamental cellular processes, performing key biological functions. These include, but are not limited to, signal transduction, cell-to-cell recognition and adhesion, and the selective transport of molecules into and out of the cell (Leth-Larsen et al., 2010).

Genomic sequencing studies indicate that 20-30% of all open reading frames (ORFs) encode for integral membrane proteins (Wallin and von Heijne, 1998; Paulsen et al., 1998). Furthermore, proteins at the cell surface represent more than 30% of all proteins in the human genome (Wallin and von Heijne, 1998; Carre, 2011). Emphasising the importance of these proteins at the cell surface is the fact that transmembrane proteins and proteins anchored to the plasma membrane are a major focus for new protein drug targets (Hopkins and Groom, 2002; Stevens and Arkin, 2000). In addition, peptides and antibodies raised against proteins of the plasma membrane have become instrumental in diagnosis and clinical treatment (Bledi et al., 2003).

The PMP of sperm is not a static entity. After leaving the testis, its components undergo alteration and redistribution as a result of additional maturation in the epididymis (Sec. 1.2) and capacitation in the female reproductive tract (Sec. 1.3). In

mammalian reproduction, sperm sequentially encounter and interact with a number of different cell types and environments in the female reproductive tract prior to encountering the oocyte at fertilization. It is indeed through their surfaces that sperm interact with, and respond to, these different cells and environments.

Functionally mature sperm first encounter the ovulated oocyte complex in the oviduct where they must contend with the cumulus layer, a mixture of follicular cells embedded in a matrix of hyaluronic acid. Sperm penetrate the cumulus layer with the assistance of cell surface hyaluronidase (Talbot *et al*., 2003) and bind the zona pellucida (ZP) triggering the acrosome reaction which helps digest a path through this structure. Sperm interact with the zona through proteins localised to the plasma membrane of the anterior head (Bleil and Wassarman, 1986). The acrosome reaction results in the loss of much of the anterior sperm head via the fusion of the outer acrosomal membrane (OAM) and the plasma membrane. After penetrating the zona, sperm encounter the plasma membrane of the egg. This interaction is believed to take place at the equatorial region of the sperm head (Yanagimachi, 1994), and results in the fusion of the two plasma membranes. Sperm surface proteins are the key mediators of these sequential interactions between the gametes.

The critical importance of constituents of the sperm surface proteome is emphasized by several mouse lines engineered to lack sperm proteins which normally localise to the cell surface. In many cases, the loss of these plasma membrane proteins leads to a significant reduction, or complete lack of, fertility (Nishimura et al., 2001; Baba et al., 1994a; Baba et al., 2002; Cho et al., 1998; Shamsadin et al., 1999).

The aim of the research contained within this chapter was to profile the surface proteome of functionally mature murine spermatozoa taken from the cauda epididymis. The surface proteome is likely to contain many functionally important proteins, the identification of which will be critical to understanding the molecular basis of how the gametes recognise and interact with each other. In addition, such a resource will represent an invaluable reference tool for future comparative type proteome studies. Such studies comparing sperm at different stages of epididymal maturation, or capacitated versus non-capacitated cells will provide a means of identifying surface proteins that are altered during epididymal maturation and/or

capacitation, proteins likely to be key players in the molecular events that underpin the process of fertilisation.

3.2 Experimental rational

Methods were developed to isolate and purify a clean, viable and membrane intact population of murine spermatozoa from the cauda region of the epididymis. Sperm were gently isolated into a modified BWW preparation, by back-flushing of cauda epididymides (Sec. 2.3.4). Sperm preparations were then purified by differential centrifugation to remove contaminating cells (Sec. 2.4.3). Cell surface proteins were then labelled with biotin to facilitate purification from intracellular proteins by resuspending in media containing a membrane-impermeable biotinylation reagent (Sec. 2.4.3). Prior to biotin labeling, the membrane integrity and acrosomal status of the cells was assessed by propidium iodide (PI) (Sec. 2.8.1) and fluorescein-conjugated peanut lectin (FITC-PNA) (Sec. 2.8.2) staining. After cell lysis, biotinylated proteins were recovered from lysates using immobilised streptavidin and resolved by SDS-PAGE (Sec. 2.5.1).

In an attempt to achieve the best possible protein sequence coverage, we chose to perform straightforward one dimensional SDS-PAGE of the enriched membrane protein sample. The lack of elaborate biochemical purification procedures ensured that there was no discrimination against classes of proteins in the sample prior to MS analysis. This is in contrast to two dimensional gel procedures which tend to selectively loose hydrophobic, very acidic, and very basic proteins (Reinders et al., 2006)

One millimetre wide gel slices were excised from the gel and subjected to in-gel proteolytic digestion (Sec. 2.7.1). Digests were then analysed by reversed phase high performance liquid chromatography (HPLC) coupled to a tandem mass spectrometer (LC-MS/MS) (Sec. 2.7.4).

Our strategy for selective chemical tagging of cell surface proteins on intact, living cells, followed by high affinity enrichment and gel-based proteomic LC-MS/MS analysis of peptides derived from the tagged proteins is depicted in figure 3.1. The specific steps of this affinity-labeling strategy involved (1) gentle, covalent biotin

tagging of proteins on live cells using the membrane-impermeant biotinylation reagent Sulfo-NHS-LC-LC-Biotin, (2) cell lysis and (3) affinity enrichment of biotin labeled proteins, (4) resolution of isoloated proteins in SDS-PAGE gels, (5) in-gel proteolytic digestion of excised gel slices and subsequent peptide and protein identification by means of reversed phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

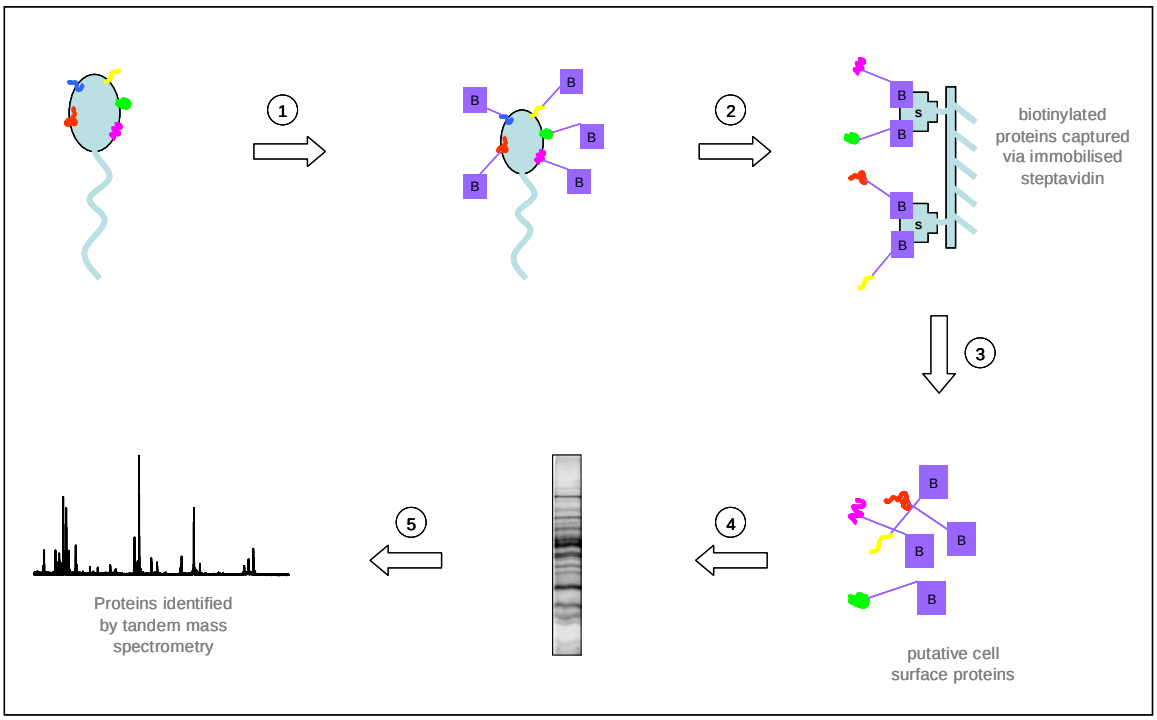


Figure 3.1. Profiling the cell surface proteome of murine spermatozoa. Identifying proteins localized to the surface of cells used a chemical tag to facilitate affinity purification of proteins and MS for identification. Sperm cell surface proteins were biotinylated with a membrane impermeant biotinylation reagent (1), labeled cells were then lysed and biotinylated proteins recovered from lysates using immobilized streptavidin (2), after extensive washing to remove unbound proteins, biotinylated proteins were eluted (3) and resolved by SDS-PAGE (4). After in gel digestion, putative cell surface proteins were identified by mass spectrometry and data base interrogation (5).

The reaction conditions were carefully optimized to maximize cell integrity and viability, as judged by propidium iodide and SYBR®14 uptake; and to minimize the

labeling of intracellular proteins, as determined by immunoblot analysis of β-tubulin in protein samples

All tandem mass spectra were searched against the mouse International Protein Index (IPI) database (Version 3.32) using the SEQUEST algotithm (Thermo Finigan San Jose, CA) to identify proteins. Only conventional tryptic peptides (allowing for two missed cleavages) displaying a cross-correlation score (Xcorr) of at least 2.0 and delta-correlation score (ΔCn) of at least 0.08 were considered for protein identification. Identified proteins were divided into two groups, protein hits based on a single peptide, group A; and those based on multiple peptides, group B. In addition, samples were analysed by western blot procedures for β-tubulin as an intra-cellular protein marker (Sec. 2.6.4).

Bioinformatic analysis of the identified proteins and the entire mouse genome were performed using several web-based algorithms and tools:

(1) Gene ontology analysis (Sec. 2.9.1). All identified proteins were mapped to PANTHER molecular function categories and compared to a reference list to statistically determine over- or under-represented PANTHER categories. Each list is compared to the reference list using the binomial test (Cho & Campbell, 2000) for each molecular function. www.pantherdb.org

(2) Tissue expression (Sec. 2.9.2)**.** WebGestalt, a web-based *Gene Set Analysis Toolkit*, maintained by Vanderbilt University, was used to determine the pattern of EST tissue distribution for protein identifications.
www.bioinfo.vanderbilt.edu/webgestalt/

For each of the identified proteins, a virtual, *in silico* generated tissue northern blot was created. The normalised expression distribution of each protein across a variety of tissues was determined using EST abundance data from the Unigene database. www.ncbi.nlm.nih.gov/unigene

(3) Cellular location (Sec. 2.9.3). Searches of the literature were used to determine the subcellular localization of each of the identified proteins. Where no document in the literature existed, gene ontology (GO) annotations were used to predict protein localizations. http://www.geneontology.org

3.3 Results

3.3.1 Sperm were acrosome intact prior to labeling of surface proteins.

Prior to studying the surface proteome of murine spermatozoa (Fig. 3.1), we sought to demonstrate that the cells were viable and acrosome intact prior to biotinylation of cell surface proteins. For this purpose, two populations of cells were used, one alive and one dead. Cells isolated from the cauda epididymis were divided into two and one half subjected to three rounds of freeze-thawing to provide a population of populations were then subjected to a triple staining protocol using fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA) in conjuction with a membrane-permeable nuclear stain, Hoechst-3342 (H342); and a viability stain, propidium iodide (PI) (Fig. 3.2). Live sperm were labeled with H342, but not with PI or FITC-PNA, i.e. $H342^{+}/PI^{-}/FITC\text{-}PNA^{-}$ (live sperm with intact acrosome). However, cells subjected to multiple rounds of freeze-thawing (dead sperm) were labeled with PI and exhibited increased FITC-PNA labeling compared to the alive cell, i.e. $H342^{+}/PI^{+}/FITC\text{-}PNA^{+}$.

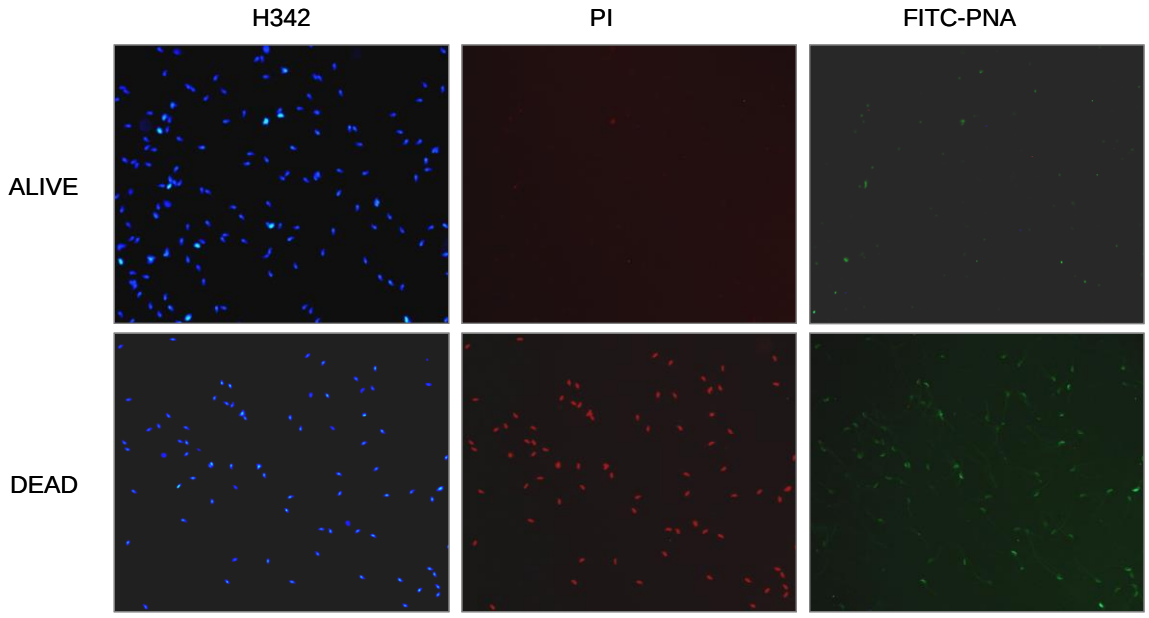


Figure 3.2. Assessment of viability and acrosomal integrity prior to surface labeling. Sperm were isolated from the caudal region of the epididymis and diluted into BWW to a final concentration of $6x10^6$ cells/ml. Cells were then stained by the addition of H342, PI and FITC-PNA to final concentrations of 2µg/ml, 8µg/ml and 5µg/ml respectively. After incubation at 37°C for 15 min in the dark, cells were washed and mounted with Vectashield mounting medium for observation by fluorescence microscopy.

3.3.2 Cells remained viable and membrane intact after biotinylation.

Next we sought to assess the affect of biotinylation on the viability of the cells. After biotinylating surface proteins as described (Sec. 2.4.3), cells were assessed for viability using a commercial kit from Molecular Probes®, LIVE/DEAD® Sperm Viability Kit (cat # L-7011) and fluorescence microscopy (Sec. 2.8.1). The kit employs two membrane-permeant nuclear dyes, the conventional dead cell stain propidium iodide (PI), and SYBR®14 which stains viable membrane intact cells. After biotinylation, cells were washed and resuspended in BWW media for viability staining. Cells were then mounted and observed with a fluorescence microscope.

Cells remained viable and membrane intact after biotinylation of cell surface proteins. Figure 3.3 indicates that more than 87% of sperm remain viable and membrane intact

as exhibited by SYBR®14 staining (Fig. 3.3B), with only a few cells showing PI staining (Fig. 3.3C).

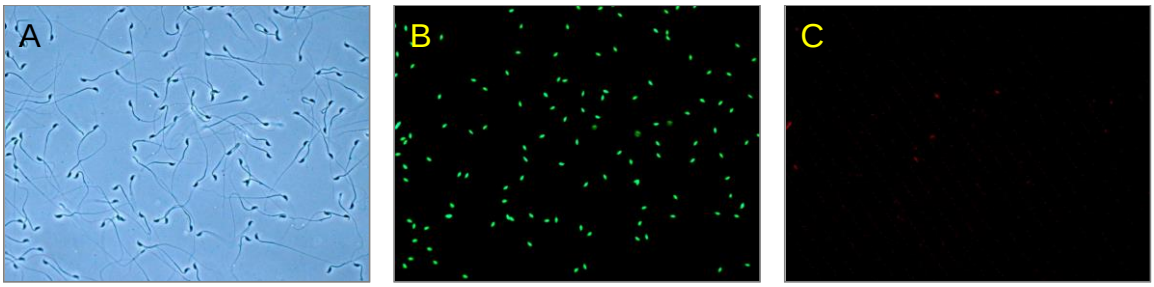


Figure 3.3. Assessment of the effect of biotinylation of membrane proteins on cell viability and membrane integrity. Epididymal sperm were biotinylated and then washed before being resuspended in 1ml of BWW media. SYBR 14 dye was added to a final concentration of 100 nM and the cells incubated for 10 min at 37°C. Propidium iodide was then added to a final concentration of 4.8 μM and cells incubated for an additional 10min in the dark. Cells were then mounted using Vectashield and observed with a fluorescence microscope. A) phase contract, B) SYBR®14, C) PI .

3.3.3 Biotin labeling procedure targets cell surface proteins.

As described in section 2.4.3 and depicted in figure 3.1, sperm surface proteins were labeled with a membrane impermeable biotinylation reagent and recovered from lysates with immobilised streptavidin. Putative surface proteins and proteins in the lysis pellet were resolved by SDS-PAGE and either visualised using CBB staining, or transferred to PVDF membrane for western analysis, (Fig. 3.4B). To confirm that the labeling protocol was specific for cell surface proteins, the presence of β-tubulin, an intracellular marker, was assayed for by western blot analysis with a β-tubulin specific antibody. One protein band consistent with the expected molecular weight of β-tubulin, 55 kDa, was detected in the cell lysis pellet but not in the purified biotinylated fractions (Fig. 3.4B). This data is consistent with labeling protocol being specific for proteins at the cell surface.

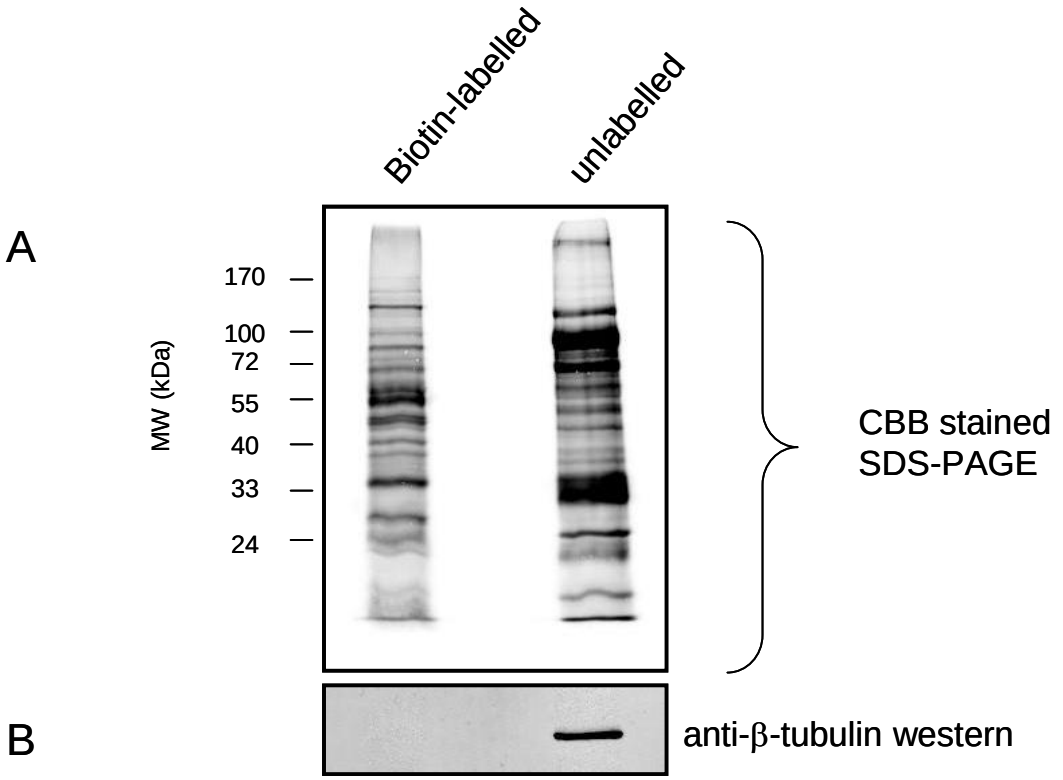


Figure 3.4. Resolution of putative cell surface proteins. Cell surface proteins on caudal epididymal sperm were labeled with a membrane-impermeant biotinylation reagent, washed and lysed in Buffer B. Biotinylated proteins were recovered from cell lysates using immobilised streptavidin and together with unlabelled proteins, resolved by SDS-PAGE. Gels were then stained with CBB or transferred to PVDF membrane for western analysis. (A) CBB stained gel of biotinylated and unlabelled proteins recovered from cell lysates, (B) biotinylated and unlabelled protein were immunoblotted with a β-tubulin specific antibody confirming that the biotinylation labeling was specific for proteins at the cell surface.

3.3.4 Identification of putative biotinylated cell surface proteins.

Following MS analysis of the membrane-enriched sample (sec. 2.7.4), we identified 186 gene products. For initial identification of proteins in the sample, only conventional tryptic peptides (allowing for two missed cleavages) displaying a cross-correlation score (Xcorr) of at least 2.0 and delta-correlation score (ΔCn) of at least 0.08 were considered (group A, appendix C, 186 protein identifications). A second filter requiring more than one peptide for identification culled the list to 32 proteins, group B (Table. 3.1).

Table 3.1. Putative sperm cell surface proteins identified by mass spectrometry (group B proteins identified from multiple peptides). Columns indicate IPI accession number (IPI reference), the protein name (Name) and gene symbol (Gene), the number of peptides identified by MS (Peptides), the percentage protein sequence coverage of the identified peptides (% sequence coverage), literature documentation of protein localization in mammalian sperm (sperm), reported protein localisation; or where none exists predictions of sub-cellular location taken from MGI gene ontology classifications (GOC) (Cellular location), literature references reporting expression and localization (Reference). Gene ontology predictions are inferred from direct assay (IDA), inferred from electronic annotation (IEA), or inferred from sequence orthology (ISO).

IPI reference	Protein name (gene)	Peptides	% Sequence coverage	Sperm	Cellular locaation	Reference
IPI00113347	Carnitine acetyltransferase (Crat)	2	4.14	yes	Mitochondria	Day-Francesconi and Casillas, 1982 Brooks, 1978
IPI00114342	Hexokinase 2 (Hk2)	2	2.51	yes	plasma membrane, Mitochondria	Baker et al., 2008a Travis et al., 2001b
IPI00114402	Sperm adhesion molecule 1 (Spam1)	2	8.01	yes	Plasma membrane, lipid rafts of head and midpiece	Griffiths et al., 2008 Sleight et al., 2005
IPI00116277	Chaperonin containing Tcp1, subunit 4 (Cct4)	3	7.79	yes	Plasma membrane, Cytoplasm (GOC-ISO), cytoskeleton (GOC-IEA), (lipid raft in other cells)	Chadwick et al., 2010 Baker et al., 2008a Redgrove et al., 2011
IPI00116283	Chaperonin containing Tcp1, subunit 3 (Cct3)	2	5.87	yes	Plasma membrane, protein complex, (lipid raft in other cells)	Chadwick et al., 2010 Baker et al., 2008a Redgrove et al., 2011
IPI00117857	Alpha-1-antitrypsin 1-3 (Serpina 1c)	2	9.95	no	Extracellular region (GOC-IDA)	Barbour et al., 2002
IPI00122684	Enolase 2 (Eno2)	2	9.84	no	Plasma membrane (GOC-IEA), Cytoplasm (GOC-IDA), (lipid raft in other cells)	Chadwick et al., 2010 Young et al., 1998
IPI00128144	A disintegrin and metallopeptidase domain 26A (Adam26a)	2	4.16	yes	Plasma membrane, head	Han et al.,2009
IPI00130391	Protease, serine, 1 (Prss1)	2	17.89	no	Extracellular space (GOC-ISO), (lipid raft in other cells)	Chadwick et al., 2010
IPI00131460	Aspartyl aminopeptidase (Dnpep)	2	8.88	yes	Cytoplasm (GOC-IEA)	Baker et al,. 2010
IPI00131695	Albumin (Alb1)	10	22.04	yes	Plasma membrane, lipid rafts	Sleight et al., 2005
IPI00133580	Fructose-bisphosphate aldolase (Aldoart2) OR Aldolase A, retroprotein 2	2	9.89	yes	Principal piece, anterior head	Arcelay et al., 2008 Baker et al,. 2010

IPI reference	Protein name (gene)	Peptides	% Sequence coverage	Sperm	Cellular locaation	Reference
IPI00134191	Solute carrier family 2 (facilitated glucose transporter), member 3 (Slc2a3)	4	10.55	yes	Plasma memebrane, lipid rafts, midpiece, principal piece	Miranda et al., 2009 Sleight et al., 2005 Urner and Sakkas, 1999
IPI00134476	Carbonic anhydrase 4 (Car4)	4	15.56	yes	Plasma membrane, lipid raft	Asano et al., 2009 Sleight et al., 2005 Ekstedt et al., 2004
IPI00137730	Phosphadtidylethanolamine-binding protein 1 (Pebp1)	3	17.65	yes	Plasma membrane	D'Amours et al., 2010 Nixon et al., 2006 Gibbons et al., 2005
IPI00153924	A disintegrin and metallopeptidase domain 1b (Adam1b)	2	5.83	yes	Plasma membrane, lipid raft	Stein et al., 2006 Nishimura et al., 2004 Kim et al., 2003
IPI00154054	Acetyl-Coenzyme A acetyltransferase 1 (Acat1)	2	8.25	no	Mitochondria (GOC-IDA), (lipid raft in other cells)	Chadwick et al., 2010 Mootha et al., 2003
IPI00224181	Aldo-keto reductase family 1, member B7 (Akr1b7)	2	9.49	yes	Plasma memebrane, lipid raft, peri-acrosomal	Nixon et al., 2009 Chapter 5
IPI00225945	Lactate dehydrogenase A-like 6B (Ldhal6b)	6	20.42	yes	Plasma membrane, lipid rafts	Sleight et al., 2005
IPI00228633	Glucose phosphate isomerase 1 (Gpi1)	3	7.35	yes	Plasma membrane (GOC-ISO), Cytoplasm (GOC-ISO)	Buehr and McLaren, 1981
IPI00276577	3-oxoacid CoA transferase 2B (Oxct2b)	8	20.00	yes	Midpiece, mitochondria	Koga et al., 2000
IPI00283611	Hexokinase 1 (Hk1)	13	17.35	yes	Plasma membrane, lipid rafts, peri-acrosomal, midpiece, principal piece	Nakamura et al., 2010 Miranda et al., 2009 Sleight et al., 2005

IPI reference	Protein name (gene)	Peptides	% Sequence coverage	Sperm	Cellular locaation	Reference
IPI00320217	Chaperonin containing Tcp1, subunit 2 (beta) (Cct2)	3	9.72	yes	Plasma membrane, protein complex, (lipid raft in other cells)	Baker et al., 2008a Chadwick et al., 2010 Redgrove et al., 2011
IPI00331174	Chaperonin containing Tcp1, subunit 7 (beta) (Cct7)	3	8.82	yes	Plasme membrane, Mitochondria (GOC-IDA)	Baker et al., 2008a Pagliarini et al., 2008 Redgrove et al., 2011
IPI00378485	ATPase, Na+/K+ transporting, alpha 4 polypeptide (Atp1a4)	6	8.53	yes	Plasma membrane, lipid raft, head, midpiece	Nixon et al., 2009 Stein et al., 2006
IPI00381611	A disintegrin and metallopeptidase domain 6B (Adam6b)	2	3.97	yes	Plasma memebrane, head	Stein et al., 2006
IPI00462072	Alpha enolase (Eno1)	3	11.75	yes	Principal piece	Jagan Mohanarao and Atreja, 2011 Khan et al., 2009 Gitlits et al., 2000
IPI00463589	ATPase, Ca++ transporting, plasma membrane 4 (Atp2b4)	3	4.10	yes	Plasma membrane, lipid raft, head, principal piece	Nixon et al., 2009 Stein et al., 2006 Okunade et al., 2004
IPI00467457	Lactate dehydrogenase C (Ldhc)	7	29.82	yes	Plasma membrane, lipid raft, head, midpiece, principal piece	Goldberg et al., 2010 Nixon et al., 2009 Sakai et al., 1987
IPI00625729	Keratin, type 2 cytoskeletal 1 (Krt1)	3	5.49	yes	Plasma membrane, lipid rafts	Sleight et al., 2005
IPI00755181	Keratin complex 1, acidic, gene 10 (Krt10)	3	6.06	no	keratin filament (GOC-IDA)	Kartasova et al., 1993
IPI00828258	Predicted gene 732 (Gm732)	2	17.31	no	Uncharacterized	

3.3.5 Bioinformatics

After applying stringent criteria for protein detection and identification we used several bioinformatics tools to gain an overview of the proteins identified.

Each of the identified proteins in group B (IDs based on multiple peptides) were analysed for known or predicted subcellular location. Literature searches were used to localise the proteins in sperm. Where no documentation of the protein in sperm existed in the literature, gene ontology (GO) annotations provided in the Mouse Genome Informatics (MGI) entry were used to describe the predicted subcellular localization of the protein. Of the proteins in table 3.1, 53% have been shown to be associated with the plasma membrane of sperm (Table 3.1). Interestingly twelve of these proteins have been described as constituents of lipid rafts in sperm while six others have been observed in lipid rafts from other cell types.

In order to facilitate a better understanding of the identified proteins, they were analysed using the PANTHER classification system (Protein ANalysis THrough Evolutionary Relationships) (http://www.pantherdb.org).

Using published scientific experimental evidence and evolutionary relationships to predict function in the absence of direct experimental evidence, the system classifies genes and proteins by their functions. The identified proteins, both Group A and Group B were classified according to molecular function (MF). In this kind of analysis it should be noted that one protein may fall into more than one category.

Figures 3.5 and 3.6 illustrate the results the PANTHER analysis of molecular function for Group B (multiple peptides) and Group A (single peptides) proteins respectively.

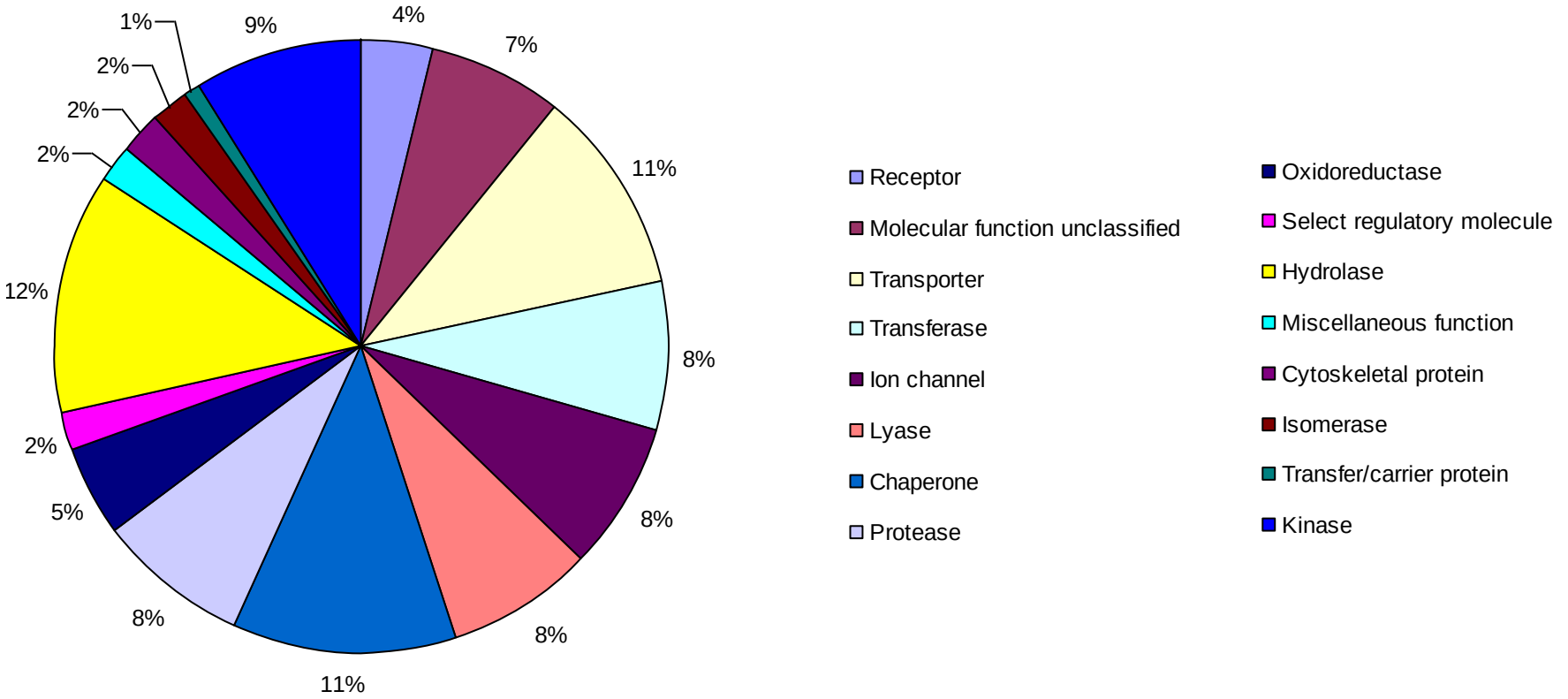


Figure 3.5. PANTHER analysis of the Molecular Function of identified proteins in group B.

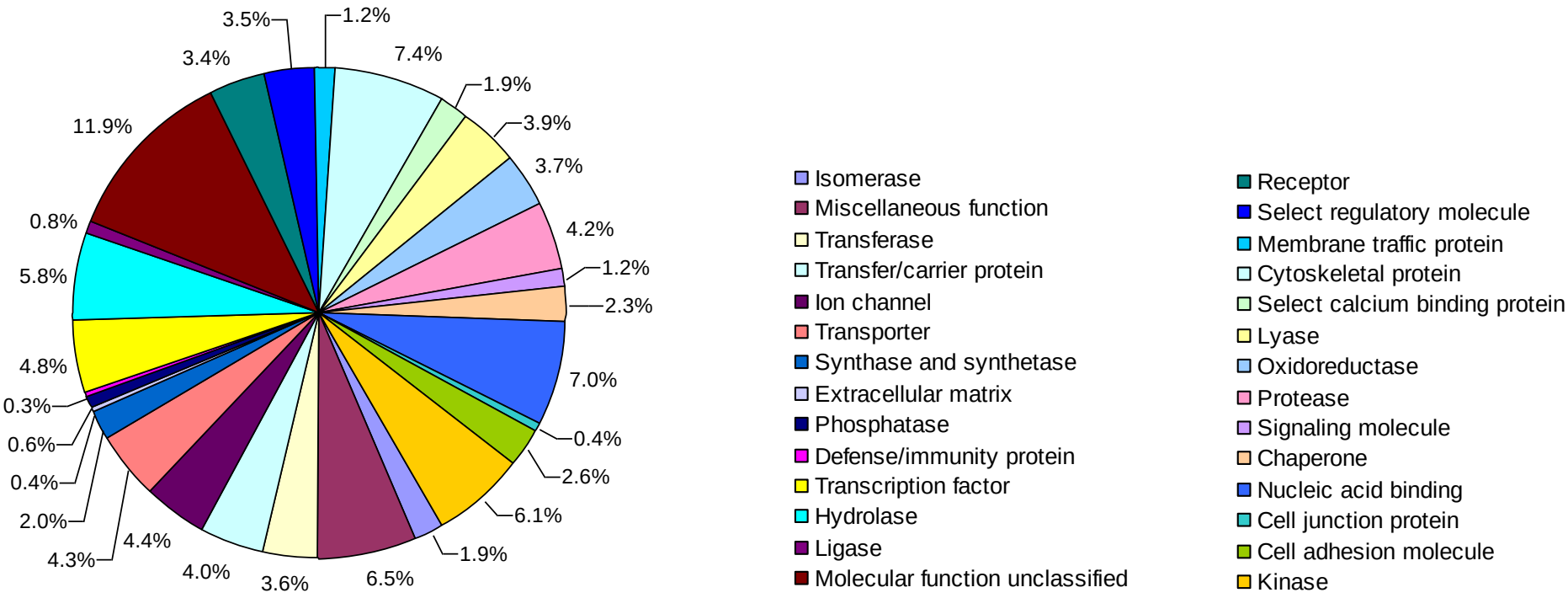


Figure 3.6. PANTHER analysis of the Molecular Function of identified proteins in group A.

Figures 3.5 and 3.6 present the PANTHER analysis for molecular function from Group B and Group A proteins respectively. In Group B, proteins were classified into 16 different categories for molecular function. 12 % are engaged in hydrolase activity, while 11 % were assigned as having a chaperone function and transport activity (Fig. 3.5). The major molecular function assigned to Group A proteins was cytoskeletal protein (7.4%). However, 11.9% of the identified protein in Group A were unclassified by PANTHER with respect to molecular function. (Fig. 3.6).

While the classification of genes or proteins into different ontology categories provides an insight into the function of the identified proteins, only a statistical comparison with the entire genome or proteome can give an indication as to whether certain classes of protein are significantly over- or under-represented in the results of an experiment. As such, a binomial statistics test (Cho & Campbell, 2000) was used to compare the classification of the group B proteins for molecular function to a reference list (NCBI *mus musculus* genes). Statistically significant ($P<0.05$) over- or under-represented classification categories were identified.

Figure 3.7. Functional analysis of Group B proteins. Genes were categorized in PANTHER molecular functions and compared to the NCBI *mus musculus* gene reference list. Each category is represented by two bars. The height of the yellow bar represents the percentage of group B proteins belonging to that category, while the green bar represents the percentage of proteins from the entire genome in each category. Statistically significant ($P<0.05$) over- or under-represented classification categories are highlighted. The percentage of gene list in the category is calculated as: # genes for the category/ # total genes in the list x 100.

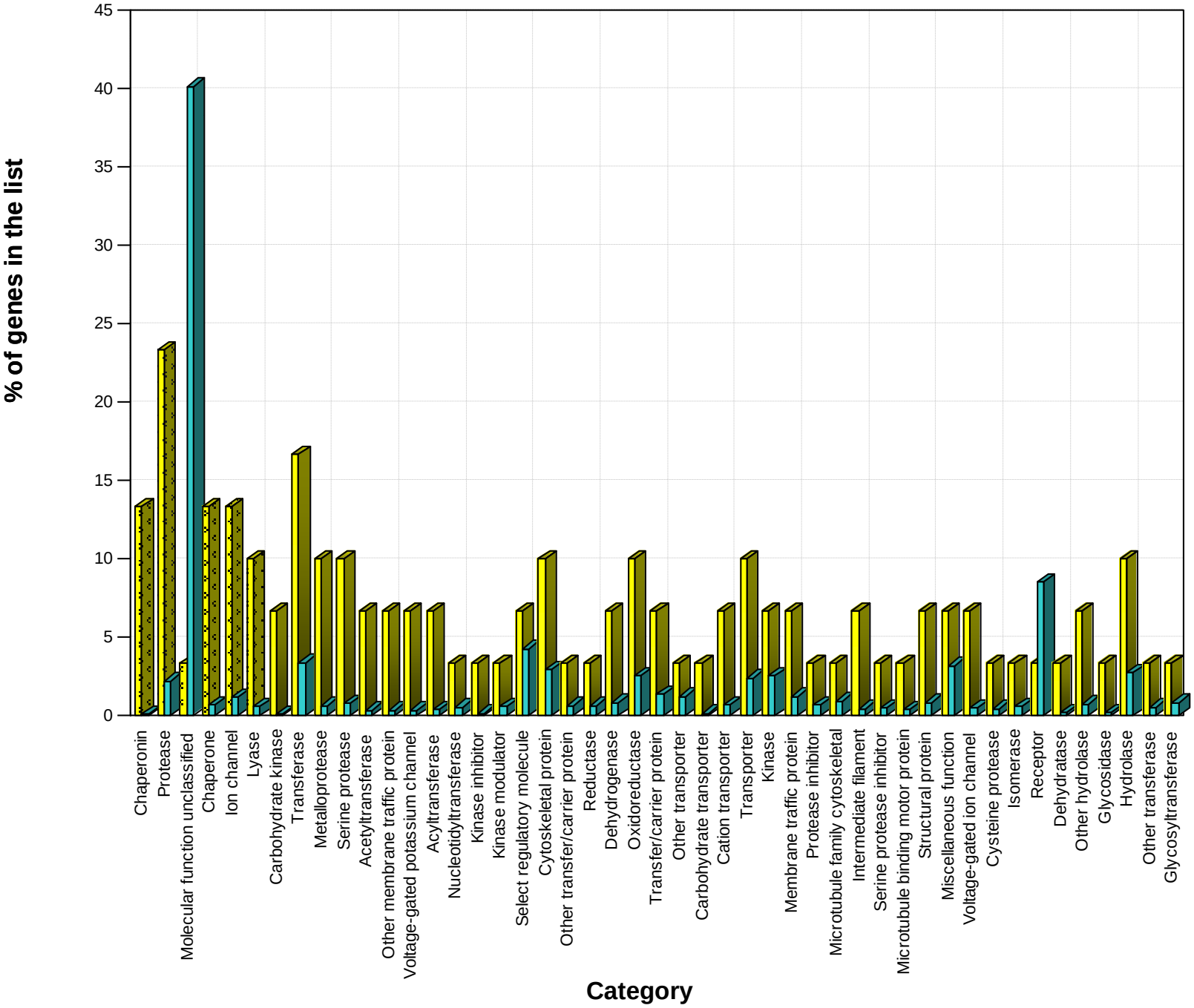

% of genes in the list
45
40
35
30
25
20
15
10
5
0
Chaperonin
Protease
Molecular function unclassified
Chaperone
Ion channel
Lyase
Carbohydrate kinase
Transferase
Metalloprotease
Serine protease
Acetyltransferase
Other membrane traffic protein
Voltage-gated potassium channel
Acyltransferase
Nucleotidyltransferase
Kinase inhibitor
Kinase modulator
Select regulatory molecule
Cytoskeletal protein
Other transfer/carrier protein
Reductase
Dehydrogenase
Oxidoreductase
Transfer/carrier protein
Other transporter
Carbohydrate transporter
Cation transporter
Transporter
Kinase
Membrane traffic protein
Protease inhibitor
Microtubule family cytoskeletal
Intermediate filament
Serine protease inhibitor
Microtubule binding motor protein
Structural protein
Miscellaneous function
Voltage-gated ion channel
Cysteine protease
Isomerase
Receptor
Dehydratase
Other hydrolase
Glycosidase
Hydrolase
Other transferase
Glycosyltransferase
Category

Figure 3.7 indicate that a number of PANTHER classification categories are over-represented in the Group B identifications. The only classification under-represented was the ‘unclassified’ category. Five categories were over represented: chaperonin, protease, chaperone, ion channel and lyase.

The normalized expressed sequence tag (EST) expression profiles of the identified proteins across a variety of tissues was determined using EST abundance data from the Unigene database. Bar charts indicating the top ten tissues possessing ESTs for the proteins in group B can be found in appendix D. Collated tissue expression profiles for the identified proteins are shown in Figures 3.8 and 3.9. for group B and A proteins respectively. Of the 186 proteins in Group A, 101 (54%) exhibit testis-expression (Fig. 3.9). In group B, 23 ESTs out of the 32 identified were in the testis (Fig. 3.8). This equates to 71% of the identified proteins with mRNA reported to be present in the testis.

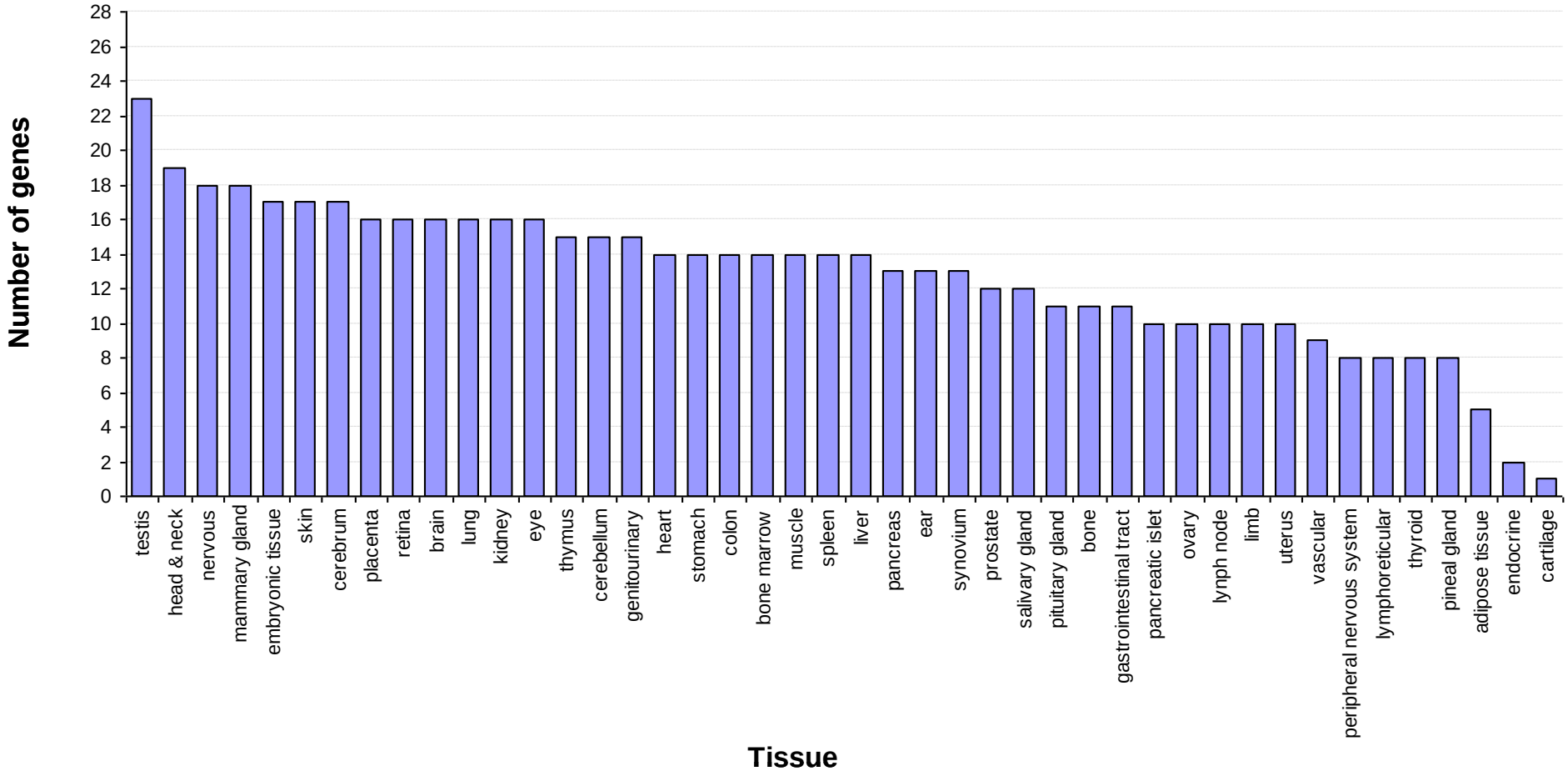


Figure 3.8. Tissue expression bar chart for proteins identified in group B. Each tissue is represented by a bar in the chart. The height of the bar represents the number of genes contained within the group B dataset that are expressed in the tissue.

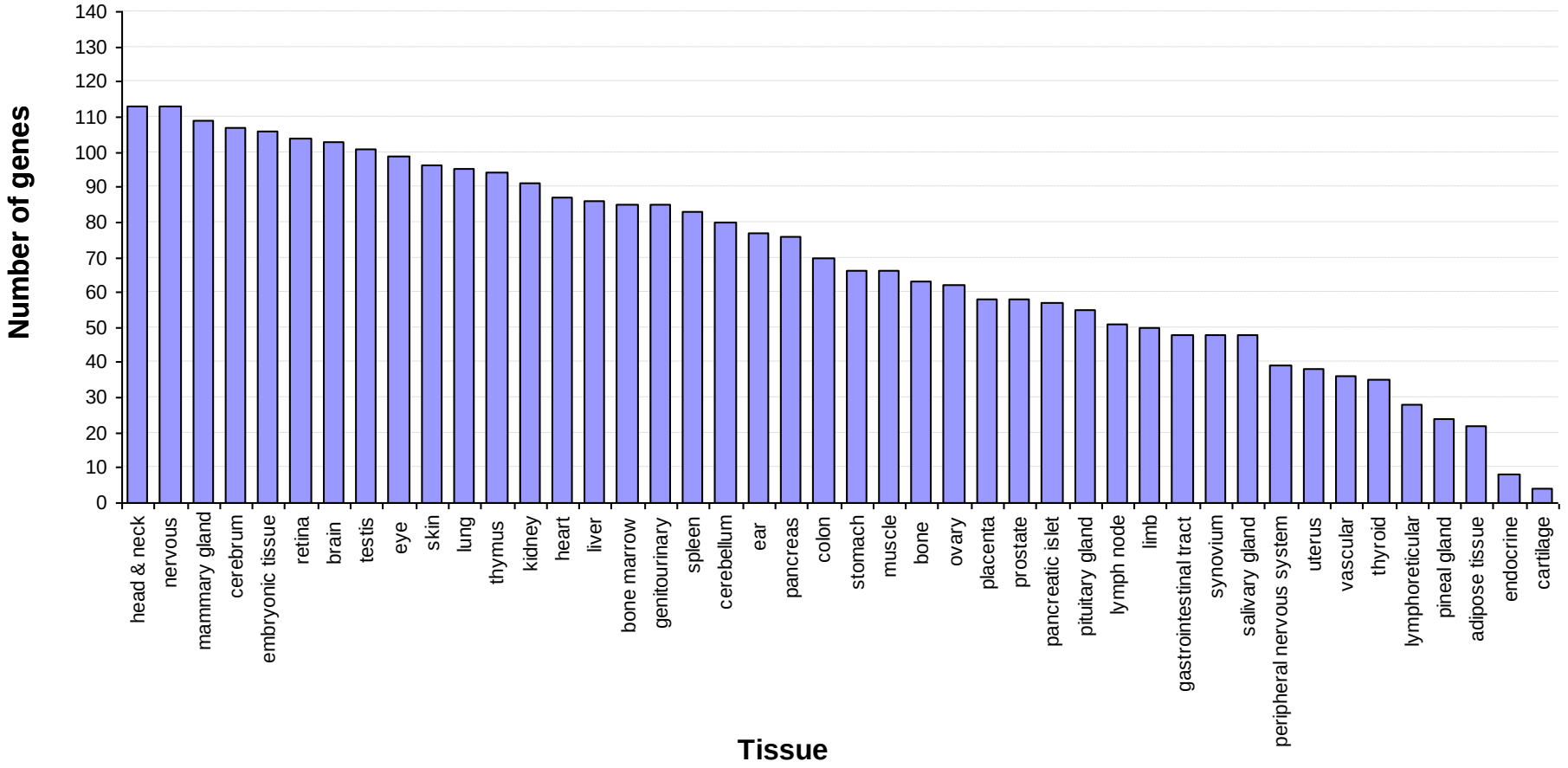


Figure 3.9. Tissue expression bar chart for proteins identified in group A. Each tissue is represented by a bar in the chart. The height of the bar represents the number of genes contained within the group A dataset that are expressed in the tissue.

3.4 Discussion

This study identified cell surface proteins of murine sperm that may be important for sperm function, particularly in regards to sperm-oocyte interactions. In total 186 proteins were identified, 32 of which were identified with a higher confidence from higher stringency filtering. These data will be of interest in future studies and provide an important reference point for comparative or differential studies. The results of this chapter could be used in studies comparing sperm at different stages of epididymal maturation, or the surface proteomes of capacitated versus non-capacitated cells. Further, the results herein may provide candidates for the development of new approaches to fertility control.

In addition, the results of this work complement the work detailed in the other chapters in this thesis which deal with one of the key issues remaining to be solved in mammalian fertilization, the mechanism by which sperm recognize and interact with the outer vestments of the oocyte. Resolving this issue is predicated on the identification of the complementary molecules on the sperm surface that orchestrate the binding of sperm to the zona pellucida.

The plasma membrane is not just merely an inert barrier between the contents of a cell and the external environment; it is a complex and dynamic structure which plays a critical role in fundamental biological processes (Leth-Larsen et al., 2010). The protein composition of the plasma membrane, the cell surface proteome, and its dynamic changes ultimately dictate how a cell responds to, and interacts with, its environment. Proteins embedded in the membrane possessing exposed, extracellular domains are crucial for inter-cellular communication, interaction with pathogens, bindings of chemical messengers and other signalling molecules, and response to environmental perturbations (von Heijne, 2007). Plasma membrane proteins confer specific cellular functions and are easily accessible. As such, they are often used as markers to classify cell and as potential drug targets (Hopkins and Groom, 2002).

The importance of plasma membrane proteins, the surface proteome, to drug discovery and development in the pharmaceutical industry is highlighted by the fact that approximately 66% of all drug targets are directed towards proteins of the plasma membrane (Hopkins and Groom, 2002). Furthermore, there is growing interest in the diagnostic and therapeutic potential of peptides and monoclonal antibodies targeted towards proteins uniquely localised at the surface of diseased cells and tissues. The surface proteome of a cell can be defined as the entire complement of plasma membrane proteins present in a cell under specific conditions at a specific time (Aebersold and Goodlett, 2001; Wilkins et al., 1996). Despite the important role cell surface proteins play, they are disproportionately under characterised biochemically, topographically and structurally (Cordwell and Thingholm, 2010). This is a consequence of their relatively high hydrophobic character, and their relative low abundance compared to other proteins present in the cell.

Historically, the identification of cell surface proteins by MS has been hindered by the difficulty in obtaining homogenous and highly enriched plasma membrane protein isolates, and the relative abundance of surface membrane proteins compared with cytoskeletal or cytosolic proteins (Josic and Clifton, 2007). In an attempt to overcome the specificity problem for membrane surface protein analysis, chemical tagging strategies have been employed in concert with subcellular fractionation.

Numerous studies have used different labels, to facilitate the localisation of membrane proteins on gels, and/or provide a means of affinity enrichment prior to gel analysis. Early investigations labelling surface molecules in sperm primarily employed enzyme-catalysed methods (Young and Goodman, 1980; Vierula and Rajaniemi, 1980; Kallajoki *et al*., 1986; Chatterjee and Majumder, 1989; Berger, 1990). In addition to enzyme-catalysed methods, Aitken and co-workers used a solid phase procedure, utilising chloroglycoluril, to label sperm surface proteins (Aitken *et al.*, 1987b). Other approaches used to selectively target cell surface proteins include the use of radioactivity (Naaby-Hansen, 1990), monitoring of selected peptides (Arnott et al., 2002), lectin-based methods (Kaji et al., 2006), cell surface shaving (Wu et al., 2003), two phase partitioning (Elortza et al., 2003), and antibody-mediated membrane enrichment (Watarai et al., 2005) strategies.

More recently, techniques exploiting the biotin-avidin system have regained popularity. This is primarily a reflection of the availability of modified labeling reagents which are reported to be membrane impermeable (Elia, 2008). In addition, these reagents are available with different chemistries permitting the targeting of several functional groups, or incorporate additional features which enhance their utility as surface labels. Biotinylation of lysine residues in the extracellular domains of plasma membrane proteins has been a popular approach to identify cell surface proteins (Nunomura et al., 2005; Rybak et al., 2005; Zhang et al., 2003). Affinity enrichment procedures using modified biotins have been employed to profile the cell surface proteome of a number of different cell types including, but not limited to, cancer cell lines (Shin *et al*., 2003), lymphocytes (Peirce *et al*., 2004), embryonic stem cells (Nunomura *et al*., 2005), and prostate epithelial cell lines (Hastie *et al*., 2005).

Fertilisation, one of the most important interactions between cells, involves signaling and recognition at cell surfaces. The role of the plasma membrane and its associated proteins, the surface proteome, in normal sperm function and the processes of fertilisation cannot be understated. It is through the repertoire of proteins at the cell surface that sperm interact with their surroundings in the male and female reproductive tracts on their journey from testis to oocyte. Proteins of the plasma membrane play important roles mediating many key molecular events during this journey which encompasses post-testicular maturation, binding to the zona pellucida, and induction of signaling cascades that culminate in the acrosome reaction.

Yet despite their importance in sperm physiology, the proteins of the plasma membrane have remained poorly characterized in the male gamete. Numerous studies have attempted to isolate the plasma membrane from sperm in several species (Millette et al., 1980; Millette and Bellve, 1980; Thomas et al., 1997), but stopped short of identifying or characterising the constituent proteins. Furthermore, one of the key issues remaining to be solved is the mechanism by which sperm recognize and interact with the outer vestments of the oocyte. Resolving this issue is predicated on the identification of the complementary molecules on the sperm surface that orchestrate the binding of sperm to the zona pellucida.

The present study was initiated to profile the surface proteome of mouse caudal epididymal spermatozoa. By resolving the composition of the sperm cell surface proteome we hoped to gain an insight into the crucial molecular events that regulate the interaction of the male and female gametes. It is anticipated that the results will be used as a reference for further comparative type studies comparing the surface proteome of capacitated sperm, and functionally incompetent sperm taken from different regions of the epididymis. Such studies may provide a means of identifying proteins that are key players in the post-testicular maturation of sperm during epididymal maturation and capacitation.

One of the goals of applying proteomic technologies to reproductive biology is to identify targets for possible contraceptive vaccination. By isolating and characterising proteins that are uniquely expressed at the cell surface, immunogenic, and testis-specific it may be possible to develop a contraceptive vaccine (Naz, 2011).

The pioneers of sperm proteomics were John Herr and colleagues who established a comprehensive human sperm protein database of 1400 spots (Naaby-Hansen et al., 1997). Later studies attempted to catalogue the repertoire of proteins exhibited on the surface of sperm. Studies by Shetty employed the biotin-avidin system in conjunction with differential protein extraction and two-dimensional polyacrylamide electrophoresis (2D-PAGE) to array surface proteins from human sperm (Shetty *et al*., 2001), however only a single protein was identified.

These studies have employed biotin labeling of surface molecules with a traditional 2D gel-based proteomics approach. This involves the resolution of proteins using 2D-PAGE followed by the identification of proteins by mass spectrometry (Santoni *et al*., 2000). However, the limitations of this approach for membrane proteins are well documented (Braun et al., 2007). The major hurdle is that of solubility. Generally, membrane proteins are hydrophobic and have an alkaline pH (Lehner *et al*., 2003) making them harder to solubilise and susceptible to precipitation during the first dimensional separation, isoelectric focusing (IEF) (Patton, 1999). Furthermore, the analysis of membrane proteins is further hampered by their low abundance in cells and the low copy numbers of individual constituents (Lopez *et al*., 2000).

Several approaches have attempted to address these problems, which render membrane proteins refractory to traditional methodologies. Most refinements have focused on sample preparation and involve the use of solubilisation buffers containing novel non-ionic and zwitterionic detergents (Carboni *et al*., 2002; Henningsen *et al*., 2002) prior to gel analysis. Other approaches have eliminated IEF altogether as membrane proteins become increasingly insoluble as they near their isoelectric point (pI) (Braun et al., 2007).

More recent work has focused on subcellular membrane fractionation as a means of enriching for protein at the cell surface. The isolation of lipid rafts, also referred to as microdomains or detergent resistant membranes (DRMs) in the literature, has become an increasingly popular means of studying the plasma membrane proteome. Lipid rafts are discrete lipid domains in the plasma membrane enriched in cholesterol and sphingolipids. In addition they have been shown to be areas where functionally important proteins are co-localized (Sprenger and Horrevoets, 2007).

Observations that capacitation causes the migration and coalescence of these microdomains at the apical region of the sperm head (Van Gestel et al., 2005; Boerke et al., 2008; Gadella et al., 2008) has led to the hypothesis that it is this process that enables zona binding and the zona-induced acrosome reaction to take place via the assembly of a zona pellucida binding protein complex (Gadella, 2008; Nixon et al., 2009). There have now been two proteomic studies targeting lipid rafts in murine sperm with 27 and 100 proteins identified, respectively (Sleight et al., 2005; Nixon et al., 2009). However, caution needs to be associated with all studies on lipid rafts. It has been suggested that lipid rafts on sperm contain not only surface membrane material, but also intracellular material (Brewis and Gadella, 2010). In addition, it is generally accepted that protocols used to isolate lipid rafts may not produce fractions that exactly mirror lipid rafts *in vivo* (Waugh and Hsuan, 2009).

To date, the most comprehensive study of the sperm plasma membrane proteome comes from Diana Myles' lab. Working on the mouse, Stein and co-workers used biotin labeling together with SDS-PAGE and MS to identify 171 putative sperm surface proteins, however, only those with a signal sequence or transmembrane domain (85 in total,) were further characterised (Stein *et al*., 2006).

In the current study we used a membrane-impermeant biotinylation reagent to selectively tag and facilitate the purification, of proteins present on the surface of sperm taken from the caudal region of the mouse epididiymis. Mass spectrometry subsequently identified 186 proteins (Group A), appendix C; 32 of which were based on more stringent filtering of identifications and thus have a higher level of confidence (Group B), Table 3.1.

To ensure that we selectively targeted proteins localized to the surface of spermatozoa, we first sought to ensure that isolated cell populations were viable and acrosome intact prior to biotinylation. Figure 3.2 demonstrates that prior to biotinylation the vast majority of sperm were viable and acrosome intact. We also demonstrated that the biotinylation procedure itself had no discernible effect on the viability of the cell population. The data in figure 3.3 indicates that after biotinylation the majority of cells were membrane intact and viable. Adding further confidence that the labeling protocol was specific for cell surface proteins, no β-tubulin, an intracellular marker protein, was detected in the purified biotinylated preparation by western blot analysis (Fig.3.4 B).

Each of the identified proteins in group B were analysed for subcellular location. Literature documentation and gene ontology (GO) annotations were used to describe the subcellular localization of each of the identified proteins in group B where 53% have been shown previously to be associated with the plasma membrane in sperm (Table 3.1). Twelve of these proteins have previously been shown to be constituents of lipid rafts in sperm, while six others have been observed in lipid rafts from other cell types.

The identified proteins, both Group A and Group B, were also classified according to molecular function using the PANTHER classification system (http://www.pantherdb.org). Figures 3.5 and 3.6 for group B and group A respectively, demonstrate that the major classifications for group B proteins were hydrolases, chaperones and transporters (Fig. 3.7). In contrast the major molecular function classification for group A proteins was “unclassified” (Fig. 3.8).

Hydrolases are enzymes catalyzing the hydrolysis of a variety of bonds, such as esters, glycosides, or peptides. Within this group of proteins, sperm adhesion molecule 1, Spam1, was contained. Previously known as hyluronidase, Spam1 is a GPI-anchored enzyme located on the sperm surface and inner acrosomal membrane. The protein enables sperm to penetrate through the hyaluronic acid-rich cumulus cell layer surrounding the oocyte (Talbot *et al*., 2003). Also classified as 'Hydrolase' was plasma membrane calcium-transporting ATPase 4 (ATP2B4). ATP2B4 belongs to a family of enzymes that remove bivalent calcium ions from eukaryotic cells against very large concentration gradients. As such it may play a critical role in intracellular calcium homeostasis (Tempel and Shilling, 2007).

Within the proteins classified as having chaperone function, four were subunits of the cytosolic chaperonin containing TCP1 (CCT) complex, namely subunits 2, 3, 4 and 7. In somatic cells, the TCP1 complex functions as a molecular chaperone, assisting the folding and assembly of proteins. The complex is comprised of two identical stacked rings, each containing eight unique protein subunits that surround a central cavity which provides an ideal environment for protein folding. Unfolded proteins enter the central cavity of the complex and are folded in an ATP-dependent manner. The TCP1 complex has been shown to play a role, *in vitro*, in the correct folding of the cytoskeletal proteins actin and tubulin (Brackley and Grantham, 2009). It has been proposed that TCP1 complexes may be involved in mediating the conformational changes in the cytoskeleton that occur during capacitation (Baker et al., 2008). Further, recent work has shown that the chaperonin containing TCP1 complex (CCT/TRiC) is involved in mediating sperm-oocyte interaction (Dun et al., 2011).

Consistent with the findings of the current study, Nixon et al. have identified several members of the TCP1 complex (CCT2, CCT6 and CCT8) on the surface of human sperm (Redgrove et al., 2011). Furthermore, in addition to the flagellum they were found to localize to the plasma membrane within the peri-acrosomal region of the sperm head, an ideal position for interaction with the zona pellucida. However, the failure of anti-CCT antibodies to inhibit sperm-ZP adhesion suggests that the complex is not direcly involved zona binding. Rather, as for HSPD1 and HSP90B1 in the current study, it has been proposed that the complex directs the assembly of a

functional zona receptor complex on the sperm surface. The characterization of TCP1 client proteins is the focus of ongoing work.

Certain classes of protein were significantly enriched in the membrane preparation, Enriched were (1) lyases: enzymes that catalyzes the breaking of various chemical bonds often forming a new double bond; (2) ion channels: proteins creating a highly selective transmembrane pore that presents a hydrophilic channel for specific ions to cross a lipid bilayer; (3) chaperonin and chaperone: proteins involved in protein assembly and (4) proteases: involved in modification and degradation (Figure 3.7).

The normalized EST expression profiles for the identified proteins was determined using EST abundance data from the Unigene database. Of the proteins in group B, three genes show EST expression restricted solely to the testis: a disintegrin and metallopeptidase domain 26A (*Adam26a*), sperm adhesion molecule 1 (*Spam1*), and a disintegrin and metallopeptidase domain 6B (*Adam6b*). Bar charts indicating the top ten tissues possessing ESTs for the proteins from group B can be found in appendix D.

It must be noted that a significant number of intracellular proteins were identified in this study. This indicates that the biotinylation labeling strategy and subsequent affinity purification scheme were not exclusively able to target proteins at the surface of the cell. It would appear that either a significant amount of sperm were membrane compromised at the time of biotin labeling, or that the biotinylation reagent was indeed membrane permeable and found its way into cells. Considering the data presented sections 3.3.4 and 3.3.5 which indicate that more than 87% of sperm remained viable and membrane intact after biotinylation, the latter scenario appears more likely. As such, the sample generated can only be regarded as a membrane-enriched sample and not a true preparation of plasma membrane proteins. Nevertheless, the list of identified proteins contains many proteins that warrant further investigation.

CHAPTER 4

ISOLATION AND LOCALISATION OF CHAPERONE INTERACTING PROTEINS DURING EPIDIDYMAL MATURATION, CAPACITATION AND THE ACROSOME REACTION: ALDOSE REDUCTASE AKR1B7

4. Isolation and Identification of proteins associated with chaperones on the surface of capacitated sperm.

4.1 Introduction

Following ejaculation, sperm require a period of residence in the female reproductive tract before they are functionally competent to fertilize the oocyte (Austin, 1951; Chang 1951). This phase of sperm maturation, termed "capacitation", is a complex multifunctional event that remains relatively poorly characterised. The mechanisms by which capacitation confers upon sperm the capacity to recognise and bind to the extracellular matrix surrounding the oocyte remain open to question. Additionally, despite decades of research and the proposition of many candidate molecules, the sperm surface receptor for the zona pellucida is yet to be unequivocally described (reviewed by Clark, 2011; McLeskey et al., 1998; Wassarman, 2009; Lyng and Shur, 2007; and Tanphaichitr et al., 2007)

Phosphorylation of sperm proteins on tyrosine residues has become an accepted correlate of mammalian sperm capacitation (Aitken et al., 1995; Visconti et al., 1995a; Visconti et al., 1995b). The significance of this cell signaling event with regard to sperm fertilising ability has, however, only recently begun to be appreciated. Asquith and colleagues demonstrated that in addition to the intracellular phosphorylation associated largely with the sperm tail, the presentation of tyrosine phosphoproteins on the surface of the murine sperm head occurs during capacitation. They suggested a causal relationship between this phosphorylation event and the capacity for zona pellucida adhesion (Asquith et al., 2004). Furthermore, two molecular chaperones, glucose-regulated protein (HSP90B1) and heat shock protein 60 (HSPD1), were both found to be tyrosine phosphorylated and localised to the surface of the mouse sperm head following capacitation (Asquith et al., 2004; Asquith et al., 2005).

Although tyrosine phosphorylated molecular chaperones may have a potential role in sperm-zona binding, evidence suggests that the specific moiety responsible for zona adhesion is not phosphorylated (Asquith et al., 2004).

The process of recognition and adhesion between the sperm and the zona pellucida has been proposed to be a multiphasic event comprising both high and low affinity interactions between multiple receptor molecules (Bleil and Wassarman, 1983; Thaler and Cardullo, 1996; Thaler and Cardullo, 2002). Asquith and colleagues have proposed that chaperone proteins form part of a receptor complex for the zona pellucida on the surface of sperm. Tyrosine phosphorylation of these chaperones during capacitation may activate the proteins and enable them to orchestrate the assembly of a functional receptor complex, initiate exposure of zona binding epitopes on the sperm surface and facilitate fertilisation.

Molecular chaperones are highly conserved, abundant proteins that facilitate the correct folding of proteins in the crowded intra-cellular environment (reviewed by Ellis, 2006; Bukau et al., 2000; Fink, 1999; Young et al., 2004). It has become progressively more apparent that chaperone activity, including HSPD1 and HSP90B1, is often the result of the co-ordinated assembly of several members of this protein family with specific cofactors to form a 'super-chaperone complex' (Nixon et al., 2009). In addition, HSP90B1 and HSPD1 have been shown to complex with a range of signal transduction proteins in somatic systems (Nishida et al., 1986; Silverstein et al., 1997; Zhang et al., 2004; Ziemiecki et al., 1986). Thus, it is possible that HSP90B1 and HSPD1 form part of a complex on the surface of sperm, combining with other chaperones, cofactors and signaling proteins, acting synergistically to mediate fertilisation. Germ cell-specific components within such a complex would be ideal targets for contraceptive targeting.

The aim of the current study was to identify and characterise chaperone binding proteins in mouse sperm, with a view to defining potential zona pellucida receptor candidates.

4.2 Experimental Rationale

Putative binding partners for the two molecular chaperones HSP90B1 and HSPD1 were investigated using co-immunoprecipitation (co-IP) coupled with a proteomics approach (Sec. 2.4.5) from lysates of capacitated mouse sperm. As such, experimental conditions most conducive to sperm capacitation were optimised. The capacitation-associated increase in the level of tyrosine phosphorylation was used to determine the optimal duration of capacitation.

Having established the conditions for capacitation to be used in preparing cells for immunoprecipitation experiments; a range of lysis buffers were tested for their suitability to co-IP experiments. The lysis buffers needed to be strong enough to liberate the two target chaperones from capacitated cells, but not so strong as to interfere with the protein-protein interactions in any multi-protein complexes or the subsequent antibody-antigen interactions. The presence of the chaperone proteins in cell lysates and immunoprecipitates was determined by western blot analysis (Sec. 2.6.4).

Co-IP of the chaperones and any associated proteins from lysates of capacitated cells was carried out using antibodies specific for HSP90B1 and HSPD1. In an attempt to limit the deficiencies associated with traditional co-IPs, the presence of the immunoprecipitating antibody in the purified material, antibodies used for precipitation were covalently immobilised to a solid support (Sec. 2.4.5); thus, allowing elution of only the target antigen (HSP90B1 and HSPD1) and any associated proteins. Two different supports with unique chemistries were trialed, an aldehyde-activated beaded agarose, and a tosyl-activated polystyrene paramagnetic bead support (Sec. 2.4.5). Both supports are designed to limit the amount of immunoprecipitating antibody contaminating the final purified protein sample.

In order to facilitate the purification of increased amounts of protein for identification, the amine reactive, water soluble, homobifunctional cross-linker 3,3´-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) was used in an attempt to stabilise and strengthen protein interactions prior to cell lysis and immunoprecipitation (Sec. 2.4.2).

Immunoprecipitated proteins were resolved by SDS-PAGE (Sec. 2.5.1) and visualised using either Coomassie brilliant blue (Sec. 2.6.1), sypro ruby (Sec. 2.6.2), or silver staining (Sec. 2.6.3) depending on the amount of protein available. Bands of interest were excised and subjected to in-gel proteolytic digestion (Sec. 2.7.1) followed by MS analysis. Tryptic digests were analysed by both matrix-assisted laser desorption ionisation - time of flight (MALDI-TOF) mass spectrometry, utilising a peptide mass fingerprinting approach (Sec. 2.7.3); and by tandem mass spectrometry incorporating liquid chromatography (LC-MS/MS) (Sec. 2.7.4). Despite several attempts, no conclusive protein identifications were made.

In light of repeated failures to identify any of the immunoprecipitated proteins with the gel-based approach, a gel-free method was considered. Instead of separating the isolated proteins by SDS-PAGE and digesting in gel, the proteins were digested in solution (Sec. 2.7.2) in what is commonly referred to as a 'shotgun' approach. The resultant digest, containing tryptic peptides from all of the immunoprecipitated proteins was analysed by LC-MS/MS. This solution-based approach afforded the identification of a number of proteins, several of which became the focus of further investigations.

4.3 Results

4.3.1 Determination of the time required to achieve maximal levels of tyrosine phosphorylation during capacitation.

Recent reports in the literature have suggested that the two molecular chaperones, HSPD1 and HSP90B1 are tyrosine phosphorylated during capacitation (Asquith et al., 2004). Moreover, it was hypothesized that the phosphorylation co-incided with the assembly or exposure of a functional receptor for the zona pellucida in the sperm plasma membrane.

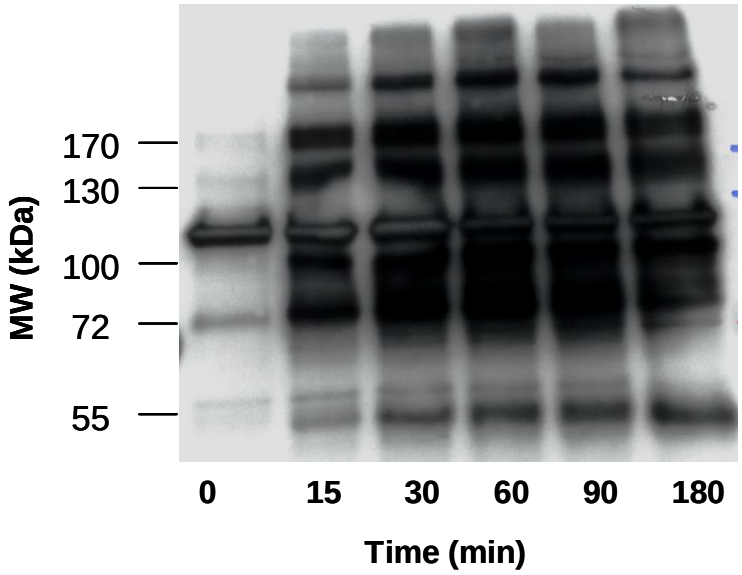


Figure 4.1. Determination of the optimal duration of capacitation. Murine spermatozoa taken from the cauda epididymis were capacitated for different periods of time in complete BWW media supplemented with 1mM dbcAMP and 1mM pentoxyfyline. After capacitation, cells were washed three times and lysed. 5µg of each sample was then analysed by western blotting using an anti-phosphotyrosine immunoglobulin.

The increased level of protein tyrosine phosphorylation associated with the capacitation of spermatozoa was used as an indicator of capacitation status. Sperm taken from the murine cauda epididymis were incubated in capacitation promoting media (Sec. 2.3.5)

for 0 to 180 minutes. Following incubation, cells were washed and lysed. An equivalent amount of protein from each incubation (5µg), was resolved by SDS-PAGE and transferred to PVDF membrane for western blot analysis. Tyrosine phosphorylated proteins were detected by immunoblotting with a phosphotyrosine-specific antibody.

A representative western blot showing the capacitation-associated increased level of protein tyrosine phosphorylation is shown in figure 4.1. The immunoblot indicates that only one protein (MW 116 kDa) was tyrosine phosphorylated at the zero time point. This protein is the constitutively tyrosine phosphorylated hexokinase that has been identified previously in mouse spermatozoa (Kalab et al., 1994). Tyrosine phosphorylation of a range of proteins (MW 50-200 kDa) increased during the 180 min incubation. Figure 4.1 also demonstrates that maximal levels of tyrosine phosphorylation are achieved by 90 minutes.

4.3.2 Optimisation of lysis conditions to be used for co-IP of chaperone-associated proteins.

Initially, three lysis buffers were tested for their ability to liberate the target chaperone proteins from capacitated cells, and for their suitability in immunoprecipitation. The buffers trialed were :

Buffer A	Buffer B	Buffer C
150 mM NaCl 20mM Tris pH7.4 0.1% Triton x-100	150mM NaCl 20mM Tris pH7.4 1% Triton x-100 0.5% NP40	M-Per lysis buffer from Pierce

Caudal sperm were capacitated for 90 min in complete BWW media. Cells were then washed three times in incomplete BWW/PVA media prior to being split three ways for lysis. The lysates generated with each buffer were used for immunoprecipitation with an anti-HSP90B1 immunoglobulin. The presence of HSP90B1 in each immunoprecipitate was determined by western blotting. Of the three lysis buffers, "Buffer B" was found to facilitate the purification of the greatest amount of HSP90B1 chaperone protein (data not shown). Buffer B was then compared to a modified RIPA buffer in the same way.

RIPA Buffer
150mM NaCl
50mM Tris pH7.4
1.0% NP40
0.5% NaDOC
0.1% SDS

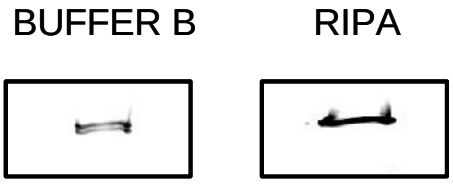


Figure 4.2. Lysis buffer trial for co-immunoprecipitation .
Mouse sperm were incubated for 90 minutes in complete BWW. Cells were then washed in BWW/PVA three times and split two ways prior to solubilisation in buffers B and RIPA buffer. Insoluble material was removed by centrifugation and lysates immunoprecipitated with a monoclonal HSP90B1 immunoglobulin. The level of HSP90B1 in the Immunoprecipitates was determined by western blot analysis.

Figure 4.2 indicated that the RIPA buffer facilitated the purification of more chaperone protein than did Buffer B. Based on this, the RIPA buffer was used for subsequent immunoprecipitation experiments.

4.3.3 Determination of HSP90B1- and HSPD1-associated proteins in capacitated sperm by co-IP.

4.3.3.1 Co-IP utilising RIPA buffer as the lysis buffer.

The two molecular chaperones HSP90B1 and HSPD1 and their associated proteins were immunoprecipitated from RIPA buffer lysates of capacitated cells with chaperone-specific antibodies. Previous experiments had demonstrated that the modified RIPA buffer was of sufficient solubilising strength to liberate both protein from sperm but did not hinder the subsequent antibody-antigen interaction critical to the immunoprecipitation.

The immunoprecipitated proteins were resolved by SDS-PAGE and visualised by silver staining for total protein content (Fig. 4.3A), or transferred to PVDF for western analysis using chaperone-specific antibodies (Fig. 4.3B). The silver stained gels in figure 4.3A indicated that one major protein, consistent with the predicted molecular weight of each chaperone; and a number of minor protein were immunoprecipitated in each case. Western analysis of the immunoprecipitates confirmed that the chaperone antigen was indeed precipitated in each case (Fig. 4.3B). Overlay analysis revealed that the major band in each of the stained gels was the target chaperone protein.

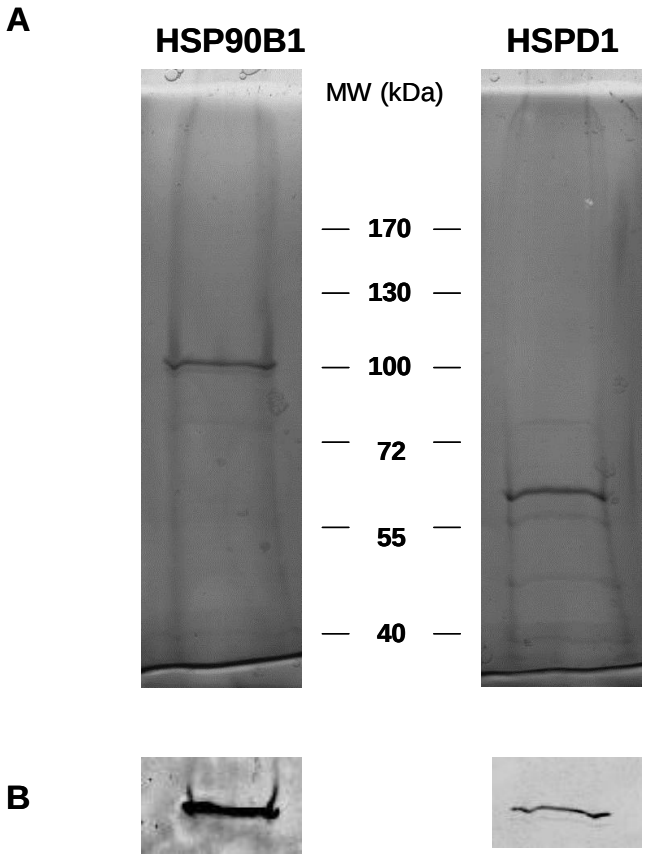


Figure 4.3. Co-immunoprecipitation of chaperone-associated proteins from capacitated sperm using a modified RIPA buffer. Caudal epididymal spermatozoa were incubated for 90 min in complete BWW, washed and lysed in a modified RIPA buffer. Lysates were immunoprecipitated with anti-HSP90B1 or anti-HSPD1 immunoglobulin. Immunoprecipitated proteins were resolved by SDS-PAGE. A) Total protein was visualised by silver staining. B) Immunoprecipitates were immunoblotted with chaperone antibodies to confirm that the precipitation was efficacious.

Taking into account the faintness of the minor bands and the fact that the gels were stained using a traditional silver staining protocol, which has a detection limit of 1-2 nanogram; it was deemed that there was not enough protein in any of the minor bands to afford identification of these putative chaperone-associated proteins by mass spectrometry.

4.3.3.2 Co-IP following a cross-linker to stabilize protein interactions

In an attempt to improve the efficiency of the co-immunoprecipitations and precipitate increased amounts of the chaperone-associated proteins, the chemical crosslinker 3,3´-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) was trialed for its ability to strengthen and stabilise the interactions of each chaperones with any binding partners prior to cell lysis.

After capacitation, but prior to cell lysis, sperm were incubated in phosphate buffered saline (PBS) containing 1mM of the cross-linker for 30 min at room temperature. After washing, cells were lysed in the modified RIPA buffer and clarified lysates used for immunoprecipitation with chaperone-specific antibodies. The immunoprecipitaed proteins were again analysed by SDS-PAGE and western blotting (Fig. 4.4). Western analysis confirmed that the target chaperones were pulled down in each case (Fig. 4.4B). However, the use of the crosslinker appeared to have little or no effect on the amount of other proteins co-purifying with the two chaperones (Fig 4.4A).

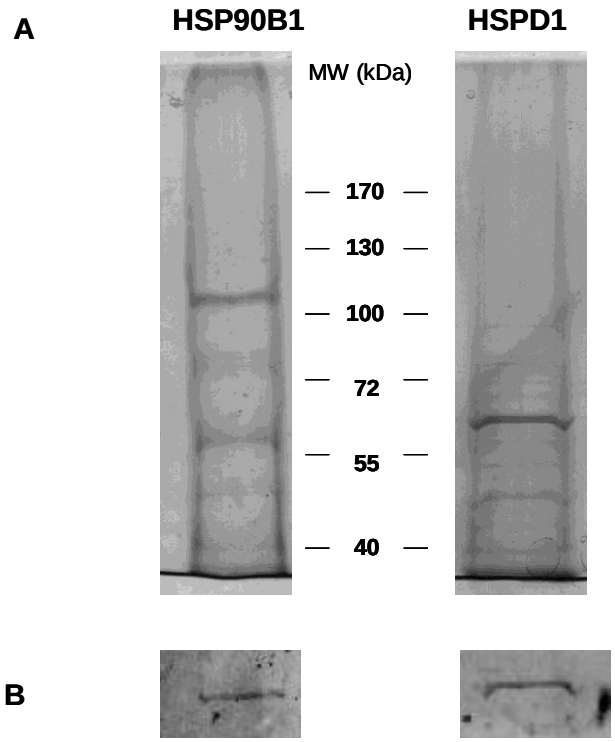


Figure 4.4. Co-IP of chaperone associated proteins from capacitated sperm using the chemical cross-linker DTSSP to stabilise protein complexes prior to cell lysis.
Caudal epididymal spermatozoa were incubated for 90 min in complete BWW, washed and then resuspended in PBS containing 1mM DTSSP for 30 min at room temperature. After washing, cells were lysed in a modified RIPA buffer. Lysates were immunoprecipitated with anti-HSP90B1 or anti-HSPD1 immunoglobulin. Immunoprecipitated proteins were resolved by SDS-PAGE. A) Total protein was visualised by silver staining. B) Immunoprecipitates were immunoblotted with anti-chaperone immunoglobulins to confirm that the precipitation was efficacious.

Notwithstanding, the limited amount of protein afforded by the crosslinking, identical samples to those depicted in figure 4.4 were run into one dimensional gels and stained with Sypro Ruby (SR). Sypro Ruby is a fluorescent stain with sensitivity approaching that of silver staining, but more importantly is compatible with downstream digestion and mass spectrometry protocols (Ball and Karuso, 2007). Protein bands were excised from SR stained gels and subjected to in-gel proteolysis with trypsin. Resultant digests were analysed by both MALDI-TOF mass spectrometry and LC-MS/MS, however no conclusive identifications were made.

In light of these results the use of the modified RIPA buffer as the lysis buffer was abandoned. Buffer B was employed as the lysis and immunoprecipitation buffer for all subsequent experiments. It was anticipated that the milder buffer would be more conducive to the preservation of protein interactions during lysis and immunoprecipitation.

4.3.3.3 Comparison of two different solid phase supports for immobilisation of antibodies utilised in imunoprecipitation.

Another variable known to affect the success of immunoprecipitation experiments of this type is the nature of the solid phase used to immobilise the immunoprecipitating antibodies (Kaboord and Perr, 2008). Two supports were tested with the same anti-HSP90B1 immunoglobulin for their ability to influence the amount of the proteins immunoprecipitated. The two supports used to immobilise the antibody were an aldehyde-activated beaded agarose (AminoLink® Plus Coupling Gel from Pierce, product number: 23600), and a tosyl-activated polystyrene paramagnetic bead (Dynabeads® M-280 tosyl-activated superparamagnetic polystyrene beads from Dynal Biotech, product number: 142.03). Both supports were designed to limit the amount of immunoprecipitating antibody contaminating the final purified protein sample.

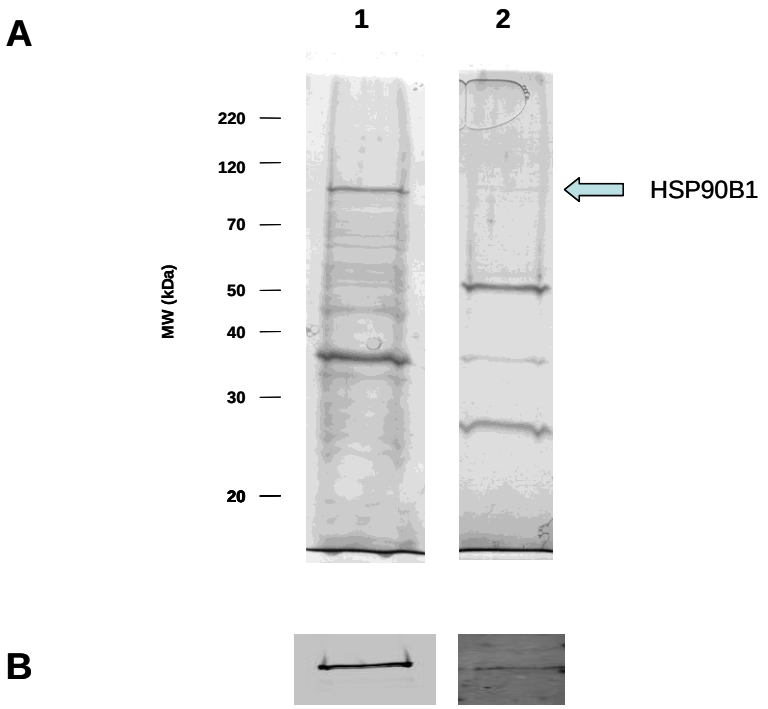


Figure 4.5 Comparison of two different solid phase supports used for immobilising antibodies in preparation for immunoprecipitation. Caudal spermatozoa were incubated for 90 min in complete BWW, washed and lysed in Buffer B. Lysates were immunoprecipitated with anti-HSP90B1 immunoglobulin covalently immobilised to (1) a beaded agarose support, and (2) a polystyrene paramagnetic bead support. Immunoprecipitated proteins were resolved by SDS-PAGE. (A) Total protein was visualised by silver staining. (B) Immunoprecipitates were immunoblotted with anti-HSP90B1 immunoglobulin to confirm the precipitation.

Both supports were used to immobilise the same amount of antibody, according to the manufacturer's instructions. Figure 4.5 indicated that the nature of the solid phase support used for immobilising antibodies did indeed influence the success of the immunoprecipitation reaction. In comparison to the magnetic bead support (Fig. 4.5, lane 2), the beaded agarose support (Fig 4.5, lane 1) facilitated the purification of significantly more protein as determined by the number and intensity of the bands detected in the silver stained gel. In addition, the beaded agarose enabled the capture and purification of significantly more of the target antigen as determined by western blot analysis using anti-HSP90B1 immunoglobulin (Fig.4.5, B).

Having tested and optimised several experimental parameters affecting the yield and fidelity of the co-IP process, experiments were scaled up to maximise the amount of

protein available for proteolytic digestion and mass spectrometric analysis. Up to 12 mice (24 epididymides) were used to harvest sperm for each immunoprecipitation reaction. Cells were capacitated in complete BWW media for 90 min and then washed several times with incomplete media to remove the exogenous bovine serum albumin (BSA) from the complete media formulation. Cells were lysed in cold lysis buffer containing protease and phosphatase inhibitors for 60 min at 4°C. Insoluble material was removed by high speed centrifugation and the lysate immunoprecipitated with either immobilised anti-HSP90B1 or anti-HSPD1 immunoglobulin. An equal amount of solid phase support devoid of the immunoprecipitating antibody was used as a negative control. Immunoprecipitated proteins were resolved by SDS-PAGE and visualised by silver or sypro ruby staining or transferred to polyvinylidene fluoride (PVDF) for western blot analysis.

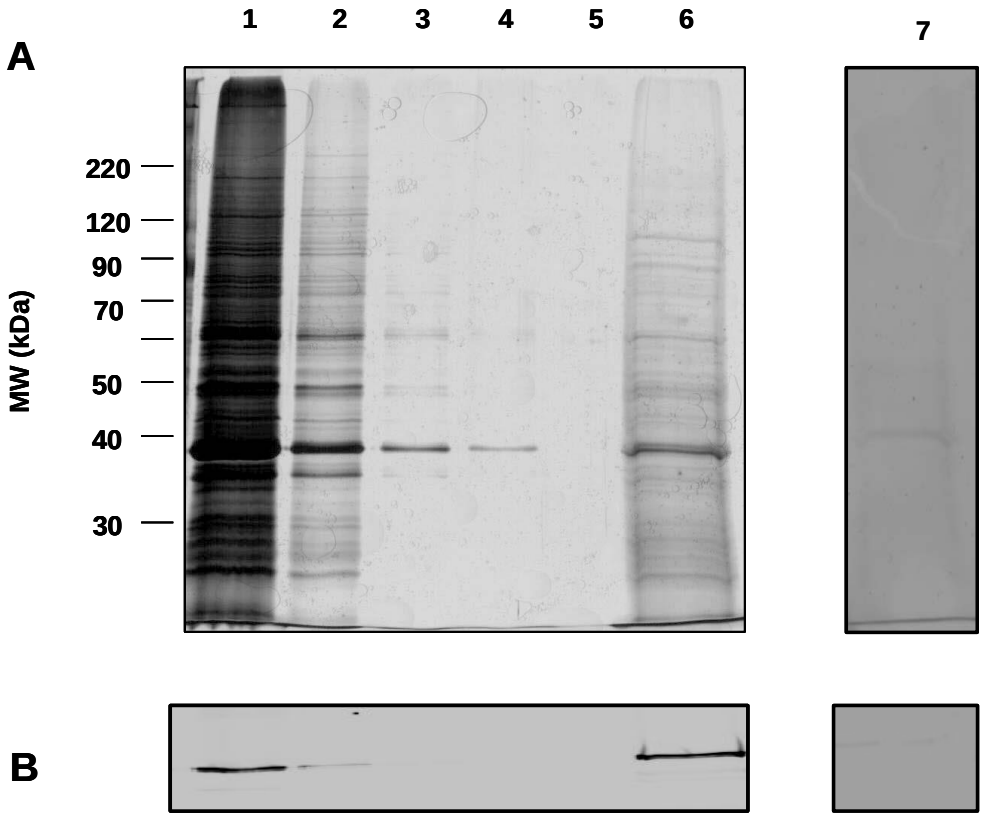


Figure 4.6. Co-IP of HSP90B1 and associated proteins using an immobilised anti-HSP90B1 immunoglobulin. Caudal spermatozoa were incubated for 90 min in complete BWW, washed and lysed in Buffer B buffer. Clarified lysates were immunoprecipitated with anti-HSP90B1 immunoglobulin or with solid phase support devoid of antibody. Immunoprecipitated proteins were resolved by SDS-PAGE. (1) capacitated sperm lysate, (2-4) successive washes of the captured antigen complex, (6) immunoprecipited proteins, (7) precipitated proteins from solid phase support devoid of antibody. (A) Resolved proteins were visualised by silver staining. B) Immunoprecipitates were immunoblotted with anti-HSP90B1 immunoglobulins to confirm that the precipitation was efficacious.

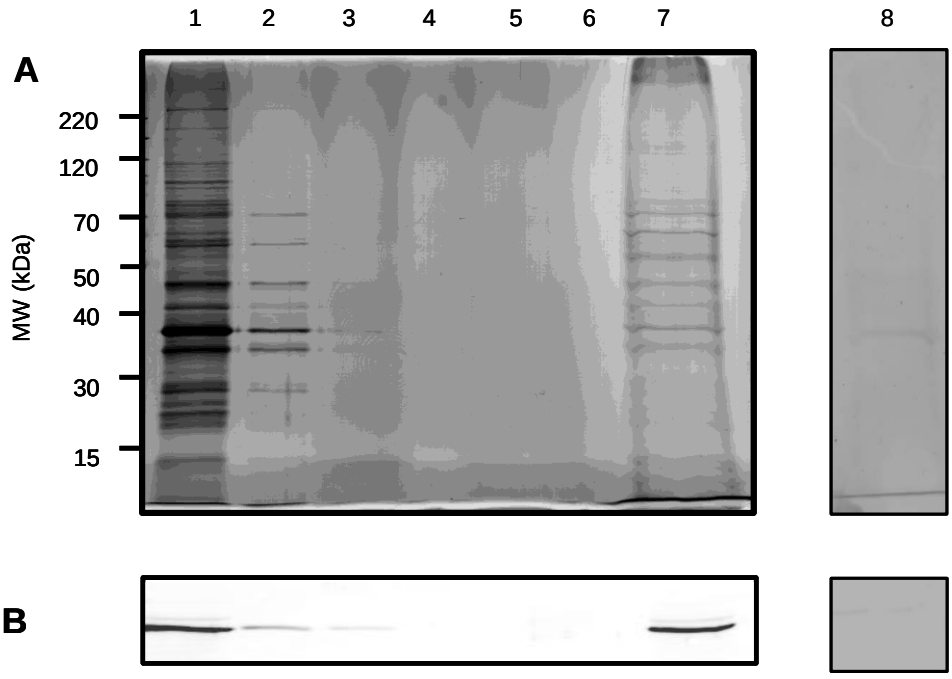


Figure 4.7. Co-IP of HSPD1 and associated proteins using an immobilised anti-HSPD1 immunoglobulin. Caudal spermatozoa were incubated for 90min in complete BWW, washed and lysed in Buffer B buffer. Clarified lysates were immunoprecipitated with immobilised anti-HSPD1 immunoglobulin or with solid phase support devoid of antibody. Immunoprecipitated proteins were resolved by SDS-PAGE. (1) capacitated sperm lysate, (2-4) successive washes of the captured antigen complex, (7) immunoprecipited proteins, (8) precipitated proteins from solid phase support devoid of antibody. (A) Resolved proteins were visualised by silver staining. B) Immunoprecipitates were immunoblotted with anti-HSPD1 immunoglobulins to confirm that the precipitation was efficacious.

Figure 4.6B showed that the co-IP protocol was successful in that it resulted in the purification of the target protein, HSP90B1, from lysates of capacitated sperm (Fig 4.6B, lane 6). More importantly, a number of proteins co-purifying with the chaperone were detected in the silver stained gel (Fig 4.6A, lane 6). Lane 7 in figure 4.6 showed that no HSP90B1, and very little protein in general, was precipitated by the control beads lacking antibody.

Similarly, Figure 4.7 demonstrated that the protocol was efficient in purifying HSPD1 from lysates of capacitated sperm (Fig. 4.7B, lane 7). Again a number of proteins co-purified with the chaperone were detected in the polyacrylamide gel (Fig. 4.7A, lane 7).

Identical gels to those shown in figures 4.6A and 4.7A were stained with sypro ruby. Protein bands co-purifying with the two chaperone proteins were excised and subjected to in-gel proteolysis using trypsin. The resultant digests were analysed by both MALDI-TOF mass spectrometry and liquid chromatography tandem mass spectrometry (LC-MS/MS). No conclusive identifications were made.

As none of the protein co-purifying with the two chaperones could be identified using the traditional approach of resolving proteins in SDS-PAGE gels prior to in-gel proteolytic digestion and mass spectrometric analysis, a gel-free shotgun approach was implemented. Instead of being resolved in gels, the immunoprecipitated proteins were digested in solution. The resultant tryptic digest was analysed by LC-MS/MS. This approach afforded the positive identification of a number of proteins co-purifying with HSPD1. Of the putative HSPD1 binding proteins identified: heat shock 10kD protein 1 (HSPE1, formally known as HSP10), an aldose reductase (AKR1B7), citron kinase (CRIK) and the proacrosin binding protein (ACRBP) were considered of high confidence and became the focus of subsequent work (Table 4.1). None of the proteins in the HSP90B1 immunoprecipitation were able to be identified.

The identifications were made by interrogating the MSDB protein database via the web-based MASCOT MS/MS ion search algorithm maintained by Matrix Science (http://www.matrixscience.com). MSDB is a non-redundant protein sequence database maintained by the Proteomics Department at the Hammersmith Campus of Imperial College London. MSDB is designed specifically for mass spectrometry applications. A mass tolerance of 50 ppm and one incomplete cleavage were allowed. Acetylation of the N-terminus, alkylation of cysteine by carbamidomethylation, and oxidation of methionine were considered as possible modifications. The criteria used to accept identifications included the extent of sequence coverage of the theoretical sequences matched within a mass accuracy of 50 ppm, the number of peptides matched, and the probabilistic score (MOWSE score), as indicated from each peptide in Table 4.1. MOWSE scores greater than 40 were deemed significant by the search algorithm and indicated identity or extensive homology.

Protein name	Accession No	MOWSE score	Peptide	Mol. Mass (Da)	
				Observed	Expected
HSP60	Q8C2C7_MOUSE	88	NAGVEGSLIVEK	1214.65	1214.65
			IQEITEQLDITTSEYEK	2039.11	2038.99
			LVQDVANNTNEEAGDGTTTATVLAR	2560.45	2559.24
Acrbp	Q62253_MOUSE	49	FFALLTPTWK	1222.68	1222.67
AKR1B7	Q5M9J9_MOUSE	82	ACDLLDAR	932.61	932.44
			HIDCAYVYHNENEVGEAIQEK	2517.07	2517.12
			IQENLQVFDFQLSEEDMAAILSFNR	2956.51	2956.43
HSP10	CH10_MOUSE	46	VLQATVVAVGSGGK	1284.63	1284.74
Citron	O88528_MOUSE	43	IQELQEKLEK	1256.51	1256.7
			AKDQGKPEVGEYSK	1535.41	1534.76
			EQEYQAQVEEMR	1539.76	1538.67
			HAMLEMNARSLQQK	1672.77	1671.82
			LQEIKEQEYQAQVEEMR	2167.18	2166.03

Table 4.1. Putative heat shock protein 60 (HSPD1) binding partners identified by co-immunoprecipitation and tandem mass spectrometry analysis. Columns indicate IPI accession number (IPI reference), the protein name (Protein name), MSDB accession number (Accession No), Mowse scores (MOWSE score), observed peptides (Peptide) and the observed and expected molecular mass of each peptide (Mol. Mass Da).

4.4 Discussion

Recent publications indicate that tyrosine phosphorylation of proteins localized to the plasma membrane overlying the acrosome during capacitation may be an important determinant of zona pellucida recognition and binding (Asquith et al., 2004). Further, the expression of phosphorylated molecular chaperones HSP90B1 and HSPD1 at this location following capacitation has been described. In order to decipher the mechanisms that underpin chaperone regulation of sperm function, chaperone binding partners in mouse sperm were investigated, with a view that HSP90B1 and HSPD1 may be members of a multi-protein receptor complex in the sperm membrane that confers upon the cell the capacity for fertilisation. Co-immunoprecipitation of sperm lysates with chaperone-specific antibodies facilitated the identification of several putative chaperone binding proteins (Table 4.1**)**

A number of proteins co-purified with HSP90B1 (Fig. 4.6A, lane 6), and HSPD1 (Fig. 4.7A, lane 7). Unfortunately, none of the protein co-purifying with HSP90B1 could be identified. The limiting factor was the amount of material available for digestion and analysis. Even though the presence of the immunoprecipitation target was repeatedly detected in immunoprecipitates by western blot analysis, it could not be detected when similar samples were digested and analysed by mass spectrometry. A significant limitation in these studies was the unavailability of high sensitivity mass spectrometry instrumentation. Digests were typically analysed several days after digestion which may have resulted in peptide losses due to adsorption. As positive identifications were obtained for HSPD1 binding partners, the persuit of the HSP90B1 associated proteins was not continued. Several putative HSPD1 binding partners were identified (Table 4.1) Further supporting the hypothesis of a multimeric complex in the sperm membrane.

Initial attempts to identify chaperone-associated proteins were unsuccessful due to the limited amount of protein being pulled down in the co-IP reactions (Fig. 4.3.). These experiments used RIPA buffer as the lysis and immunoprecipitation buffer. Western blot analysis indicated that the buffer was successful in liberating the chaperone

proteins from cells (Fig. 4.3B). Only protein bands corresponding to the chaperones were detected in silver stained gels. It was interpreted that while the RIPA buffer had not interfered with the antibody-antigen interaction, it may have had disrupted the protein-protein interactions between each chaperone and its binding proteins.

In an attempt to rectify this situation, the chemical crosslinker DTSSP was used to covalently stabilise the protein interactions within chaperone containing protein complexes prior to exposure to RIPA buffer. DTSSP is only 12 Angstrongs in length and should therefore only crosslink proteins in very close proximity to each other, proteins likely to be part of a multi-protein complex. The use of the crosslinker also permits more stringent washing of captured immunocomplexes thereby decreasing the amount of non-specific protein purification. The crosslinker contains an intramolecular disulfide bond which facilitates separation of crosslinked protein under the reducing conditions of SDS-PAGE. In reality the use of DTSSP had little effect on the amount of protein co-purifying with the two chaperone proteins (Fig 4.4A).

In light of this, the use of RIPA buffer as the lysis and immunoprecipitation buffer was abandoned and 'Buffer B' was used for all subsequent experiments. In addition to affording the precipitation of both chaperone proteins as determined by western blot (Fig 4.6B, lane 6 and Fig. 4.7B, lane 7), the buffer facilitated the detection of proteins co-purifying with the chaperons (Figs 4.6A and 4.7A). Importantly, negative controls using only the solid phase support (no antibody) for immunoprecipitation were clear (Fig 4.6, lane 7; and Fig 4.7, lane 8). This means that the proteins co-purifying with each of the chaperones were not binding non-specifically to the solid phase support because of the decrease strength of Buffer B compared to the previously used RIPA buffer.

The solid phase used to immobilise anti-chaperone immunoglobulins for use in co-immunoprecipitation experiment was an aldehyde-activated beaded agarose gel. Before adopting this support it was compared to a polystyrene magnetic bead support. Both supports are reported to covalently immobilise antibodies for use in immunoprecipitation reactions. The advantage of this is that in theory, the amount of immunoprecipitation antibody in the final purified material should be limited. Figure 4.5 shows the results of this direct comparison. The agarose support afforded the

purification of the chaperone (Fig. 4.5B, lane 1) and a number of co-purifying proteins (Fig. 4.5A, lane 1). In contrast, while the magnetic bead support afforded isolation of the chaperone (Fig. 4.5B, lane 2), only negligible amounts of co-purifying material were detected (Fig.4.5A, lane 2). The two major bands detected here correspond to the predicted molecular weight of antibody heavy and light chains. The presence of these heavy and light chains would hinder the identification of the putative chaperone interacting proteins. As such the beaded agarose support was adopted for use.

The traditional approach to protein identification in these types of experiments, the resolution of isolated proteins in polyacrylamide gels followed by in-gel proteolysis and mass spectrometric analysis, proved unsuccessful and no identifications could be made. As such, a solution-based enzymatic digestion was trialed in place of the gel-based approach. It was hoped that avoiding the peptide losses normally associated with the in-gel based approach would facilitate the identification chaperone-associated proteins.

In summary, co-immunoprecipitation using the method described herein resulted in the successful identification of several HSPD1 binding proteins; an aldose reductase AKR1B7, the molecular chaperone HSPE1, the proacrosin binding protein ACRBP and a kinase, citron kinase. The confirmation/testing of these interactions is the topic of subsequent chapters.

CHAPTER 5

LOCALISATION OF CHAPERONE INTERACTING PROTEINS DURING EPIDIDYMAL MATURATION, CAPACITATION AND THE ACROSOME REACTION: ALDOSE REDUCTASE AKR1B7

5. Localisation of chaperone interacting proteins during epididymal maturation, capacitation and the acrosome reaction: Aldose Reductase AKR1B7.

5.1 Introduction

The molecular basis of how the gametes recognise and interact with each other remains unclear. Although it is well established that the sperm binding activity of the zona pellucida resides on the glycan side chains of the ZP3 glycoprotein (Florman and Wassarman, 1985), the complementary ligand on the surface of sperm remains elusive. A number of potential candidate molecules for the primary zona receptor have been proposed including galactosyltransferase, sp56 and zona receptor kinase (Sec. 1.4.3).

Recently, Asquith and colleagues investigated the relationship between the capacitation-associated increase in the level of protein tyrosine phosphorylation and the ability of mouse sperm to interact with the zona pellucida. They demonstrated a strong correlation between this phosphorylation event and the ability of capacitated sperm to bind the zona (Asquith et al., 2004). In addition, they identified two chaperone proteins localised on the surface of capacitated mouse sperm, “heat shock protein 90kDa beta member 1” (HSP90B1) and “heat shock protein 1” (HSPD1). Both HSP90B1 and HSPD1 proteins were tyrosine phosphorylated and localised to the plasma membrane of the sperm head overlying the acrosome, an ideal position for participation in zona binding. These observations led the authors to propose a novel hypothesis. Although not directly involved in binding ZP3, it was proposed that "activation" of the chaperones by tyrosine phosphorylation during capacitation may trigger conformational changes or a change in localisation facilitating the assembly and/or exposure of a functional zona pellucida receptor on the surface of sperm (Asquith et al., 2004).

Molecular chaperones are highly conserved, abundant proteins that facilitate the correct folding of proteins in the crowded cellular environment (Ellis and van der Vies, 1991). HSPD1 constitutes one of the major and best characterised molecular chaperones in prokaryotic and eukaryotic organisms (Ellis and van der Vies, 1991; Hartl et al., 1992; Craig et al., 1993). In bacteria, HSPD1 (also referred to as GroEL or cpn60) is involved in the folding and assembly into oligomeric complexes of other proteins, as well as their transport across the plasma membrane (Craig et al., 1993; Goloubinoff et al., 1989; Langer and Newpert, 1991).

In order to further our understanding of the role of chaperone proteins in the process of fertilization, putative chaperone-associated proteins or "binding partners" were identified (chapter four). Examination of the localisation pattern of the putative chaperone-interacting proteins during sperm maturation and fertilization is essential if we are to understand their role in the reproductive process.

In this chapter, I sought to confirm the interaction between HSPD1 and AKR1B7 and to define the localisation of the AKR1B7 protein during epididymal maturation, capacitation and the acrosome reaction as an initial step to characterising the function of the protein in the maturation of spermatozoa and fertilization.

5.2 Experimental Rationale

Putative binding partners for the molecular chaperones HSPD1 were identified using a proteomics approach coupled with co-immunoprecipitation (co-IP) from lysates of capacitated mouse sperm (chapter four). From the list of putative HSPD1 binding proteins, several including AKR1B7, an aldose reductase, were selected for further characterisation.

AKR1B7-specific antibodies were used to confirm the interaction of AKR1B7 and HSPD1. The reciprocal co-IP was performed with AKR1B7-specific antibodies and the presence of HSPD1 in the immunoprecipitated material demonstrated by western blot analysis (Sec. 2.6.4). The same antibodies were used to determine the relative level of the AKR1B7 protein in sperm during epididymal maturation.

Localisation of the AKR1B7 prtotein on the cell surface was demonstrated with a membrane impermeable biotinylation reagent (Sec. 2.4.3) in conjunction with western blotting (Sec. 2.6.4). The results of the cell surface biotinylation experiments were confirmed by flow cytometry (Sec. 2.6.6).

The effect of capacitation (Sec. 2.3.5) and the acrosome reaction (Sec. 2.3.6) on the localisation of the protein were investigated using indirect immunofluorescence techniques (Sec. 2.6.5).

5.3 Results

5.3.1 Confirmation of HSPD1 interaction with AKR1B7 by reciprocal co-IP and western blot analysis.

Confirmation of the interaction between HSPD1 and AKR1B7 was demonstrated by performing the reciprocal co-immunoprecipitation experiment (Sec. 2.4.5) utilising an AKR1B7-specific antibody and detecting the presence of HSPD1 in the precipitated material by western blotting (Fig. 5.1).

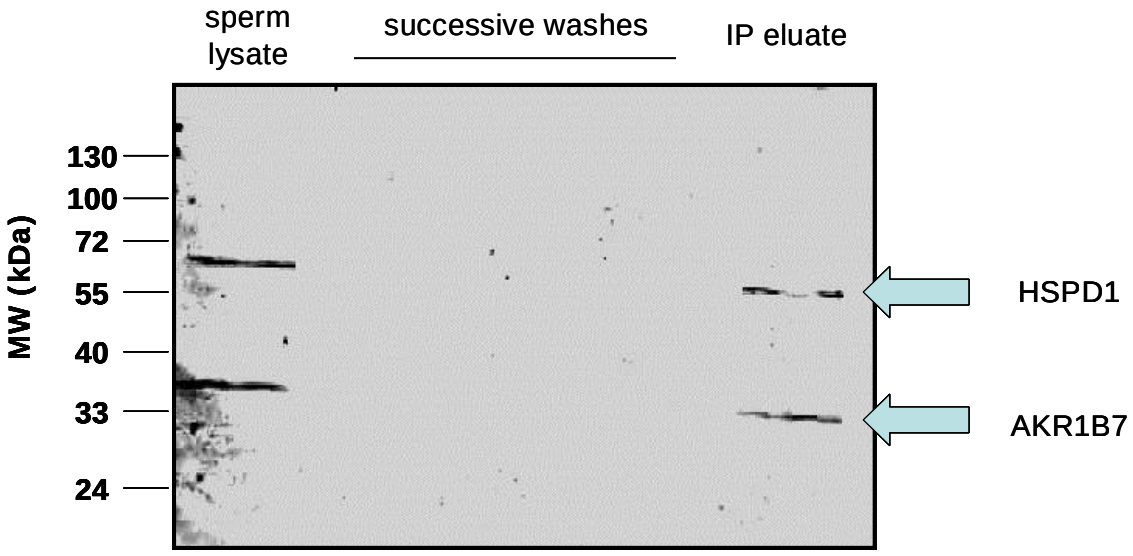


Figure 5.1. Confirmation of HSPD1-AKR1B7 protein interaction by reciprocal co-IP. Caudal spermatozoa were capacitated for 90 min in complete BWW, washed and lysed in Buffer B. Clarified lysates were used for immunoprecipitation with a specific AKR1B7 antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membrane for western blot analysis. The presence of both AKR1B7 and HSPD1 were detected with protein-specific antibodies.

The molecular chaperone HSPD1 was detected in proteins immunoprecipitated with an AKR1B7-specific antibody from lysates of capacitated caudal sperm by western

blot analysis (Fig. 5.1). This data strongly suggests that AKR1B7 is a *bona fide* HSPD1 binding partner.

5.3.2 Mammalian sperm acquire AKR1B7 as they mature in the epididymis.

In order to define the origin of AKR1B7 in sperm, an adult testis extract and purified populations of epididymal sperm were assayed for the presence of AKR1B7 by western blot analysis. Western analysis revealed that the aldose reductase AKR1B7 is acquired by sperm in the corpus epididymis during epididymal transit (Fig. 5.2). The aldose reductase antibody detected a single protein of approximately 35 kDa, consistent with the predicted size of the AKR1B7 protein, in lysates derived from corpus and caudal sperm cells. The use of β-tubulin as a loading control indicated that the relative abundance of the reductase increased as spermatozoa moved from the corpus to the cauda epididymis. The protein was not detected in sperm isolated from the caput epididymis or in total testicular protein extracts.

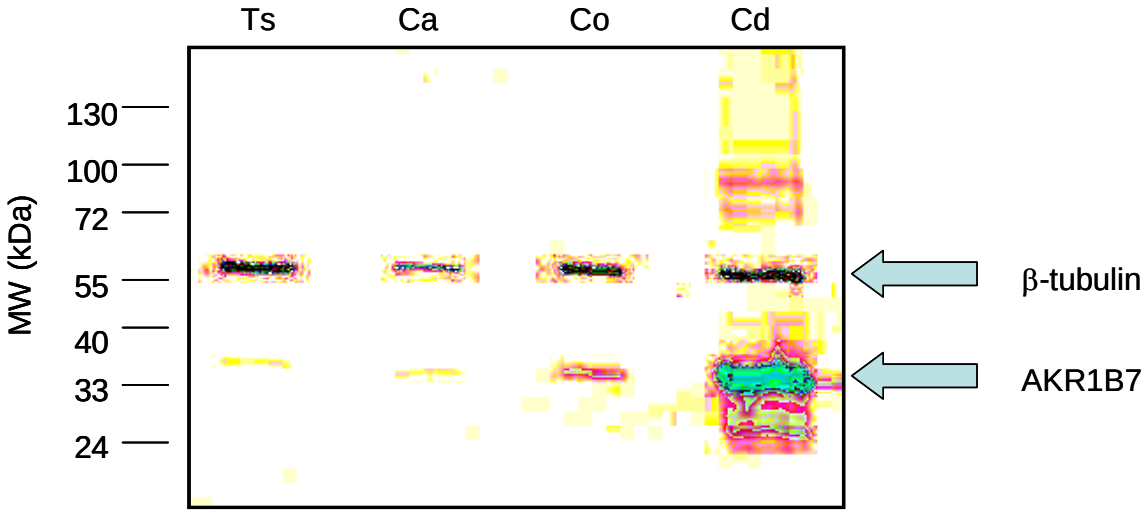


Figure 5.2. Western analysis of aldose reductase (AKR1B7) in the mouse testis and epididymal spermatozoa. 5µg of cell extracts were resolved by SDS-PAGE and immunoblotted with an AKR1B7-specific antibody. Blots were also probed with a β-tubulin-specific antibody to confirm equal protein loading in each lane. Mouse testis total cell extract **(Ts)**, purified sperm from mouse caput **(Ca)**, corpus **(Co)** and cauda **(Cd)** epididymides.

5.3.3 Verification of surface localisation of AKR1B7 in live capacitated sperm cells.

Since the co-IP experiments that pulled down AKR1B7 were designed to isolate chaperone-associated proteins from the surface of capacitated spermatozoa, experiments were performed to confirm the surface localisation of AKR1B7. Sperm surface proteins were labeled with a membrane impermeable biotinylation reagent (Sec. 2.4.3) and recovered from lysates with immobilised streptavidin. Putative surface proteins were separated by SDS-PAGE and the presence of AKR1B7 was demonstrated by western analysis with an AKR1B7-specific antibody (Fig. 5.3).

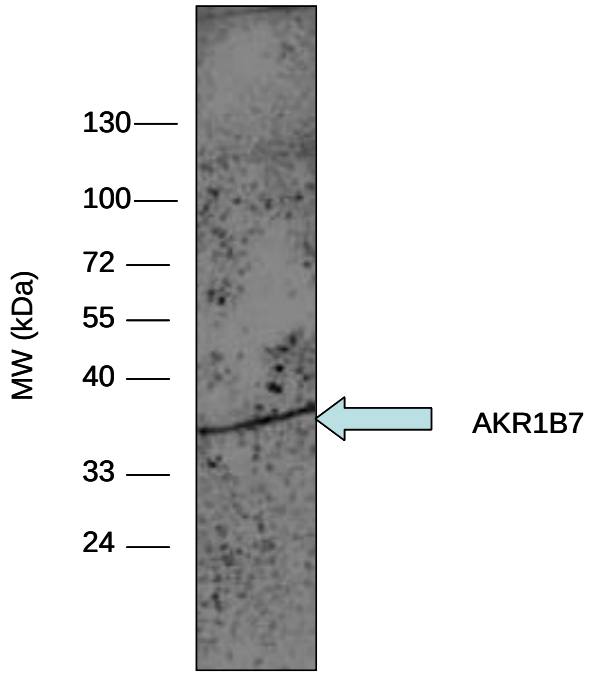


Figure 5.3. Aldose reductase AKR1B7 protein was localised on the surface of spermatozoa. Cauda epididymal spermatozoa were capacitated for 90 min in complete BWW. Surface proteins were biotinylated, affinity purified and resolved by SDS-PAGE. Aldose reductase was identified by immunoblotting with an AKR1B7-specific antibody. One protein of approximately 35 kDa, the predicted size, was identified.

The presence of AKR1B7 on the cell surface was ultimately confirmed by flow cytometry. It is well documented that the process of capacitation is accompanied by a remodeling of the plasma membrane and can result in the redistribution of sperm surface proteins (Lopez and Shur, 1987; Della Giovampaola et al., 2001; Focarelli et al., 1998). To determine whether capacitation influenced the level of AKR1B7 surface localisation, both uncapacitated and capacitated spermatozoa were analysed by flow cytometry (Sec. 2.6.6).

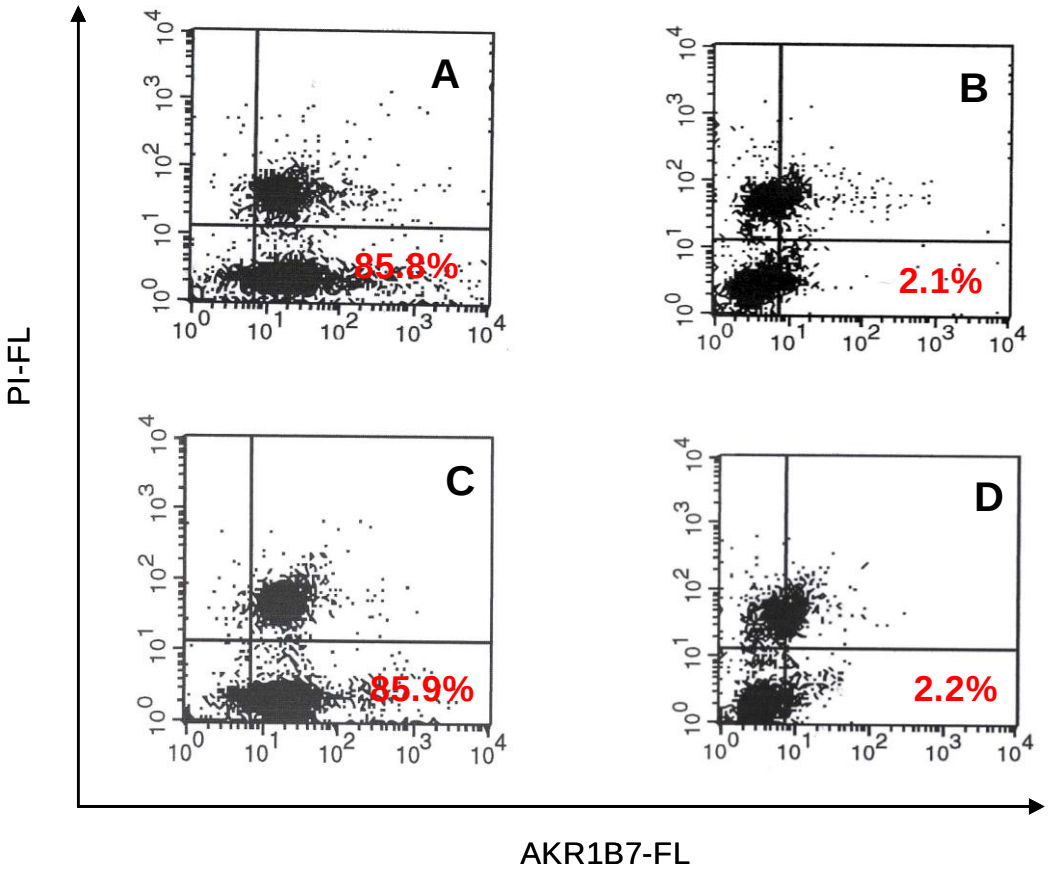


Figure 5.4. Flow cytometric analysis of AKR1B7 on the surface of mouse spermatozoa. Surface localisation of AKR1B7 on live sperm was demonstrated by flow cytometry. Spermatozoa were recovered from the cauda epididymis and either labeled as described in Sec. 2.6.6 or capacitated in complete BWW media for 45 min prior to labeling. Surface AKR1B7 was bound using an AKR1B7-specific antibody and visualized using an FITC-labeled secondary antibody (AKR1B7-FL). Cells were then treated with propidium iodide to label dead cells which were subsequently gated out of the analysis (PI-FL). The percentage of sperm with surface labeling is indicated in the bottom right hand quadrant. **A** and **B**) Uncapacitated spermatozoa. **C** and **D**) Capacitated spermatozoa. **B** and **D**) Control experiments omitting the primary AKR1B7 antibody.

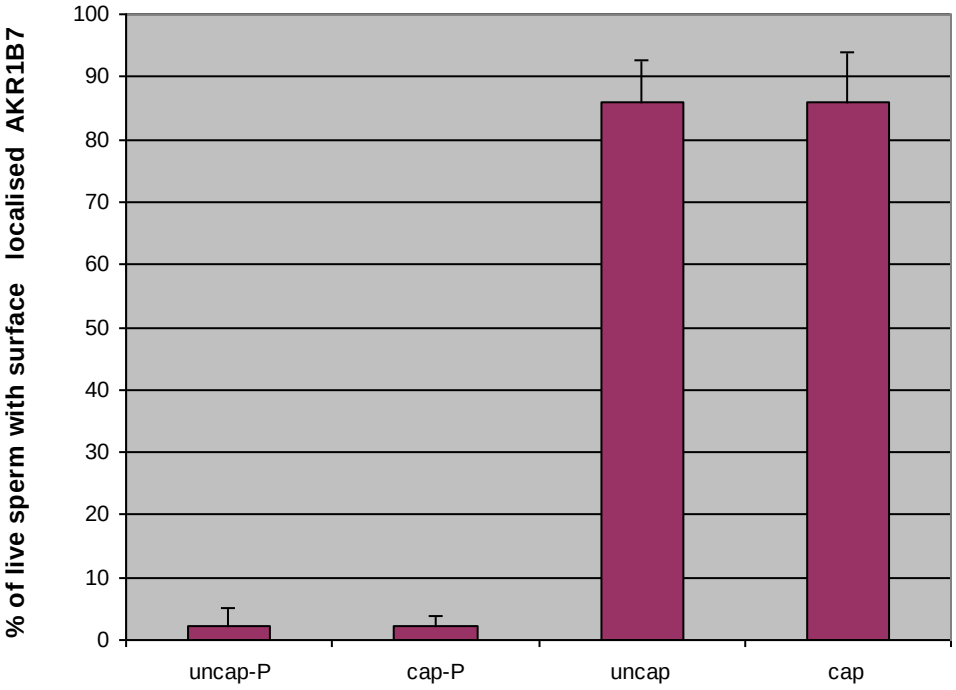


Figure 5.5. Flow cytometric analysis showing exposure of AKR1B7 on the surface of live mouse spermatozoa. Spermatozoa were recovered from the cauda epididymis and either labeled as described, or capacitated in complete BWW media for 45 min prior to labeling. Surface localised AKR1B7 was bound using a goat polyclonal AKR1B7-specific antibody and visualized using FITC-labeled rabbit anti-goat antibody. Cells were then treated with propidium iodide to label dead cells which were subsequently gated out of the analysis (n=3). uncap-P: uncapacitatd sperm control omitting the primary antibody, cap-P: capacitated sperm control omitting the primary antibody, uncap: uncapacitated sperm, cap: capacitated sperm.

Since live (propidium iodide negative) sperm would exclude antibody entry into the cytoplasm, any fluorescence would be indicative of AKR1B7 surface localisation. In Fig. 5.4, the AKR1B7-FL channel measured fluorescein isothiocyanate (FITC) labeling of cells (AKR1B7 localisation), and the PI-FL channel measured propidium iodide staining (membrane compromised, dead cells). As demonstrated in Figs. 5.4 and 5.5, when both uncapacitated and capacitated spermatozoa were probed with the AKR1B7 antibody, positive staining was observed in 85.8% of live, uncapacitated

cells; and in 85.9% of live, capacitated cells (A and C, lower right quadrants). As anticipated, spermatozoa in the control population, prepared in the absence of primary antibody, displayed only background levels of fluorescence; uncapacitated (2.1%) and capacitated (2.2%) (B and D, lower right quadrants). These data not only confirmed the surface localization of AKR1B7, but also indicate that the process of capacitation had no effect on the level of surface exposure of the protein

5.3.4 AKR1B7 localised to the peri-acrosomal region of mammalian sperm.

Having confirmed the presence and surface exposure of AKR1B7 in epididymal sperm, we next sought to examine the cellular localization pattern of the protein. AKR1B7 was immunolocalised on fixed mouse spermatozoa taken from different regions of the epididymis, using indirect immunofluorescence (IIF) (Sec. 2.6.5). The protein was not detected in sperm isolated from the caput (Fig. 5.6 A) or corpus (Fig. 5.6 B) regions of the epididymis, however, AKR1B7 immunofluorescence was detected in the peri-acrosomal region of spermatozoa taken from the cauda epididymis (Fig. 5.6 C). No labeling was observed in controls in which the primary antibody was omitted (not shown).

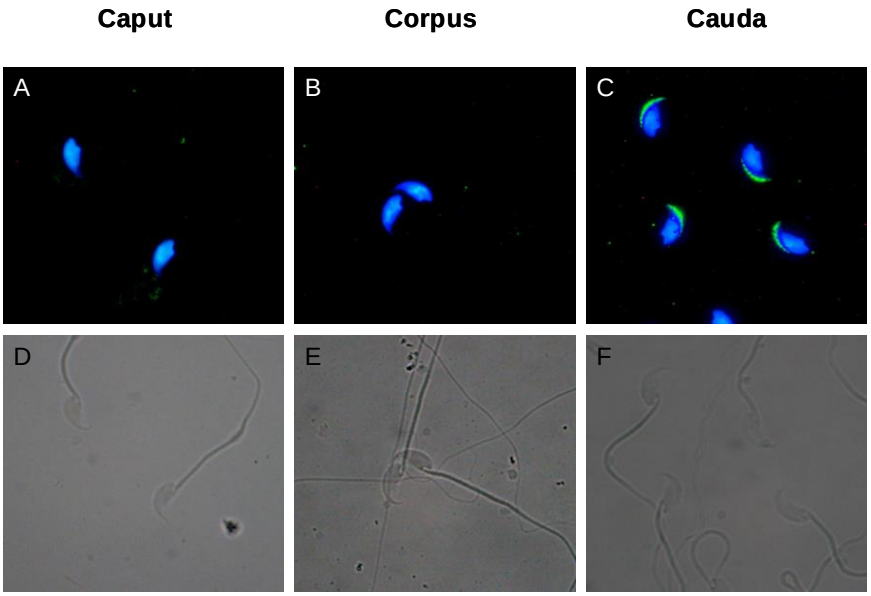


Figure 5.6. Indirect immunofluorescence localisation of AKR1B7 in epididymal spermatozoa. As described in section 2.6.5, epididymal sperm were incubated with anti-AKR1B7 immunoglobulin followed by an FITC-conjugated secondary antibody. **A** and **D**) caput spermatozoa; **B** and **E**) corpus spermatozoa; **C** and **F**) caudal spermatozoa. **A**, **B**, and **C**) labeled with an anti-AKR1B7 antibody (green) and DAPI (blue); **D**, **E**, and **F**) phase contrast. Control experiments omitting the primary antibody displayed negligible staining (not shown).

These data indicate that AKR1B7 immunoreactive products are acquired by sperm as they pass through the cauda epididymis.

5.3.5 Acrosomal loss was correlated with the loss of AKR1B7 from the sperm head.

Additional support for the surface localisation of aldose reductase was achieved by following the fate of AKR1B7 subsequent to induction of acrosomal exocytosis. For these studies, cauda epididymal were capacitated and the acrosome reaction induced using calcium ionophore, A23187 (Sec. 2.3.6). Both capacitated and acrosome reacted spermatozoa were probed with an AKR1B7-specific antibody followed by an FITC-

conjugated secondary antibody (green labelling in Fig. 5.7). DNA was labelled with DAPI (blue labeling in Fig. 5.7).

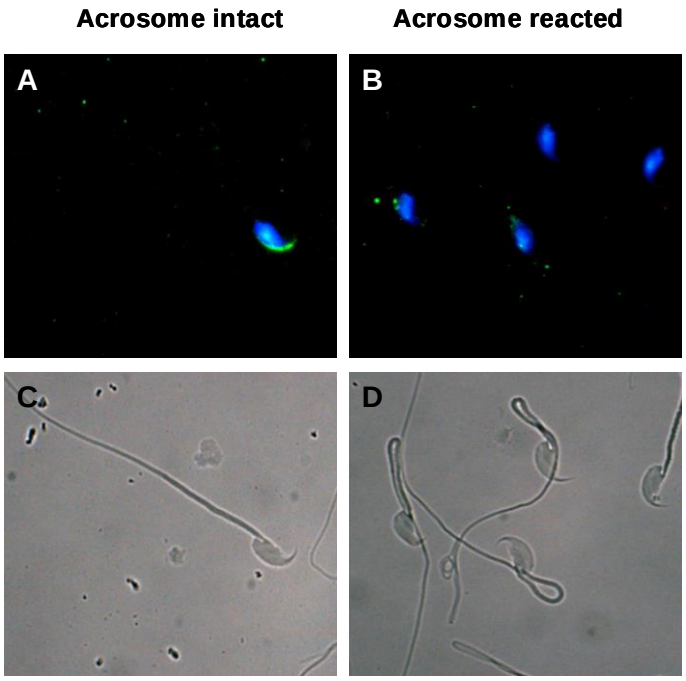


Figure 5.7. Indirect immunofluorescence demonstrating localisation of AKR1B7 in capacitated and acrosome reacted spermatozoa. Cauda epididymal sperm were capacitated (Sec 2.3.5), acrosome reacted (Sec 2.3.6) and incubated with anti-AKR1B7 immunoglobulin followed by an FITC-conjugated secondary antibody (Sec 2.6.5). **A** and **C**) capacitated spermatozoa; **B** and **D**) acrosome reacted cells. **A** and **B**) labeled with an anti-AKR1B7 antibody (green) and DAPI (blue); **C** and **D**) phase contrast.

A comparison of Fig. 5.7 A (capacitated) and Fig. 5.6 C (non-capacitated) indicated that capacitation had no disernable affect on the localisation of aldose reductase. The protein was detected in the acrosomal region of capacitated caudal spermatozoa (Fig. 5.7A), however, cells that had undergone ionophore-induced acrosomal exocytosis lost their aldose reductase labeling (Fig. 5.7B).

5.4 Discussion

Mammalian spermatozoa are highly differentiated cells when they leave the testis, but lack the capacity for forward motility and to fertilise oocytes. They acquire both of these functions during consecutive phases of post-testicular maturation; epididymal maturation in the male, and capacitation in the female reproductive tract.

Recent literature describes a close relationship between the exhibition of tyrosine phosphorylated phosphoproteins on the surface of spermatozoa following capacitation and an ability to interact with, and bind, the outer vestments of the oocyte, the zona pellucida (Asquith et al., 2004; Asquith et al., 2005).

Immunolocalisation studies indicate that in addition to the flagellum, tyrosine phosphorylated proteins appear on the surface of sperm in the plasma membrane overlying the acrosome in response to capacitation. The importance of this novel phosphorylation pattern in zona binding was highlighted in immunofluorescence studies of sperm bound to the zona pellucida. Every single sperm cell bound to the zona pellucida was found to express phosphorylated tyrosine residues on the head.

Analysis of the surface phosphoproteome of capacitated mouse cauda epididymal sperm identified two molecular chaperones, heat shock protein 90kDa beta member 1 (HSP90B1) and heat shock protein 60 (HSPD1) as proteins that were tyrosine phosphorylated during the process of capacitation (Asquith et al., 2004; Asquith et al., 2005). Although tyrosine phosphorylated molecular chaperones appear to have an important role in gamete interactions, evidence suggests that the zona receptor responsible for adhesion is not phosphorylated itself (Asquith et al., 2004). It was suggested however, that the chaperone proteins may be instrumental in the assembly and/or exposure of a receptor complex on the surface of sperm that mediates zona recognition.

These previous data prompted an investigation into proteins associated with the molecular chaperones on the surface of capacitated spermatozoa (chapter 4). A

number of putative HSPD1-associated proteins were identified in chapter four, several of which were selected for further characterisation. From the identified proteins, the putative HSPD1 binding protein aldose reductase AKR1B7 (table 4.1, chapter 4) was selected for further characterisation.

Although the AKR1B7 sequence has been identified in the mouse genome and localised to the male reproductive tract (Baumann et al., 2007), no literature regarding the role or activity of the enzyme in specific sperm function has been published. As other members of the AKR1B subfamily have however been shown to play a role in the maturation of spermatozoa in other species (Girouard et al., 2009), a characterisation of AKR1B7 has been pursued. As an initial step towards characterising the role of this protein in sperm function, the current study sought to confirm the interaction between HSPD1 and AKR1B7, and describe the localisation of the protein in spermatozoa during epididymal maturation, capacitation and the acrosome reaction.

Data presented in this chapter documents for the first time the surface localisation of the aldose reductase AKR1B7 in mouse spermatozoa.

The mouse aldose reductase AKR1B7 belongs to the aldo-keto reductase (AKR) superfamily of proteins. The AKRs are a group of 120 enzymes currently composed of 15 sub-families from a wide variety of organisms including plants, animals, and prokaryotes (reviewed by Bhatnagar & Srivastava, 1992; Di Luccio et al., 2006). In almost all cases they catalyse the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of aliphatic and aromatic carbonyl compounds to their corresponding alcohols (Petrash, 2004). The various AKRs show broad overlapping substrate specificities for aldehydes and ketones, including xenobiotics in addition to endogenous compounds (Bohren et al., 1989).

Historically, members of the superfamily were identified by names based on substrate specificity (Jez and Penning, 2001); however, at the 8th International Symposium on the Enzymology and Molecular Biology of Carbonyl Metabolism, a nomenclature system for the AKR superfamily was proposed to unify naming (Jez et al., 1997). In addition, the Human Genome Organisation (HUGO) Gene Nomenclature Committee

has adopted the AKR superfamily nomenclature (www.gene.ucl.ac.uk/nomenclature/AKR.shtml). The general format for aldo-keto reductase names includes the root symbol 'AKR' for *Aldo-Keto Reductase*; an Arabic number designating the family; a letter indicating the subfamily; and an Arabic numeral representing the unique protein sequence. The AKR1 family includes the several subfamilies including; (A) mammalian aldehyde reductases; (B) mammalian aldose reductases; (C) hydroxysteroid dehydrogenases; and (D) Δ^4-3-ketosteroid-5β-reductases.

In the AKR superfamily, the aldose reductases (AKR1B) have been the focus of much interest because of their possible role in the development of secondary diabetic complications (Kinoshita and Nishimura, 1988). Consistent with its broad substrate specificity, aldose reductase has been implicated in a number of biological processes including osmotic homeostasis (Bagnasco et al., 1986), steroid conversion (Warren et al., 1993), and detoxification of xenobiotic and endogenous aldehydes (Grimshaw, 1992).

Aldose reductase catalyses the first step in the polyol pathway of glucose metabolism (Fig. 5.8) in a number of tissues including the testes, pancreas, brain, and the lens of the eye. In this pathway, AR uses NADPH as an electron donor to reduce glucose to sorbitol. In the second step of the pathway sorbitol dehydrogenase uses NAD^+ as an electron acceptor to generate fructose (Oates, 2002).

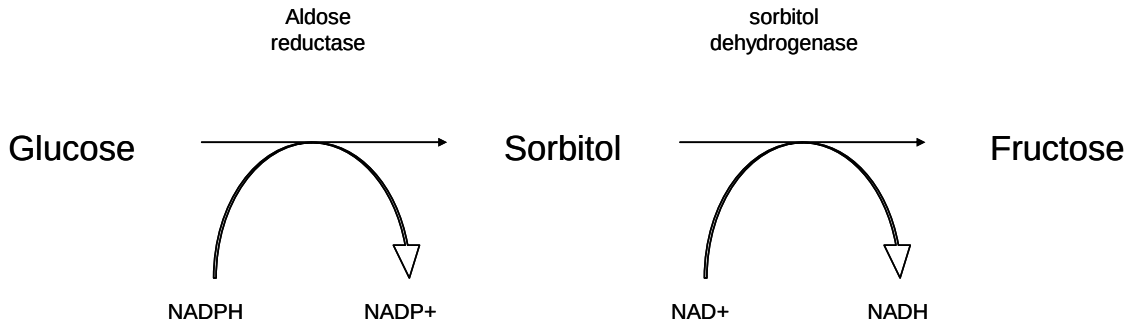


Fig. 5.8 The polyol pathway of glucose metabolism.

Nucleotide sequencing of cDNA libraries and clones constructed from human, rat and mouse tissues indicate that the AKR1B subfamily of AKRs contains several proteins with very high sequence and structural similarity (Petrash, 2004). Two human *AKR1B* genes, *AKR1B1* and *AKR1B10* have been identified, while the mouse genome contains three transcriptionally active *Akr1b* genes: *Akr1b3*, *Akr1b7*, and *Akr1b8* (Mindnich and Penning, 2009). The *Akr1b7* gene encodes an aldose reductase that is highly expressed in the vas deferens epithelium and zona fasciculata of the adrenal cortex (Martinez et al., 2001).

The data in figure 5.1 supports the interaction between HSPD1 and AKR1B7. An anti-AKR1B7 antibody was used to precipitate the aldose reductase from lysates of capacitated mouse spermatozoa, and western analysis confirmed the presence of HSPD1 among the precipitated proteins. The identification of an aldose reductase as a binding partner of a cell surface chaperone has not been previously been reported.

Aldose reductase AKR1B7 is acquired by mouse sperm during epididymal maturation as they travel through the corpus and cauda epididymis. Using testis, caput, corpus and cauda sperm extracts, the presence of AKR1B7 was detected by western analysis (Fig. 5.2). The protein was not be detected in testis lysates or in sperm taken from the caput epididymis, however, the protein was present in sperm isolated from the corpus and cauda regions of the epididymis (Fig. 5.2). Although first acquired in the corpus region, the protein was localized in presumably higher concentrations on sperm taken from the cauda region. No change in the observable molecular weight of the protein was detected between epididymal regions where the protein was present. Thus, within the limits of detection of this method, AKR1B7 does not undergo any post-translational modification or processing as sperm pass from the corpus to the cauda epididymis.

The amino acid sequence of AKR1B7, as deduced from the nucleotide sequence of its cDNA, does not contain the signal sequence that normally triggers the translocation of secretory protein across the endoplasmic reticulum membrane (Manin et al., 1995). This suggests that the protein is mainly a cytosolic protein and that it has a role inside the cell. However, surface biotinylation and flow cytometric analysis of spermatozoa

demonstrated that the protein is localised on the surface of caudal spermatozoa (Sec. 5.3.3). The mechanism by which spermatozoa in the epididymis acquire new surface proteins remains unclear, however recent reports have suggested that epididymosomes and what have been termed “dense bodies” in the epididymal lumen may be involved in the process (Sullivan et al., 2007; Oh et al., 2009).

Epididymosomes are membranous secretory vesicles of epididymal origin implicated in the transfer of new proteins to sperm during epididymal transit (Sullivan et al., 2007). In bulls, proteins such as glycosylphosphatidylinositol (GPI)-anchored protein P25b, macrophage migration inhibitory factor (MIF), and aldose reductase (AKR1B1), are transferred to spermatozoa from epididymosomes during the epididymal maturation process (Girouard et al., 2009).

Members of the AKR1B subfamily of aldose reductases have been identified as constituents of epididymosomes in other species, including humans and bulls.
(Frenette et al., 2003; Sullivan et al., 2007).

In the male reproductive tract, three mechanisms of secretion have been described: merocrine, apocrine and release of membrane-limited vesicles termed prostasomes or epididymosomes. Apocrine secretion entails the protrusion of a section of plasma membrane into the lumen, which is then pinched off and released as an apical bleb. In the lumen, the membrane surrounding the blebs breaks down, releasing the contents. Apical blebs have been reported in the epididymis from species including bulls, monkeys, hamsters, and humans (reviewed by Aumuller et al., 1999; Hermo and Jacks, 2002). This suggests that the proteins contained by the blebs may play a role in the maturation of spermatozoa, whether through the development of functional capacity or in the protection of gametes as they transit the region. Several apical bleb proteins have been identified including glutathione transferase, ubiquitin and aldose reductase AKR1B7 (Sullivan et al., 2007; Manin et al., 1995)

Epididymal dense bodies, first described by Asquith and colleagues (Asquith et al., 2005), are discrete structures suspended in the epididymal fluid juxtapositioned to, but independent of sperm. Distinguished from epididymosomes by their lack of membrane and increased size, they have been shown to be a rich source of HSPD1

and first appear in the corpus epididymis and persist throughout the cauda epididymis and vas deferens (Asquith et al., 2005)

The origin of the dense bodies remains open to question. It has been proposed that they represent the accretion of epididymal luminal proteins, including the contents of apical blebs, into multi-molecular complexes (Asquith et al., 2005). Such a process could be mediated by HSPD1 evident within the dense bodies. Accumulation into a single mass could be a way of efficiently and simultaneously delivering a number of proteins through the mediation of HSPD1. The dense bodies may be instrumental in a mechanism by which proteins secreted via apical blebs and the apocrine pathway, interact with spermatozoa.

Indirect immunofluorescence (IIF) studies revealed that AKR1B7 localises to the periacrosmal region of sperm from the cauda epididymis (Fig. 5.6). The AKR1B7 protein could not be detected on sperm taken from the caput or corpus regions of the epididymis. This is inconsistent with detection of the protein in lysates of sperm from the corpus region of the epididymis (Fig 5.2). This is likely to be a reflection of differences in the sensitivities of IIF and western blotting. Less likely, AKR1B7 is not surface orientated in the corpus but becomes readily available in the cauda epididymis. The epitope may be masked in the corpus epididymis, but subsequently unmasked in the cauda as a consequence of the remodeling of the plasma membrane architecture during the epididymal maturation process. This pattern of localisation is inconsistent with that reported for HSPD1 which localises to the acrosomal region of sperm throughout the epididymis (Asquith et al., 2005). This suggests that components of HSPD1 containing protein complexes are progressively acquired.

If HSPD1-associated proteins, including AKR1B7, have a role in the molecular interactions that take place between the gametes, they should be localised on the surface of capacitated spermatozoa. Cell surface biotinylation experiments employing a membrane impermeable biotinylation reagent indicated that the aldose reductase AKR1B7 was indeed surface localised in caudal spermatozoa (Fig 5.3). The results of these experiments were confirmed by flow cytometry which demonstrated that both uncapacitated and capacitated spermatozoa exhibit the protein at the cell surface, although there was little difference between the percentage of cells demonstrating

surface localisation between the capacitated and uncapacitated samples (Fig. 5.4 & 5.5). Approximately 80% of live cell in both uncapacitated and capacitated samples exhibit the protein. This is significantly more that the percentage of spermatozoa reported to exhibit HSPD1 at the cell surface, 6% and 25% for uncapacitated and capacitated cells respectively (Asquith et al., 2005). The fact that there is little difference in the level of surface localisation between uncapacitated and capacitated cells is inconsistent with the protein having a role in direct binding of the zona pellucida. Previous reports demonstrate an increased ability of sperm to bind the zona pellucida after capacitation (Asquith et al., 2005) suggesting that the exhibition of the actual receptor for the zona pellucida is altered as a consequence of capacitation.

Considering AKR1B7 appeared in the acrosomal region, it is possible that the protein would be lost or spatially redistributed as spermatozoa capacitated or underwent acrosomal exocytosis. The process of capacitation appeared to have no effect on the localisation of AKR1B7 which localised to the same region seen in capacitated cells (Fig. 5.7 A). This localisation however, was lost once sperm have undergone calcium ionophore A23187-induced acrosomal exocytosis (Fig 5.7 B). The loss of the protein during the acrosome reaction is consistent with the observed peri-acrosomal localisation, Furthermore, these data taken together suggest that AKR1B7 is a constituent of the plasma membrane overlying the acrosome in murine sperm. This is supported by the data in chapter three of this thesis which identified AKR1B7 as a putative cell surface protein and a constituent of lipid rafts (Table 3.1 and references therein).

Although aldose reductase has been reported not to be present in rat testicular and epididymal spermatozoa (Kobayashi et al., 2002), recent proteomic investigations by a collaborator identified another aldose reductase (AKR1B3) in murine sperm (Baker et al., 2008b). The same study also produced data suggesting that the polyol pathway is activated in sperm as they capacitate. This would provide sperm with fructose, their preferred substrate for glycolysis (Manin et al., 1995). The polyol pathway has previously been shown to function in seminal vesicles (Hers, 1960). In addition, sperm contain a sorbitol dehydrogenase, and it has been shown that a fructose transporter is expressed in human spermatozoa (Burant et al., 1992). Recently,

Sullivan and colleagues demonstrated that the polyol pathway plays a role in human and bovine sperm physiology (Frenette et al., 2006; Sullivan et al., 2007).

Cell surface AKR1B7 may function via the polyol pathway to modulate sperm motility during epididymal transit. In the polyol pathway, the rate determining step is the reduction of glucose to sorbitol by aldose reductase. Extracellular aldose reductase (AKR1B7) would favour the accumulation of sorbitol within the epididymal fluid. Sorbitol being membrane impermeable would accumulate in the epididymal lumen and contribute to the relatively high osmotic pressure of the epididymal fluid, and deprive the sperm intracellular compartment of an energy source.

Sorbitol is known to be an osmotically active organic solute (Kaneko et al., 1990), which suggests AKR1B7 may play an osmoregulatory role. It has been hypothesized that sorbitol in the epididymal lumen acts as an osmolyte required for volume regulation of the sperm cell (Pruneda et al., 2006). By its accumulation in the excurrent duct luminal compartment, aldose reductase activity, via sorbitol production could thus contribute to sperm transient immobilization within the epididymis.

Although a function of AKR1B7 in conjunction with sorbitol dehydrogenase is to produce fructose, it may fulfill other roles in the male reproductive tract. AKR1B7 could also play a detoxification role, as it has been shown *in vitro* that 4-hydroxynonenal (HNE) is a substrate of the enzyme (Martinez et al., 2001). HNE is a highly toxic and harmful compound (Dianzani et al., 1999; Pizzimenti et al., 2002) whose detoxification into less reactive metabolites is of considerable importance for cellular survival.

Martinez and co-workers demonstrated that HNE can be efficiently detoxified by AKR1B7 in adrenocortical cells and other steroidogenic tissues (Martinez et al., 2001; Vander Jagt et al., 1995). HNE is a product of the lipid peroxidation process. The formation of the highly reactive HNE occurs through reactive oxygen species (ROS) induced peroxidation of polyunsaturated fatty acids (Yi et al., 1997; Comporti, 1998). Lipid peroxidation generates several unsaturated aldehydes that are involved in many of the pathophysiological effects associated with oxidative stress in cells and tissues (Stadman and Berlett, 1997). HNE, a major aldehyde product of membrane lipid

peroxidation is believed to be responsible for the cellular pathological effects observed during oxidative stress *in vivo* (Esterbauer et al., 1991).

Oxidative stress is known to play a major role in the aetiology of defective sperm function through induction of peroxidative damage to the plasma membrane (Aitken and Clarkson, 1987). Moreover, since the membranes of spermatozoa contain higher concentrations of polyunsaturated fatty acids in comparison with other cell types (Aitken, 1994), the presence of AKR1B7 in distal regions of the excurrent ducts, in addition with anti-oxidant systems described in the epididymis (Tramer et al., 1998) may be involved in the protection of spermatozoa against peroxidative damage.

The conversion of glucose to fructose (the polyol pathway) results in the utilisation of both NADPH and NAD^+. Consequently, this could lead to a depletion of NADPH, accumulation of NAD and a shift in the redox state of spermatozoa. It has been shown that both capacitation and the acrosome reaction are redox regulated processes (de Lamirande and Gagnon, 2003; Baker and Aitken, 2004). In addition, polyol pathway-mediated alterations in pyridine nucleotides have been linked to the activation of protein kinases (Nishikawa et al., 2000).

Mounting evidence suggests that aldose reductases interact in some way with signaling cascades involving protein kinase C (PKC). Additionally, studies indicate that the antioxidant and signaling roles of aldose reductase are interlinked and that aldose reductase regulates PKC via a redox sensitive mechanism. It has been proposed that the increase in NADH due to polyol pathway activity increases the production of diacylglycerol (DAG) from dihydroxyacetone phosphate (Thomas et al., 1994). DAG is the physiological activator of PKC whose presence and activity has been demonstrated in mammalian spermatozoa (Baldi et al., 2002; Breitbart et al., 1992; Breitbart and Naor, 1999) indicating a possible involvement in the acrosome reaction. Further support for the notion that PKC plays a role in the activation of sper comes from studies with physiological sperm ligands. It has been demonstrated that both ZP3 and progesterone stimulate phosphoinositide turnover and the acrosome reaction in mammalian spermatozoa (Roldan et al., 1994). Moreover, progesterone stimulated protein phosphorylation is mediated by PKC (O'Toole et al 1996).

It has also been suggested that sorbitol is an inhibitor of phosphatidylinositol turnover, leading to alteration in cell functions that are regulated by DAG and inositol phosphates (Vander Jagt et al., 1990). In this way, an extracellular production of sorbitol may be important, since Ca^{2+} plays a major role in the physiology of spermatozoa.

The mechanism by which AKR1B7 may regulate sperm function remains open to question. It has been proposed that HSPD1-associated proteins form part of a macromolecular protein complex in the sperm plasma membrane and that tyrosine phosphorylation of chaperones during sperm capacitation may then signal the activation of the complex and stimulate a signaling cascade leading to the acquisition of zona binding ability. The data presented in this chapter are in line with the notion that AKR1B7 maybe a constituent of just such a macromolecular protein complex.

Knockout mice deficient in AKR1B7 however, were found to be viable and no reproductive phenotype was observed (Baumann et al., 2007), which indicates that the protein is dispensable for mouse development and reproductive success. Furthermore, it is difficult to envisage such a role for the protein based on its functions in other tissues and cell types where it is involved in glucose metabolism and the detoxification of cytotoxic carbonyl compounds.

CHAPTER 6

LOCALISATION OF CHAPERONE INTERACTING PROTEINS DURING EPIDIDYMAL MATURATION, CAPACITATION AND THE ACROSOME REACTION: HSPE1.

6. Localisation of chaperone interacting proteins during epididymal maturation, capacitation and the acrosome reaction: HSPE1.

6.1 Introduction

As discussed in chapter four, heat shock protein 10 (HSPE1) was identified as a putative binding partner of the molecular chaperone HSPD1 on the surface of capacitated spermatozoa using a co-IP approach with HSPD1-specific antibodies (chapter four).

Historically, HSPE1 (formally HSP10) has been considered only as a partner of HSPD1 in the HSPD1/ HSPE1 protein folding machinery (Zeilstra-Ryalls et al., 1991; reviewed in Hartl, 1996). The HSPD1/HSPE1 complex is believed to be responsible for mediating the correct folding of proteins imported into mitochondria, as well as the refolding of denatured proteins (Czarnecka et al., 2006). Recent data however, suggest that HSPE1 may not only be a component of the folding machine but an active player of the cell signaling network, influencing cell cycle, nucleocytoplasmic transport and metabolism (Czarnecka et al., 2006). As such HSPE1 was selected for further study.

As with the previous chapter, the present study sought to confirm the interaction of the two chaperone proteins and, as an initial step to characterising the function of HSPE1 in the maturation of spermatozoa and fertilization, determine the localisation of the HSPE1 protein during epididymal maturation, capacitation and the acrosome reaction.

6.2 Experimental Rational

In order to confirm the interaction between HSPD1 and HSPE1, an anti-HSPE1 immunoglobulin was used to perform the reciprocal co-IP. The reciprocal co-IP was performed with an HSPE1-specific antibody and the presence of HSPD1 in the immunoprecipitated material demonstrated by western blot analysis (Sec. 2.6.4). The same antibodies were used to determine the localisation of the HSPE1 protein in spermatozoa during epididymal maturation (Sec. 2.6.5).

The localization of HSPE1 on the cell surface was investigated using a membrane impermeable biotinylation reagent (Sec. 2.4.3) in conjunction with western blotting (Sec. 2.6.4). The results of the biotinylation experiments were confirmed by flow cytometry (Sec. 2.6.6).

The effect of capacitation (Sec. 2.3.5) and the acrosome reaction (Sec. 2.3.6) on the localisation of the protein was investigated using an indirect immunofluorescence technique (Sec. 2.6.5).

6.3 Results

6.3.1 Confirmation of HSPD1 interaction with HSPE1 by reciprocal co-immunoprecipitation and western blot analysis.

Confirmation of the interaction between HSPD1 and HSPE1 was demonstrated by performing the reciprocal co-immunoprecipitation experiment utilising an HSPE1-specific antibody and detecting the presence of HSPD1 in the precipitated material by immunoblotting (Fig. 6.1), as described in Sec. 2.4.5.

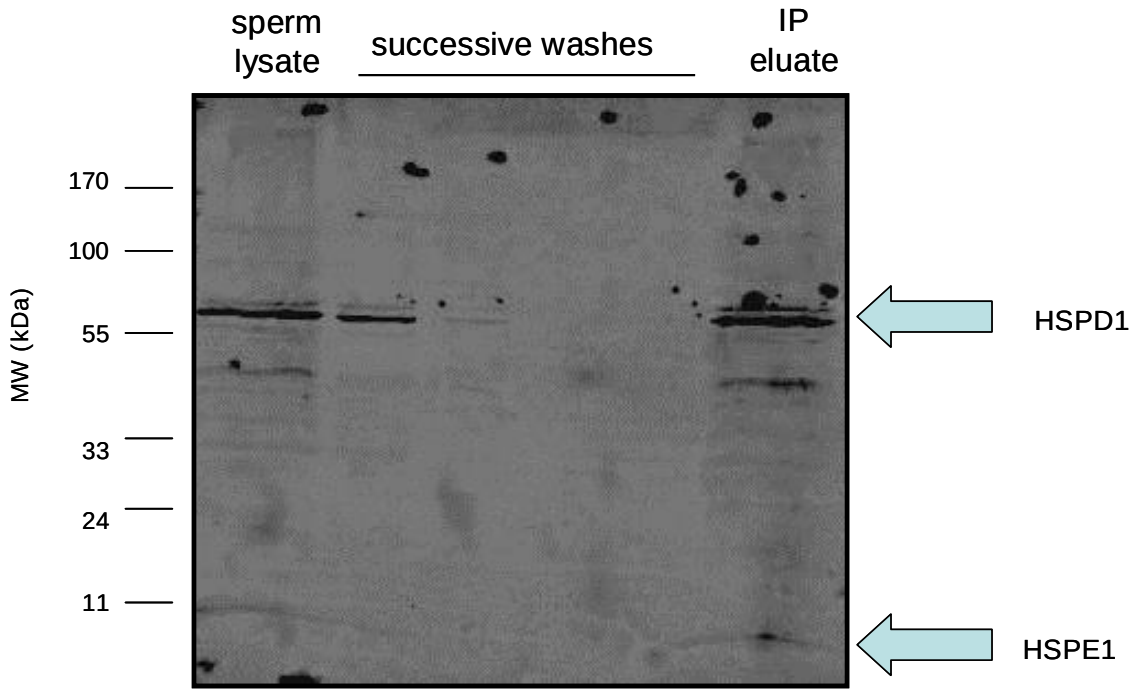


Figure 6.1. Confirmation of the HSPD1-HSPE1 protein interaction by reciprocal co-IP. Caudal spermatozoa were incubated for 90 min in complete BWW supplemented with 1 mM pentoxifyline and 1 mM dibutyryl cAMP, washed and lysed in Buffer B. Clarified lysates were used for immunoprecipitation with an HSPE1-specific antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane for western analysis. The presence of both HSPD1 and HSPE1 were detected with protein-specific antibodies.

HSPD1 was detected by western blot analysis in proteins immunoprecipitated by an HSPE1-specific antibody from lysates of capacitated spermatozoa (Fig. 6.1).

6.3.2 HSPE1 was localised to spermatozoa from the mouse epididymis.

Sperm from the caput, corpus and cauda regions of the epididymis were isolated. Lysates of sperm from different regions of the epididymis, and a total adult testis extract were assayed for the presence of HSPE1 by western analysis.

An HSPE1-specific antibody detected a protein of approximately 10 kDa in lysates derived from caput, corpus and caudal sperm cells, which is consistent with the predicted molecular weight of HSPE1 (Dickson et al., 1994) (Fig. 6.2). Similarly, a protein of the predicted molecular mass was detected in lysates prepared from whole mouse testis. The use of β-tubulin as a loading control indicated that the relative abundance of the chaperone remained constant in sperm as they migrate through the epididymis. These data suggest that HSPE1 is produced and incorporated into sperm within the testis.

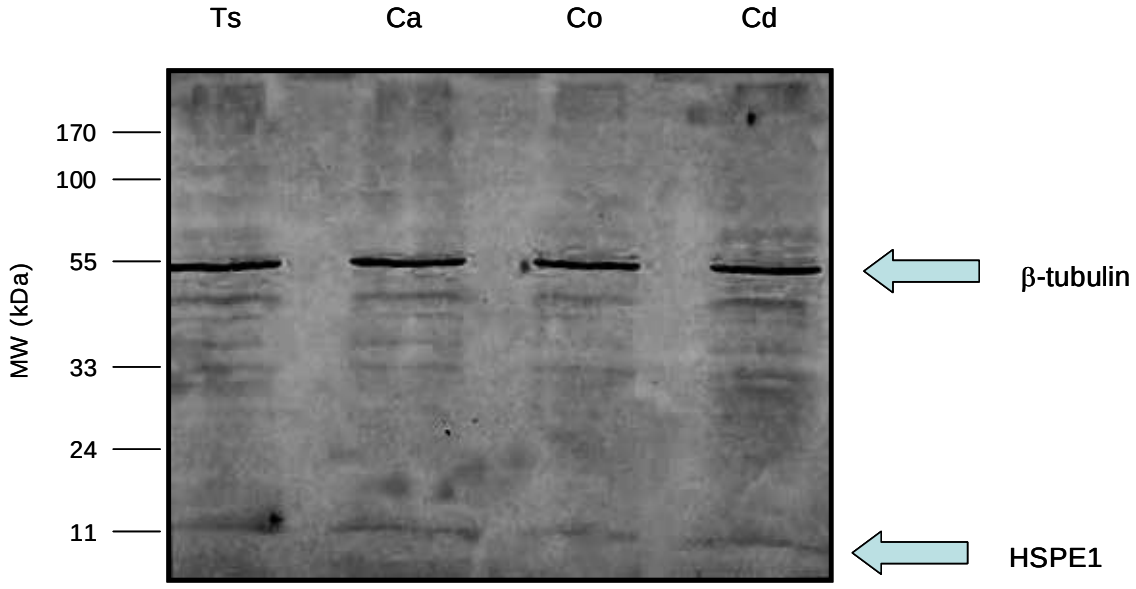


Figure 6.2. Western analysis of HSPE1 in the mouse testis and epididymal spermatozoa. 5μg of cell extracts were resolved by SDS-PAGE and immunoblotted with a specific-HSPE1 antibody. Blots were also probed with a β-tubulin antibody to assess relative protein loading in each lane. Mouse testis total cell extract **(Ts)**, purified sperm from mouse caput **(Ca)**, corpus **(Co)**, and cauda **(Cd)** epididymides.

6.3.3 The molecular chaperone HSPE1 was localised on the surface of capacitated mouse spermatozoa.

Since the co-IP experiments that pulled down the HSPD1-HSPE1 complex were designed to isolate proteins from the surface of capacitated spermatozoa, experiments were performed to assess the surface localisation of HSPE1. As described in Sec. 2.4.3, sperm surface proteins were labeled with a membrane impermeable biotinylation reagent and recovered from lysates with immobilised streptavidin. Putative surface proteins were resolved by SDS-PAGE and the presence of HSPE1 was demonstrated by western analysis with an HSPE1-specific antibody. A single protein band of the predicted molecular weight for HSPE1 was detected in the purified plasma membrane protein preparation (Fig. 6.3).

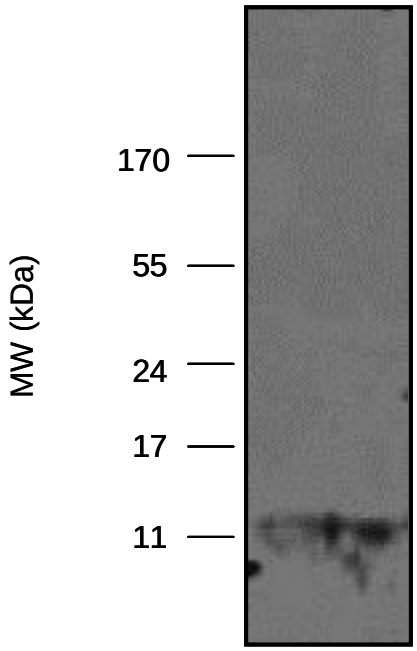


Figure 6.3. Heat shock protein 10 (HSPE1) protein was localised on the surface of capacitated spermatozoa. Caudal epididymal spermatozoa were capacitated for 90 min in complete BWW. Surface proteins were biotinylated, affinity purified and resolved by SDS-PAGE. HSPE1 was identified by immunoblotting with a specific HSPE1 antibody. One band of the predicted size, 10 kDa, was identified.

The presence of HSPE1 on the cell surface was ultimately confirmed by flow cytometry (Fig. 6.4 and 6.5). The process of capacitation is accompanied by a remodeling of the plasma membrane and can result in the redistribution of sperm surface proteins (Lopez and Shur, 1987; Della Giovampaola et al., 2001; Focarelli et al., 1998). To investigate whether capacitation influenced the percentage of sperm that exhibited surface HSPE1, both uncapacitated and capacitated spermatozoa were analysed using a flow cytometric strategy.

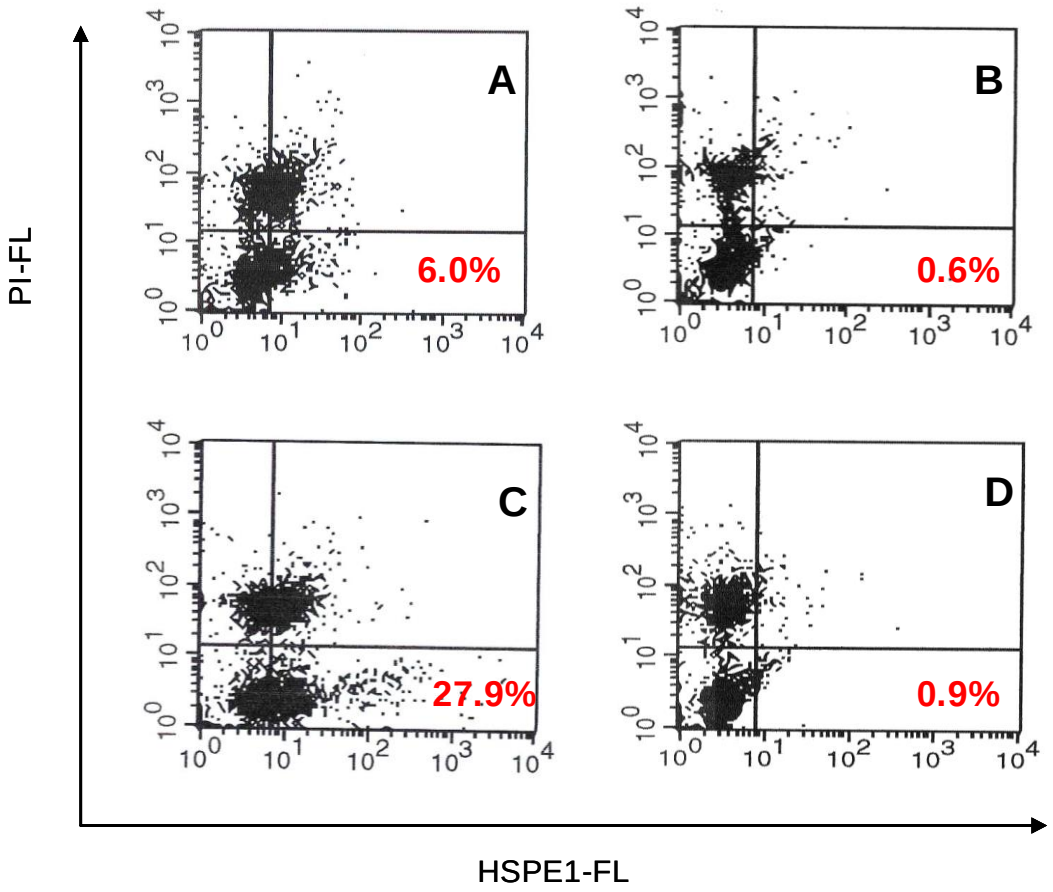


Figure 6.4. Flow cytometric analysis of HSPE1 on the surface of mouse spermatozoa. Surface localisation of HSPE1 on living sperm was demonstrated by flow cytometry. Spermatozoa were recovered from the cauda epididymis and either labeled, as described in Sec. 2.6.6, or capacitated in complete BWW media for 45 min prior to labeling. Surface HSPE1 was bound using an HSPE1-specific antibody and visualized using an FITC-labeled antibody (HSPE1-FL). Cells were then treated with propidium iodide to label dead cells which were subsequently gated out of the analysis (PI-FL). The percentage of sperm with surface labeling is indicated in the bottom right hand quadrant. **A** and **B**) Uncapacitated spermatozoa. **C** and **D**) Capacitated spermatozoa. **B** and **D**) Control experiments omitting the primary HSPE1 antibody.

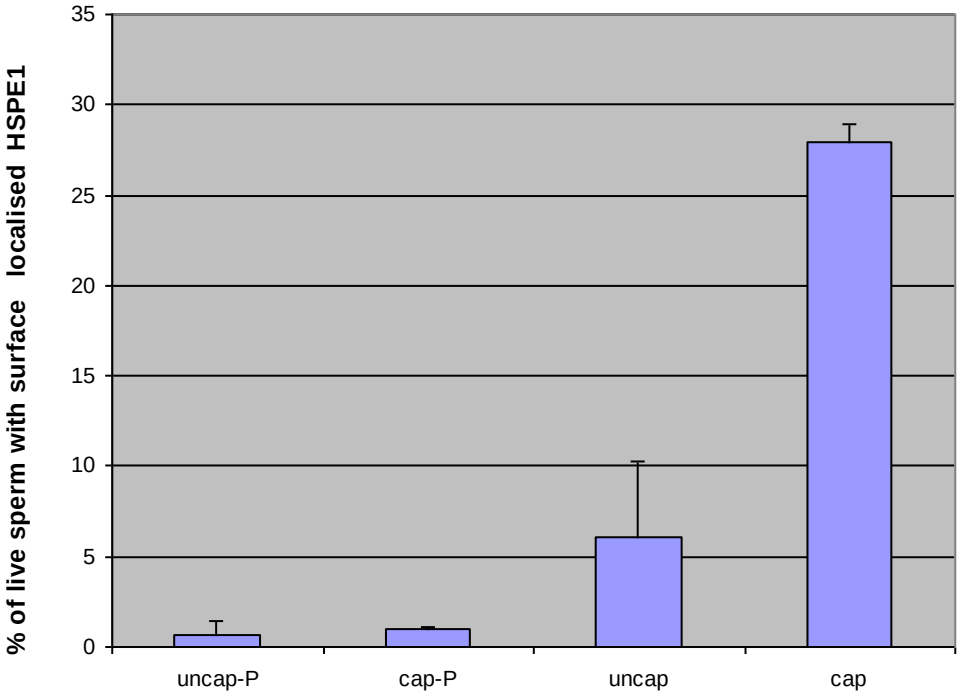


Figure 6.5. Flow cytometric analysis showing capacitation-dependent exposure of HSPE1 on the surface of live mouse spermatozoa. Spermatozoa were recovered from the cauda epididymis and either labeled, as described, or capacitated in complete BWW media for 45 min prior to labeling. Surface localised HSPE1 was bound using an HSPE1-specific antibody and visualized using an FITC-labeled antibody. Cells were then treated with propidium iodide to label dead cells which were subsequently gated out of the analysis (n=3). uncap-P: uncapacitatd sperm control omitting the primary antibody, cap-P: capacitated sperm control omitting the primary antibody, uncap: uncapacitated sperm, cap: capacitated sperm.

Propidium iodide staining was used to stain non-viable cells and facilitated the exclusion of dead and membrane compromised cells from the analysis. Since live, membrane intact sperm (PI negative) would exclude antibody entry into the cytoplasm, any fluorescence would be indicative of HSPE1 surface localisation. In Fig. 6.4, the HSPE1-FL channel measured fluorescein isothiocyanate (FITC) labeling of cells (HSPE1 localisation), and the PI-FL channel measured propidium iodide staining (membrane compromised, dead cells).

As demonstrated in Figs. 6.4 and 6.5, in uncapacitated sperm 6% of live cells were positively labeled for HSPE1. After capacitation however, 28% of live cells bound the antibody. These data not only confirmed the surface localization of HSPE1, but also indicate that the process of capacitation is correlated with a significant increase in the level of surface exposure of HSPE1. As anticipated, spermatozoa in the control population, prepared in the absence of primary antibody, displayed only background levels of fluorescence; uncapacitated (0.6%) and capacitated (0.9%) (Fig. 6.4 B and D, lower right quadrants; Fig. 6.5).

6.3.4 HSPE1 localised to the peri-acrosomal region of mouse sperm.

In order to determine the cellular localization pattern of the protein, localisation studies were performed on sperm isolated from different regions of the epididymis by indirect immunofluorescence (IIF) (Sec. 2.6.5).

HSPE1 was detected in the peri-acrosomal region of sperm taken from the caput and corpus epididymis (green labeling in Fig 6.6 A and B respectively). In mature sperm isolated from the cauda region however, HSPE1 was observed in the principal piece of the tail in addition to stronger labeling in the peri-acrosomal region of the sperm head (green labeling in Fig. 6.6 C). No labeling was observed in controls in which the primary antibody was omitted (not shown).

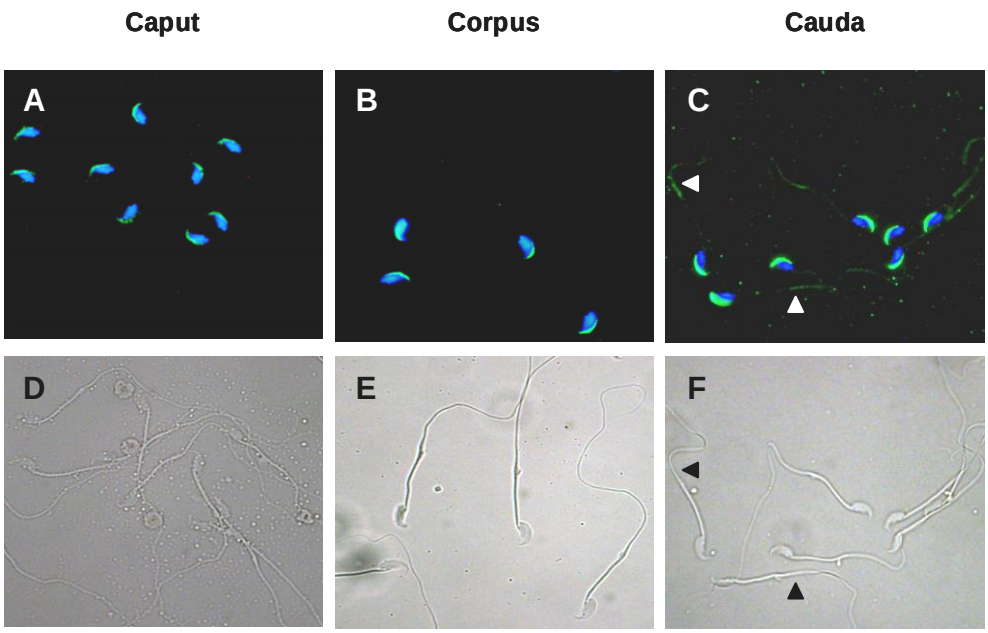


Figure 6.6. Indirect immunofluorescence localisation of HSPE1 on epididymal spermatozoa. As described in section 2.6.5 epididymal sperm were incubated with anti-HSPE1 immunoglobulin followed by an FITC-conjugated secondary antibody. HSPE1 labeling was present in the acrosomal region of spermatozoa taken from all regions of the epididymis and in the principal piece of the tail in spermatozoa isolated from the cauda epididymis (arrow heads). **A** and **D**) caput spermatozoa; **B** and **E**) corpus spermatozoa; **C** and **F**) caudal spermatozoa. **A**, **B**, and **C**) labeled with an HSPE1 antibody (green) and DAPI (blue); **D**, **E**, and **F**) phase contrast. Control experiments omitting the primary antibody displayed negligible staining (not shown).

These data indicate that HSPE1 immunoreactive products were present in the acrosomal region of spermatozoa taken from all regions of the mouse epididymis. However, the protein was only detected in the principal piece of the tail in mature

cells isolated from the cauda epididymis (Fig. 6.6 C). This peri-acrosomal localization pattern was supportive of a role for HSPE1 in capacitation-dependent acquisition of zona binding ability.

6.3.5 Acrosomal loss was correlated with loss of HSPE1 from the sperm head.

Additional support for the peri-acrosomal localisation of HSPE1 was achieved by following the fate of the protein subsequent to induction of acrosomal exocytosis. For these studies, cauda epididymal sperm were capacitated and the acrosome reaction induced using calcium ionophore, A23187 (Sec. 2.3.6). Both capacitated and acrosome reacted spermatozoa were probed with the HSPE1 polyclonal antibody followed by FITC-conjugated secondary antibody (green labeling in Fig. 6.7). DNA was labeled with DAPI (blue labeling in Fig. 6.7).

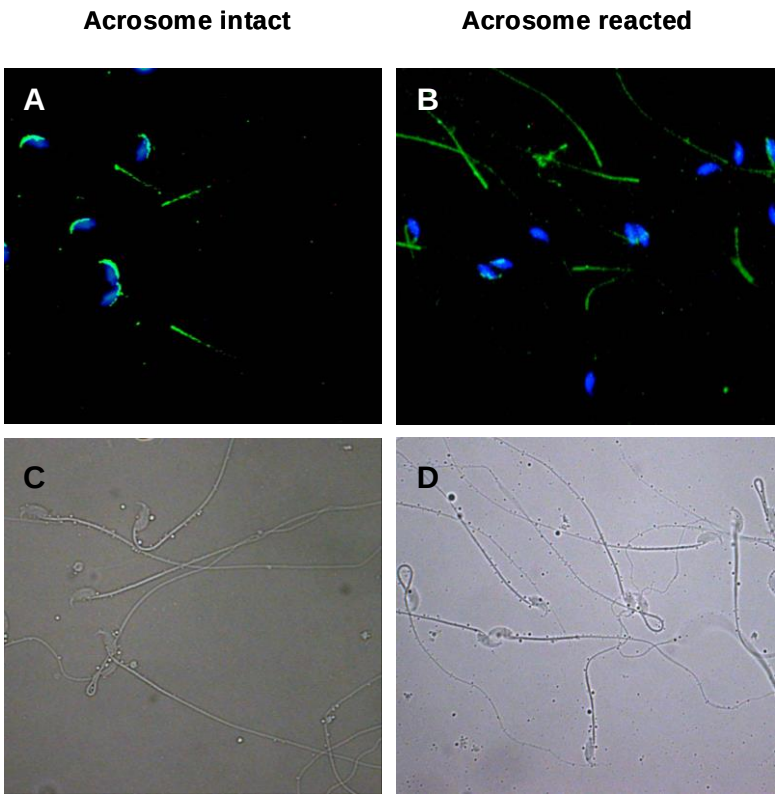


Figure 6.7. Indirect immunofluorescence demonstrating localisation of HSPE1 in capacitated, acrosome intct versus capacitated, acrosome reacted spermatozoa. Cauda epididymal sperm were capacitated (Sec. 2.3.5), acrosome reacted (Sec. 2.3.6) and incubated with anti-HSPE1 immunoglobulin followed by an FITC-conjugated secondary antibody (Sec. 2.6.5). **A** and **C**) capacitated acrosome intact spermatozoa; **B** and **D**) capacitated acrosome reacted cells. **A** and **B**) labeled with an HSPE1 antibody (green) and DAPI (blue); **C** and **D**) phase contrast.

Figure 6.7 indicates that the process of capacitation had no effect on the localization of HSPE1 in mouse caudal spermatozoa. The same localization pattern observed with uncapacitated cells (Fig 6.6 C) was evident with capacitated spermatozoa (Fig. 6.7 A). The protein localised to the peri-acrosomal region of the sperm head and the principal piece of the flagellum. Although acrosomal exocytosis had no observable effect on the localization of HSPE1 in the sperm tail, the strong acrosomal staining observed in sperm taken from all regions of the epididymis (Fig. 6.6 A, B and C; Fig. 6.7 A) was absent in sperm that have undergone ionophore-induced acrosomal exocytosis (Fig. 6.7 B).

These data further support the localization of HSPE1 to the peri-acrosomal region of the sperm head. Collectively, these data and the FACS data suggest that HSPE1 is localized to the plasma membrane overlying the acrosome, an ideal position for a proposed role in zona recognition and interaction.

6.4 Discussion

Data presented in this chapter documents for the first time the surface localisation of the molecular chaperone HSPE1 in mouse spermatozoa.

Reciprocal co-immunoprecipitation confirmed the association of HSPE1 with HSPD1 in capacitated sperm. Western and indirect immunofluorescence analysis demonstrated that the protein was present in sperm isolated from all regions of the epididymis but its localization is influenced by the degree of epididymal maturation the cells have attained. In spermatozoa from all regions of the epididymis, HSPE1 localised to the peri-acrosomal region of the head, a position consistent with the protein having a role in fertilization related events. However, only in mature cells derived from the cauda region of the epididymis was the protein detected in the principal piece of the sperm tail. The tail localization suggests that HSPE1 may have a role in regulating the motility of spermatozoa. Localisation of HSPE1 to the acrosomal region is suggestive of a role in interaction with the oocyte. Furthermore, the fact that this pattern of localization was lost once spermatozoa have lost their acrosome further strengthens the concept that HSPE1 is localised on the surface of capacitated sperm and may be involved in oocyte interactions.

Molecular chaperones are abundant, highly conserved cellular proteins with roles in facilitating the correct folding of proteins. Many chaperones were originally termed "heat shock" proteins due to their up-regulation after environmental stress, however, it is now well established that these proteins are vital for correct cellular function at all times. It is likely that most, if not all, cellular proteins will interact with chaperone proteins at some stage of their life cycle (Ellis and van der Vies, 1991; Ellis, 2000). The basic paradigm of chaperone function is the recognition and selective binding of the hydrophobic residues of immature proteins to form stable complexes, the dissociation of which is often stimulated by the binding and hydrolysis of ATP. Different members of this protein superfamily utilise distinct strategies to prevent the misfolding and aggregation of newly synthesized proteins in the crowded cellular environment. Further, it is becoming increasingly apparent that chaperone activity is

often the result of the cooperation of several different members of this protein class, as well as a variety of cofactors, to form a 'super-chaperone complex'.

The major families of molecular chaperones are the 40kDa heat shock protein (HSP40; the DnaJ family), 60 kDa heat shock protein (HSPD1, including GroEL and the T-complex polypeptide 1 (TCP-1) ring complexes), 70 kDa heat shock protein (HSP70) and 90 kDa heat shock protein (HSP90) families. Chaperones have been implicated in a range of pathological states, highlighting the clinical relevance of this class of proteins and their regulators. For further consideration of chaperone structure and function the reader is directed to the following reviews (Bukau et al., 2000; Fink, 1999; Young et al., 2004; Ellis, 2007)

Several somatic and germ cell-specific molecular chaperones have been identified in mammalian spermatozoa (Miller et al., 1992; Kamaruddin et al., 2004; Ikawa et al., 1997; Nixon et al., 2005), however, the functional significance of many of these proteins in the process of reproduction remains unclear. The most well characterised sperm chaperone is calmegin, which was originally identified as a calcium-binding protein present specifically in male germ cells and expressed from mid-pachytene through to late spermatids (Tanaka et al., 1997; Watanabe et al., 1994; Yoshinaga et al., 1999). Despite the fact that calmegin is not found in mature sperm, it has been identified as a critical molecule in sperm-zona interaction through its role during spermatogenesis. Calmegin helps establish the correct sperm architecture by ensuring the appropriate folding of nascent endoplasmic reticulum glycoproteins destined for the acrosomal matrix or the plasma membrane of mature spermatozoa (Ikawa et al., 2001; Ikawa et al., 1997; Yamagata et al., 2002). Targeted disruption of the calmegin gene led to impaired male fertility that appeared to be based solely on the loss of spermatozoa's ability to adhere to the zona pellucida (Ikawa et al., 1997). Further studies demonstrated that sperm from calmegin knockout mice do not ascend into the oviduct *in vivo*, indicating an additional problem with motility (Ikawa et al., 2001). Absence of signaling proteins or antigenic determinants from the surface of sperm was proposed as a mechanism for this impaired sperm transport. Spermatozoa from calmegin null mice also lack fertilin β, a protein implicated in sperm-egg plasma membrane binding and fusion (Ikawa et al., 2001). Thus, the chaperone function of

calmegin may facilitate the correct function or placement of a diversity of sperm molecules.

In addition to calmegin, male germ cells contain a number of members of the heat shock protein 70 (HSP70) family including the testis-specific isoform HSP70-2, the expression of which is developmentally regulated during spermatogenesis (Bohring et al., 2001; Huszar et al., 2000; Miller et al., 1992). Originally identified as a putative creatine kinase M-isoform, this protein has been proposed as a marker of sperm quality in several species (Huang et al., 2000; Huszar et al., 1994; Sidhu et al., 1998). Targeted disruption of the Hsp70-2 gene renders male mice infertile because of a lack of post-meiotic spermatids and mature spermatozoa (Dix et al., 1996). This study suggests that Hsp70-2 has a critical function in the synaptonemal complex during male germ cell meiosis.

HSP90 has been identified in both human and mouse sperm as a key phosphoprotein activated during capacitation (Ecroyd et al., 2003; Ficarro et al., 2003). The localisation and mechanism of action of HSP90 in mature sperm, however, remains to be elucidated. While both HSP90B1 and HSPD1 have been reported in the testis (Aguilar-Mahecha et al., 2001; Meinhardt et al., 1995; Werner et al., 1997), it was only recently demonstrated that both chaperones are present in mature spermatozoa (Asquith et al., 2004).

Although chaperones were initially thought to be restricted to organelles within the cell, a growing body of literature describes roles for these proteins at the cell surface and the extracellular space. Calnexin, calreticulin, GP96, GRP74, HSP72, HSP70, HSPD1, HSP54, HSP27 and protein disulfide isomerase (PDI) have all been reported on the surface of cell types including cancer cells, fibroblasts and blood cells (Akagi et al., 1988; Altmeyer et al., 1996; Essex et al., 1995; Ferrarini et al., 1992; Goicoechea et al., 2000; Okazaki et al., 2000; White et al., 1995; Wiest et al., 1997; Weist et al., 1995). A recent study has revealed the major proteins present on the surface of certain cancer cells are molecular chaperones (Shin et al., 2003).

Consistent with the findings of the current study, chaperone proteins have been detected on the oocyte (Calvert et al., 2003). HSP90B1, GRP78, calreticulin and HSP90 were identified on the surface of the mature mouse oocyte, providing the first

suggestion of a role for surface chaperones in mammalian fertilisation. Interestingly, both GRP78 and HSPD1 have been identified on the surface of bovine oviductal epithelial cells and found to strongly associate with spermatozoa, implying a role for these proteins in the events preceding fertilisation (Boilard et al., 2004).

Chaperones may interact to form multi-component complexes. This phenomenon is thought to facilitate the complete folding of polypeptide substrates without their exposure to the cytosol (Frydman and Höhfeld, 1997). A number of co-chaperone molecules have now been described and new candidates continue to be reported. The formation of a multi-chaperone complex between HSP90 and HSP70 is regulated by a number of cofactors including p23, the BCL2-associated athanogene-1 (BAG-1), HSP70-interacting protein (HIP) and HSP-organising protein (HOP), activator of HSP90 ATPase (AHA1) and carboxyl terminus of HSC-70 interacting protein (CHIP) (Ballinger et al., 1999; Chen and Smith, 1998; Hernandez et al., 2002; Morishima et al., 2003; Panaretou et al., 2002; Pratt and Toft, 2003). The HSP70/HSP90 multi-chaperone machinery can be adapted by specific co-chaperone molecules to perform alternative functions in the sorting of proteins to specific cellular compartments, or to the proteasome for subsequent degradation (Young et al., 2004).

Heat shock protein 10 (HSPE1) has been identified as a co-chaperone for HSPD1, forming a seven membered ring that binds to the HSPD1 oligomer and stimulates release of the client protein into the central cavity of HSPD1 (Richarson et al., 1998). Following correct folding HSPE1 is released, allowing exit of the mature substrate protein. The work detailed in this chapter documents for the first time the presence of co-chaperones in mammalian spermatozoa.

Work presented in Chapter four of this thesis identified HSPE1 as a putative HSPD1-interacting protein on the surface of capacitated mouse spermatozoa. The data in Fig. 6.1 confirms the physical association of the two chaperone proteins in capacitated mouse spermatozoa. In the reciprocal co-immunoprecipitation experiment, HSPD1 was detected in the material immunoprecipitated by an antibody specific for HSPE1.

As described earlier, the physical association of HSPE1 and HSPD1 is not without precedent. The association of HSPD1 and HSPE1 is well documented in other cells.

In *E.coli*, the homologous proteins GroEL (HSPD1) and GroES (HSPE1) function as a team in the protein folding and assembly process (Gething and Sambrook, 1992). Both proteins exhibit a seven-fold axis of symmetry with GroES forming a heptameric ring of identical subunits that binds to each end of GroEL to form a functional heterodiamer. In eukaryotic organisms the HSPD1/ HSPE1 protein folding machinery is thought to be restricted to subcellular organelles such as mitochondria and chloroplasts (Ellis and van der Vies, 1991; Langer and Neupert, 1991; Craig et al., 1993). The current study is the first report of an HSPD1/ HSPE1 interaction on the surface of mammalian spermatozoa.

Having confirmed the presence and interaction of HSPD1 and HSPE1 in capacitated caudal spermatozoa, I sought to examine the localization of HSPE1 during epididymal maturation, capacitation and the acrosome reaction as an initial step to characterising the role of the protein in sperm function.

The relative levels of HSPE1 in spermatozoa at different stages of epididymal maturation was determined. For this purpose lysates of sperm taken from different regions of the epididymis, together with a testis total cell extract were compared. Western blot analysis of these extracts detected a protein of the predicted size for HSPE1, 10.8 kDa. As indicated in Fig. 6.2, HSPE1 was detected in spermatozoa at all stages of maturation and the relative level of the protein was unchanged. In addition, no change in the molecular weight of the protein was detected between sperm taken from different regions of the epididymis.

Indirect immunofluorescence studies localised HSPE1 to the peri-acrosomal area of the sperm head in spermatozoa taken from all regions of the epididymis (Figs. 6.6 & 6.7). In addition, the protein localises to the principal piece of the flagellum in sperm from the cauda epididymis. Whether this represents a redistribution of the protein or the unmasking of an epitope during epididymal maturation remains to be established.

If HSPE1 plays a role in the interaction of the gametes, it should be localised on the surface of functionally competent capacitated caudal spermatozoa. Biotinylation experiments targeting proteins of the plasma membrane and flow cytometry have demonstrated that HSPE1 is indeed localized to the cell surface in mature mouse

spermatozoa. In uncapacitated sperm populations, HSPE1 antibody bound to the surface of 6% of viable cells (Figs. 6.4 & 6.5). Following capacitation, there was a significant increase ($P<0.01$) in the proportion of spermatozoa expressing HSPE1 at the cell surface (Figs. 6.4 & 6.5) with 28% of viable cells binding HSPE1 antibody. Control experiments where the primary HSPE1 antibody was omitted displayed negligible levels of fluorescence. This increase in the superficial localisation of HSPE1 during capacitation not only implicates the protein in the ability of spermatozoa to bind to the zona pellucida, but also highlights the potential use of the protein as a marker to monitor the capacitation status of sperm. Pleasingly, the change in the level of surface expression of HSPE1 following capacitation is consistent with that reported for HSPD1 where 6% of uncapacitated cells expressed the protein at the cell surface compared to 27% of capacitated cells (Asquith et al., 2005).

This surface localisation of HSPE1 is not surprising considering the growing body of literature describing the extra-mitochondrial localisation of chaperone proteins, including the cell surface (Kaur et al., 1993; Soltys and Gupta, 1996). HSPE1 has been described at several extra-mitochondrial sites including zymogene granules, hormone granules, secretory granules, and mature red blood cells (Sadacharan et al., 2001). HSPE1 has also been detected in the cytoplasm of cancer cells (Sadacharan et al., 2001; Cappello et al., 2003; Hansen et al., 2003). In addition, both HSPD1 and HSPE1 have been described extracellularly. HSPD1 has been identified in the peripheral circulation of normal individuals (Pockley et al., 1999), while HSPE1 has been detected in the serum and urine of pregnant women during the first and second trimesters (Morton et al., 1977). Termed early pregnancy factor (EPF), this extracellular form of HSPE1 has been characterised to be a major immunosuppressive agent during pregnancy (Morton et al, 1992). More recently the protein has been shown to exhibit growth factor properties (Morton, 1998).

A number of possible roles for HSPE1 at the cell surface may be proposed. HSPE1 may be acting as an accessory molecule to HSPD1. This suggests a direct transposition of HSPD1-HSPE1 function to the plasma membrane where it could function to improve the efficiency of the folding and assembly of surface molecules. Similar levels of HSPD1 surface exposure in uncapacitated and capacitated cells are consistent with this notion.

The mechanism by which HSPE1 may regulate sperm function remains open to question. It has been proposed that HSPD1-associated proteins form part of a macromolecular protein complex in the sperm plasma membrane and that tyrosine phosphorylation of chaperones during sperm capacitation may then signal the activation of the complex and stimulate a signaling cascade leading to the acquisition of zona binding ability.

If HSPE1 plays a role in the interaction of the gametes, as we have hypothesized for the chaperones HSPD1, then these proteins would be expected to colocalize on the apical surface of the head of live, capacitated sperm. Indirect immunofluorescence, surface biotinylation and flow cytometry demonstrated that HSPE1 does indeed localise to the apical surface of the head of live, capacitated spermatozoa, where HSPD1 has been localised. Moreover, the process of capacitation, where sperm realize their potential to engage the zona pellucida results in similar levels of increase in the cell surface exhibition of both HSPE1 and HSPD1. Taken together, these findings implicate HSPE1 in the capacitation-dependent acquisition of zona binding.

Further support for HSPE1 having a role in fertilisation was demonstrated when it was reported that HSPE1 antibodies inhibited the sperm-zona pellucida interaction in a concentration-dependent manner (Walsh et al., 2008). However, the possibility that HSPE1 acts as a direct receptor for the zona pellucida was rejected on the basis that recombinant HSPE1 protein failed to adhere to the zona pellucida and competitively inhibit sperm interaction.

It was interpreted that HSPE1 is localized within close proximity of the zona receptor, and suggested that the chaperone may be involved in chaperone-mediated assembly of a zona-receptor complex, a notion that is consistent with the data described in this chapter. The data within this chapter was incorporated into Walsh et al., 2008 (I am equal first author, appendix E).

CHAPTER 7

LOCALISATION OF CHAPERONE INTERACTING PROTEINS DURING EPIDIDYMAL MATURATION, CAPACITATION AND THE ACROSOME REACTION: CITRON KINASE

7. Localisation of chaperone interacting proteins during epididymal maturation, capacitation and the acrosome reaction: Citron kinase.

7.1 Introduction

As discussed in chapter four, citron kinase (CRIK) was identified as a putative binding partner of the molecular chaperone HSPD1 on the surface of capacitated spermatozoa using a co-IP approach with HSPD1-specific antibodies (chapter four).

To date, the characterization of proteins that undergo tyrosine phosphorylation during capacitation has been limited. Furthermore, the identification of protein kinases involved in the signaling pathways that culminate in the capacitation-associated increases in tyrosine phosphorylation and acrosomal exocytosis are yet to be resolved. As such CRIK was selected for further study.

As with the previous chapters, the present study sought to confirm the interaction of the two proteins. As an initial step toward characterising the function of CRIK in spermatozoa and fertilization, determine the localisation and relative level of the protein during epididymal maturation, capacitation and the acrosome reaction.

7.2 Experimental Rationale

A CRIK antibody was obtained from a commercial source and used in an attempt to confirm the interaction of CRIK with HSPD1. The reciprocal co-immunoprecipitation was performed with a CRIK-specific antibody and the presence of HSPD1 in the immunoprecipitated material was assayed by western blot analysis (Sec. 2.6.4). The same antibody was then used to determine the localisation of the CRIK protein in spermatozoa at different stages of epididymal maturation (Sec. 2.6.5).

The localization of CRIK on the cell surface was investigated using a membrane impermeable biotinylation reagent (Sec. 2.4.3) in conjunction with western blotting (Sec. 2.6.4). The results of the biotinylation experiments were confirmed by flow cytometry (Sec. 2.6.6).

The effect of capacitation (Sec. 2.3.5) and the acrosome reaction (Sec. 2.3.6) on the localisation of CRIK were investigated using an indirect immunofluorescence technique (Sec. 2.6.5).

7.3 Results

7.3.1 Confirmation of HSPD1 interaction with citron kinase by reciprocal co-immunoprecipitation and western blot analysis.

Co-immunoprecipitation experiments using an HSPD1 antibody identified the rho effector protein citron kinase (CRIK) as a putative HSPD1 interacting protein in capacitated sperm (chapter four). In an attempt to confirm the interaction between HSPD1 and CRIK the reciprocal co-immunoprecipitation experiment utilising a CRIK-specific antibody was performed. Heat shock protein 60 (HSPD1) was not detected by western blotting in the immunoprecipitated material. More importantly, the target protein, in this case citron kinase, was not detected in the immunoprecipitated material (Fig. 7.1). These data suggest that the CRIK antibody was not capable of immunoprecipitating CRIK.

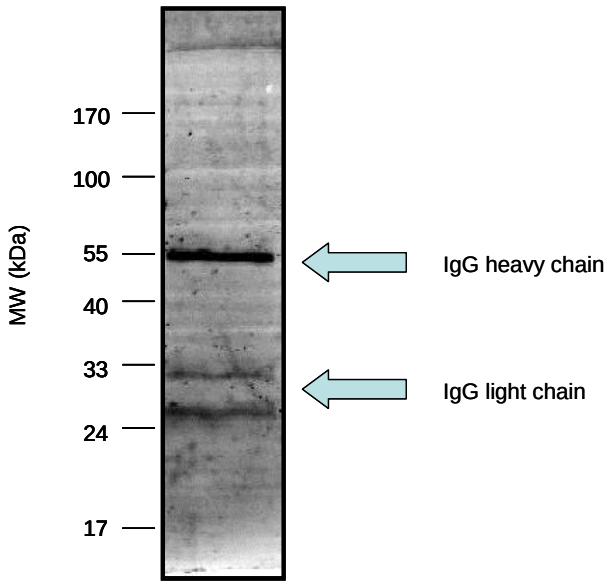


Figure 7.1. Reciprocal co-IP. Caudal spermatozoa were incubated for 90 min in complete BWW supplemented with 1 mM pentoxifyline and 1 mM dibutyryl cAMP, washed and lysed in Buffer B. Clarified lysates were used for immunoprecipitation with a CRIK-specific antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membrane for western analysis. The presence of neither HSPD1 nor CRIK could not be shown with protein specific antibodies.

The bands present in the western blot shown in Fig. 7.1 were determined to be heavy and light chain fragments of the immunoprecipitating antibody as they were also detected when the antibody alone was subjected to gel electrophoretic and western analysis (data not shown). This result suggested that the epitope recognized by the anti-CRIK antibody was masked or inaccessible to the antibody during immunoprecipitaion, which is not unusual for proteins that are constituents of multi-protein complexes. Alternatively, the antibody-antigen complex may have had a half life so short that any bound CRIK may have been lost in the washing prior to elution of proteins. BIACORE analysis would be capable of determining if this was indeed the case.

7.3.2 CRIK was localised to spermatozoa from the mouse epididymis.

In order to determine the origin of CRIK in sperm, an adult testis extract and sperm isolated from different regions of the epididymis were assayed for the presence of CRIK by western blot analysis. A CRIK-specific antibody detected three proteins in lysates. In the testis lysate two proteins were detected, one with a molecular weight of approximately 230 kDa (band 1, Fig.7.2) and another of about 55 kDa (band 2, Fig.7.2). However, in lysates generated from epididymal sperm, a 55 kDa protein was observed in the sample derived from caput sperm (band 3, Fig.7.2), while in the corpus and caudal derived sample, a protein of approximately 85 kDa was detected (bands 4 and 5 respectively, Fig.7.2). CRIK may exist in five different isoforms having molecular masses of 239, 235, 230, 183.5 and 55.6 kDa. The use of β-tubulin as a loading control indicated that a comparable amount of protein was used in each sample. A~55 kDa band consistent with the predicted mass of β-tubulin was detected in all samples.

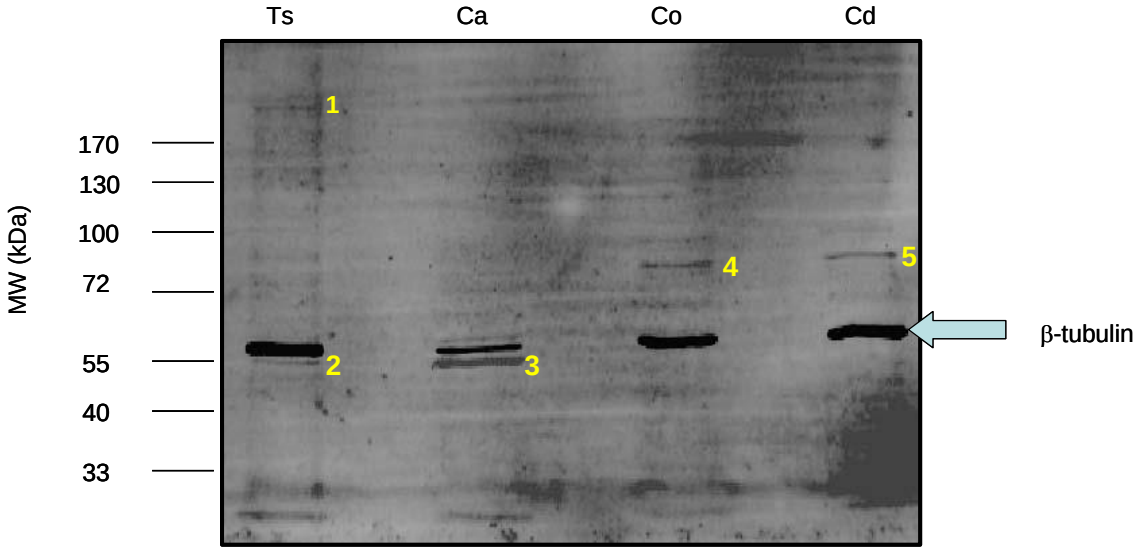


Figure 7.2. Western analysis of CRIK in the mouse testis and epididymal spermatozoa. 5µg of cell extracts were resolved by SDS-PAGE and immunoblotted with a CRIK-specific antibody. Blots were also probed with a β-tubulin antibody to confirm equal protein loading in each lane. Mouse testis total cell extract **(Ts)**, purified sperm from mouse caput **(Ca)**, corpus **(Co)**, and cauda **(Cd)** epididymides. A number of proteins (1-5) with varying masses were deected.

7.3.3 Citron Kinase localizes to the surface of capacitated mouse spermatozoa.

Since the co-IP experiments that pulled down the HSPD1-CRIK protein complex were designed to isolate chaperone-associated proteins from the surface of capacitated spermatozoa, experiments were performed to assess the surface localisation of CRIK. As described in Sec. 2.4.3, sperm surface proteins were labeled with a membrane impermeable biotinylation reagent and recovered from lysates with immobilised streptavidin. Putative surface proteins were resolved by SDS-PAGE and the presence of CRIK assayed for by western blot analysis with a CRIK- specific antibody. Two proteins of about 80 and 90 kDa were detected in the purified plasma membrane protein preparation (Fig. 7.3).

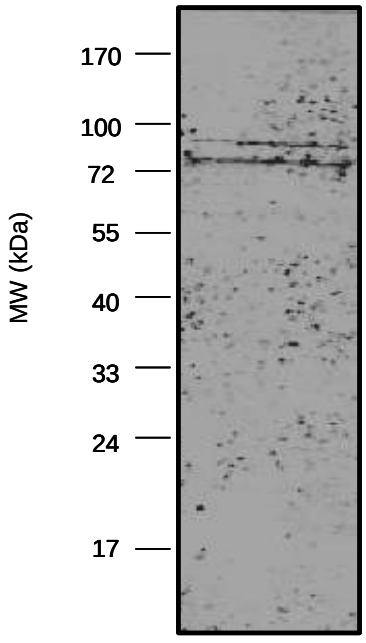


Figure 7.3. Citron kinase (CRIK) protein was localised on the surface of capacitated spermatozoa. Caudal epididymal spermatozoa were capacitated for 90 min in complete BWW. Surface proteins were biotinylated, affinity purified and resolved by SDS-PAGE. CRIK was identified by immunoblotting with a CRIK-specific antibody. Two bands of about 80 and 90 kDa, were identified.

To investigate whether capacitation influenced the level of CRIK surface localisation, both uncapacitated and capacitated sperm were analysed by flow cytometry (Sec. 2.6.6).

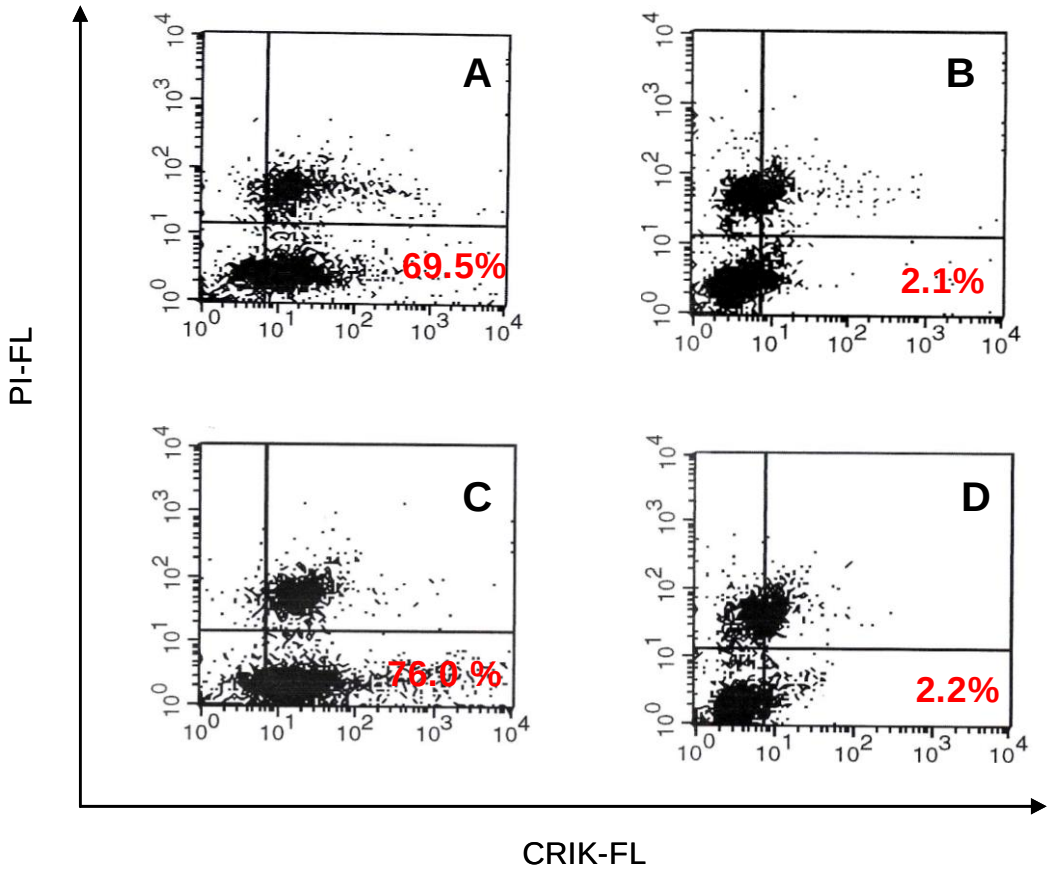


Figure 7.4. Flow cytometric analysis of CRIK on the surface of mouse spermatozoa. Surface localisation of CRIK on living sperm was demonstrated by flow cytometry. Spermatozoa were recovered from the cauda epididymis and either labeled as described in section 2.6.6 or capacitated in complete BWW media for 45 min prior to labeling. Surface CRIK was bound using a CRIK-specific antibody and visualized using an FITC-labeled secondary antibody (CRIK-FL). Cells were then treated with propidium iodide to label dead cells which were subsequently gated out of the analysis (PI-FL) (n=3). The percentage of sperm with surface labeling is indicated in the bottom right hand quadrant. **A** and **B**) Uncapacitated spermatozoa. **C** and **D**) Capacitated spermatozoa. **B** and **D**) Control experiments omitting the primary CRIK antibody.

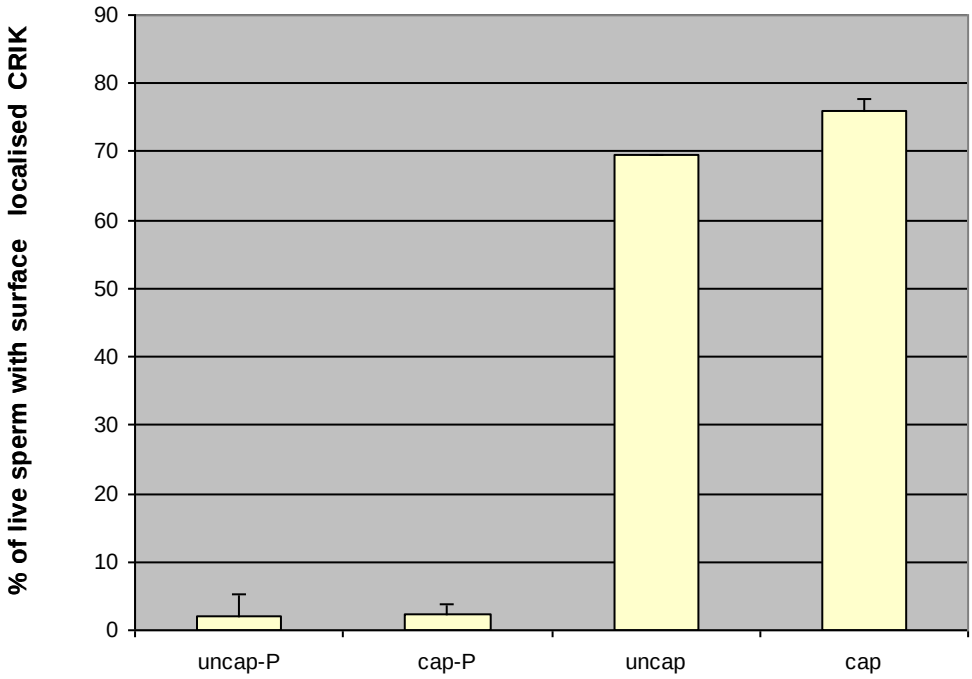


Figure 7.5. Flow cytometric analysis showing exposure of CRIK on the surface of live mouse spermatozoa. Spermatozoa were recovered from the cauda epididymis and either labeled as described, or capacitated in complete BWW media for 45min prior to labeling. Surface localised CRIK was bound using a goat polyclonal CRIK-specific antibody and visualized using FITC-labeled rabbit anti-goat antibody. Cells were then treated with propidium iodide to label dead cells which were subsequently gated out of the analysis (n=3). uncap-P: uncapacitatd sperm control omitting the primary antibody, cap-P: capacitated sperm control omitting the primary antibody, uncap: uncapacitated sperm, cap: capacitated sperm.

Propidium iodide (PI) staining was used to facilitate the exclusion of dead and membrane compromised cells from the analysis. Since live, membrane intact sperm (PI negative) would exclude antibody entry into the cytoplasm, any fluorescence would be indicative of CRIK surface localisation. In Fig. 7.4, the CRIK-FL channel measured fluorescein isothiocyanate (FITC) labeling of cells (CRIK localization), and the PI-FL channel measured propidium iodide staining (membrane compromised, dead cells).

As demonstrated in Figs. 7.4 and 7.5, in uncapacitated sperm the CRIK antibody bound 69.5% of live cells. After capacitation, 76.0% of live cells bound the antibody. In control experiments where the primary antibody was omitted, both uncapacitated (2.1%) and capacitated (2.2%) cells displayed only background levels of fluorescence (Fig. 7.4 B and D, lower right quadrants; Fig. 7.5). These data not only confirmed the surface localization of CRIK, but also indicate that the process of capacitation had no effect on the level of surface exposure of the protein.

7.3.4 CRIK localised to the peri-acrosomal region of mouse spermatozoa.

Having confirmed the presence and surface exposure of CRIK in epididymal sperm, I next sought to determine the localization of the protein in sperm. CRIK was immunolocalised on fixed mouse spermatozoa taken from different regions of the epididymis, using indirect immunofluorescence (IIF) (Sec. 2.6.5). CRIK immunofluorescence was detected in the peri-acrosomal region of sperm taken from all regions of the epididymis (Fig 7.6 A, B and C). No labeling was observed in controls in which the primary antibody was omitted (not shown).

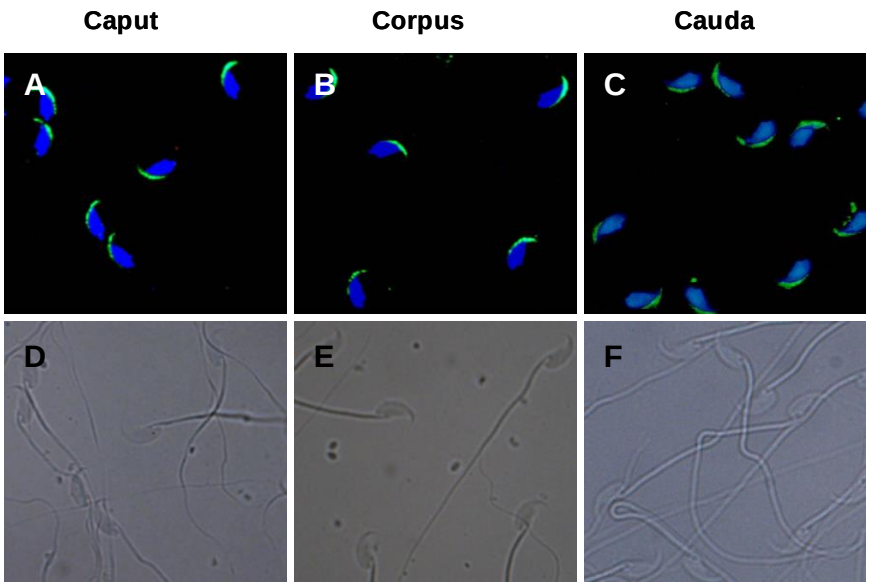


Figure 7.6. Indirect immunofluorescence localisation of CRIK in epididymal spermatozoa. As described in section 2.6.5 epididymal sperm were incubated with anti-CRIK immunoglobulin followed by an FITC-conjugated secondary antibody. **A** and **D**) caput spermatozoa; **B** and **E**) corpus spermatozoa; **C** and **F**) caudal spermatozoa. **A**, **B**, and **C**) labeled with an anti-CRIK antibody (green) and DAPI (blue); **D**, **E**, and **F**) phase contrast. Control experiments omitting the primary antibody displayed negligible staining (not shown).

These data indicate that CRIK-immunoreactive products are present in the acrosomal region of spermatozoa taken from all regions of the mouse epididymis.

7.3.5 Acrosomal loss was correlated with the loss of CRIK from the sperm head.

Additional support for the peri-acrosomal localisation of CRIK was achieved by following the fate of the protein subsequent to induction of acrosomal exocytosis. For these studies, cauda epididymal sperm were capacitated and the acrosome reaction induced using calcium ionophore, A23187 (Sec. 2.3.6). Both capacitated and acrosome reacted spermatozoa were probed with a CRIK-specific antibody followed by an FITC-conjugated secondary antibody (green labeling in Fig. 7.7). DNA was labeled with DAPI (blue labeling in Fig. 7.7).

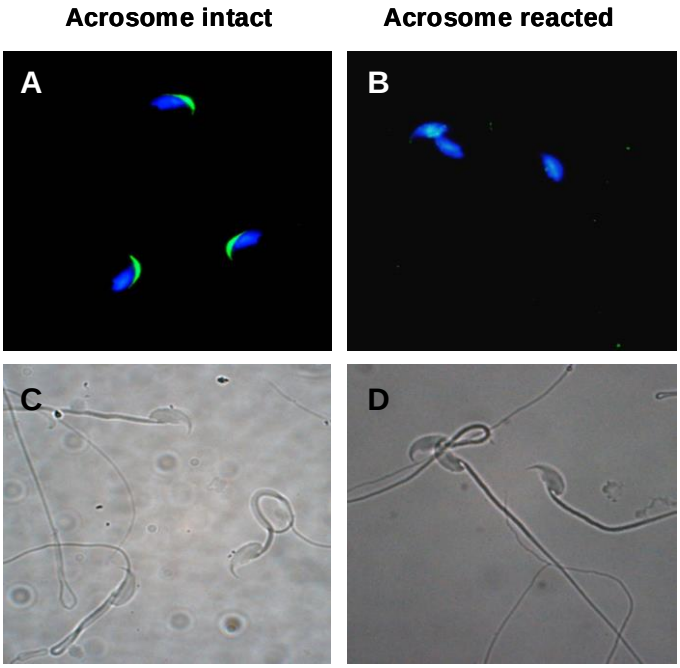


Figure 7.7. Indirect immunofluorescence demonstrating localisation of CRIK in capacitated and acrosome reacted spermatozoa. Cauda epididymal sperm were capacitated (Sec. 2.3.5), acrosome reacted Sec. 2.3.6) and incubated with anti-CRIK immunoglobulin followed by an FITC-conjugated secondary antibody (Sec. 2.6.5). **A** and **C**) capacitated spermatozoa; **B** and **D**) acrosome reacted cells. **A** and **B**) labeled with an anti-CRIK antibody (green) and DAPI (blue); **C** and **D**) phase contrast.

Comparing Fig. 7.7 A (capacitated) and Fig. 7.6 C (uncapacitated) indicated that the process of capacitation had no distinguishable affect on the localization of CRIK. The same localization pattern seen with uncapacitated cells (Fig. 7.6 C) was observed with capacitated spermatozoa (Fig. 7.7 A). The protein localized to the peri-acrosomal region of the sperm head. The acrosomal staining observed in sperm taken from all regions of the epididymis (Fig. 7.6 A, B and C; Fig. 7.7 A) however, was absent in sperm that had undergone ionophore-induced acrosomal exocytosis (Fig. 7.7 B).

These data further support the localization of CRIK immunoreactive products to the peri-acrosomal region of sperm. Collectively, these data and the FACS data suggest that CRIK is localized to the plasma membrane overlying the acrosome, an ideal position for a proposed role in zona recognition and interaction.

7.4 Discussion

Although citron kinase (also referred to as CRIK and Cit-K), an effector protein of the small GTPase Rho, has been identified in the male germ line of the mouse as playing a role in cytokinesis of spermatogenic precursors (Di Cunto et al., 2002); no literature regarding the role, or activity of the protein in functionally competent, mature spermatozoa has been published. Data presented in this chapter documents for the first time the localization of CRIK in mouse spermatozoa. Other Rho effector proteins, including Rho-associated kinase (ROCK), which exhibits a high degree of structural similarity to CRIK (suggesting a similarity in function) have been localized to the acrosomal region of sperm from a number of different species (Ducummon and Berger, 2006) suggestive of a role for the proteins in gamete interactions.

In mammalian cells, the Rho family of GTPases have been characterized as having a role in the regulation and maintenance of the cytoskeleton (Kaibuchi et al., 1999). All eukaryotic cells contain an internal supporting framework of minute filaments and tubules known as the cytoskeleton. The cytoskeleton is composed of three major types of protein filaments: microtubules, microfilaments and intermediate filaments (Schmidt and Hall, 1998); as well as an array of accessory proteins responsible for linking these structures to one another, to the plasma membrane and to the membrane of intracellular organelles (Stossel, 1993; Winsor and Schiebel, 1997). The most abundant cytoskeletal components, microfilaments, also called actin filaments, are assemblies of the protein actin (Kaibuchi et al., 1999). Cytoskeletal proteins include actin, actin binding proteins such as specrin and various tubulins (Hein et al., 2000). In somatic cells, these proteins lay beneath the plasma membrane and form a cortical network involved in many cellular processes.

Within the cell, actin exists either in monomeric (G-actin) or in polymeric form (F-actin) (Schmidt and Hall, 1998), with approximately half the actin kept in monomeric form. The polymerization of actin is a dynamic process (Xiao et al., 2007), each actin molecule binds ATP, which is hydrolyzed to ADP after incorporation of the actin molecule into the polymer (Schmidt and Hall, 1998).

In addition to providing the structural framework around which cell shape and polarity are defined, the dynamic properties of actin mediate numerous biological processes (Cooper, 1991). It mediates changes to cell shape during mitosis (Kunda and Baum, 2009); it is required for motility and migration (Stossel, 1993); is essential for surface remodeling of the cell (Schmidt and Hall, 1998); plays a role in the separation of daughter cells by the contractile ring during cytokinesis (Fishkind and Wang, 1995); controls cell-cell and cell-substrate interactions (Hall, 2009) and participates in transmembrane signal transduction (Zigmond, 1996). However, the role and function of the actin cytoskeleton in mammalian male germ cells is not as clearly defined as it is in somatic cells (Howes et al., 2001b; Breitbart et al., 2005)

All of these critical functions of the actin cytoskeleton are governed and regulated by signaling pathways. The Rho family of signaling molecules are small guanosine triphosphatases (GTPases) and have emerged as key regulators of the actin cytoskeleton (Spiering and Hodgson, 2011; Narumiya et al., 1997; Hall, 1998; Ridley, 2001; reviewed by Lee and Dominguez, 2010). Present in all eukaryotic cells, Rho GTPases function as binary molecular switches alternating between an inactive GDP-bound and active GTP-bound state. In the active (GTP-bound) state, GTPases recognise their target proteins, referred to as effector proteins, and generate a response until GTP hydrolysis returns the switch to the 'off' state. The Rho signaling pathway is involved in stimulating actin polymerization, a dynamic process that regulates many cellular functions including motility (Hall, 1998; Ridley, 2001), cell adhesion (Wettschureck and Offermanns, 2002), and cytokinesis (Narumiya et al., 1997).

Considering the involvement of Rho GTPases in such a diverse array of cellular processes, it is not surprising that a great deal of effort has been directed toward identifying and characterizing their cellular targets or effector proteins. Several target or effector proteins of Rho have been identified by their ability to interact with the activated GTP-bound form of the protein in affinity chromatography (Matsui et al., 1996) and yeast two-hybrid studies (Tapon and Hall, 1997). These include the protein kinase C related Ser/Thr kinases PKN and PRK2 (Amano et al., 1996b; Quilliam et al., 1996); and the rhophilin, rhotekin (Reid et al., 1996), and citron proteins (Madaule et al., 1995). However, the function and downstream targets of these effector proteins remain to be clarified.

The most extensively studied and best characterized Rho effector protein is the Rho-binding serine/threonine kinase, Rho kinase (ROCK) (Fujisawa et al., 1996). ROCK was the first effector of Rho to be discovered and is highly expressed in the heart, lung, skeletal muscle, kidney and pancreas and to a lesser extent in the placenta and liver, with only trace amounts found in the brain (Leung et al., 1996; Nakagawa et al., 1996). The kinase activity of ROCK is stimulated upon binding to the activated form of Rho (Ishizaki et al., 1996). ROCK phosphorylates a range of effector proteins (Ishizaki et al., 1996; Leung et al., 1995). In addition, activated Rho has been reported to promote the translocation of ROCK to the plasma membrane facilitating the recognition of specific substrates (Leung et al., 1995; Kimura et al., 1996). The ROCK family of kinases have been implicated in the regulation of smooth muscle contraction (Amano et al., 1996a), the formation of stress fibers and focal adhesions (Ishizaki et al., 1997), neurite retraction (Katoh et al., 1998), and in the progression of cytokinesis (Yasui et al., 1998).

ROCK is a multi-domain ser/thr protein kinase containing an amino-terminal catalytic kinase domain, a 600 amino acid long central putative coiled-coil region encompassing a leucine zipper domain, and a C-terminal pleckstrin homology (PH) domain which is split by a cystein-rich zinc finger region (Leung et al., 1995). PH domains have been shown to mediate both protein-protein and protein-phospholipid interactions (Musacchio et al, 1993). The pleckstrin-homology domain is thought to mediate protein localization through lipid binding (Riento and Ridley, 2003). GTP-bound Rho interacts with the C-terminal portion of the coiled-coil domain and activates the phosphotransferase activity of ROCK (Matsui et al., 1996).

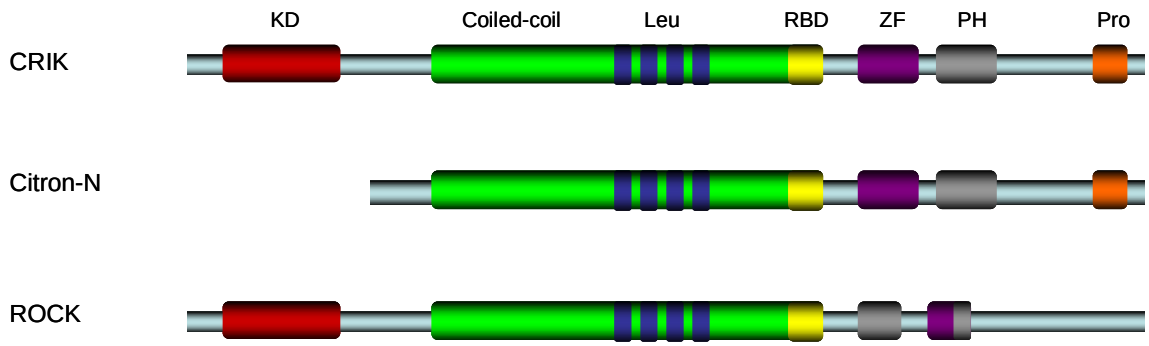


Figure 7.8. Schematic representation of the domain organisation and features of ROCK, CRIK and Cit-N. Indicated are the kinase domain (red, KD), the coiled-coil region (green), the leucine zipper domain (Leu), the Rho binding domain (RBD, yellow), the zinc finger region (ZF, purple), the plekstrin homology domain (PH, grey) and the proline-rich region (PH, orange).

In addition to ROCK, a number of other proteins have been proposed as putative downstream Rho effectors based on their specific association with the activated GTP-bound form of Rho (Madaule et al., 1995; Mukai et al., 1996; Reid et al., 1996; Vincent and Settleman, 1997). Citron kinase (CRIK), another target of activated Rho (Di Cunto *et al.*, 1998; Madaule *et al.*, 1998, 2000), is a 240 kDa Ser/Thr kinase and resembles ROCK in its overall domain structure and organization (Leung et al., 1996; Matsui et al., 1996; Nakagawa et al., 1996) suggesting a similarity in function. In contrast to ROCK, the zinc finger and pleckstrin homology domains are independent of each other, and the protein contains a C-terminal proline rich domain (Fig 8.8). CRIK is ubiquitously expressed in most tissues and cell types with a cell cycle-dependant localization (Madaule *et al.*, 1998) and has been reported to play a key role in cytokinesis (Madaule *et al.*, 1998; Eda et al., 2001).

As a consequence of differential splicing, a truncated form of CRIK lacking the amino-terminal kinase domain exists (Fig 7.8) (Madaule et al., 1995). Referred to as Citron-N (CIT-N), the protein is specifically expressed in the nervous system by differentiating and differentiated nerve cells, where it has been associated to the Golgi

(Camera et al., 2003) and to the post-synaptic densities (Furuyashiki et al., 1999; Zhang et al., 1999).

The role of CIT-N as an effector protein of Rho remains unclear as the protein does not contain an active kinase domain. It has been suggested however, that the protein may function as a scaffolding molecule on Golgi membranes, organizing Rho-mediated actin polymerization locally by ochestrating the assembly of actin polymerizing protein complexes (Camera et al., 2003). It has been demonstrated that CIT-N redirects the actin-polymerising machinery, bringing together activated Rho, ROCK and the actin-binding, neuron-specific protein, Profilin-IIa (PIIa). Profilins, first described by Carlsson and colleagues in 1976 (Carlsson et al., 1976; Carlsson et al., 1977) are actin-binding proteins involved in the regulation of cytoskeleton dynamics and have been implicated in the maintenance of cytoskeletal integrity in a variety of organisms such as *Dictyostelium discoideum* (Haugwitz et al., 1994), yeast (Haarer et al., 1990), and *Drosophila melanogaster* (Verheyen and Cooley, 1994). Futhermore, Profilins have been implicated in the actin dynamics that control membrane trafficking events such as vesicle endo- and exocytosis (Wiedemann, 2007; Gareus et al., 2006).

There is a molecular association between CIT-N and the post-synaptic scaffold protein PSD-95/SAP-90 at glutamatergic synapses in the thalamus. PSD-95/SAP-90 is a member of the membrane-associated guanylate kinase protein family (MAGUK) (Furuyashiki et al., 1999; Zhang et al., 1999). Membrane-associated guanylate kinase (MAGUK) family members function as molecular scaffolds for the assembly of multi-protein complexes localizing to the plasma membrane (Fanning and Anderson, 1999). This interaction involves signaling pathways essential for neural plasticity and post-synaptic signal transduction events and it has been suggested that it may link the Rho signalling cascades to NMDA receptor complexes (Furuyashiki et al., 1999; Zhang et al., 1999).

Both ROCK and CRIK have been implicated in the control of cytokinesis in somatic cells downstream of Rho (Madaule et al., 2000). It has been demonstrated that CRIK is highly enriched in the cleavage furrow and in the midbody of dividing HeLa cells

(Madaule et al., 1998). In addition, Rho is also localized to the cleavage furrow and the midbody (Madaule et al., 1998; Kosako et al., 1999).

The over-expression of a kinase-deficient mutant of CRIK resulted in multi-nucleated cells, whereas that of a constitutively active kinase resulted in abnormal contraction during cytokinesis (Madaule et al.,1998). Di Cunto et al. (2000) subsequently demonstrated through the production of a knockout mouse, that CRIK is essential for cytokinesis only in specialised cells, like proliferating neuronal precursors. The mice were affected by a complex neurological syndrome caused by cytokinesis block and apoptosis of neuronal precursors. In addition, the mice displayed a severe testicular phenotype, with embryonic and post-natal loss of undifferentiated germ cells and a complete absence of spermatocytes. Spermatogenic precursors displayed a dramatic cytokinesis defect resulting in the production of multi-nucleated cells and apoptosis, indicating that CRIK is specifically required for cytokinesis of the male germ line (Di Cunto et al., 2002).

Work presented in chapter four of this thesis identified CRIK as a putative HSPD1-interacting protein on the surface of capacitated mouse spermatozoa. The reciprocal co-immunoprecipitation was unable to support the physical association of the two proteins as the CRIK-specific antibody failed to precipitate CRIK, however, this result does not rule out a physical association between the two proteins. A technique for characterizing protein complexes not reliant on the availability of an epitope, such as Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE), may be useful here.

I next sought to compare the relative level of CRIK in spermatozoa at different stages of epididymal maturation. For this purpose, lysates of sperm taken from different regions of the epididymis, together with a testis total cell extract. As indicated in figure 7.2, western blot analysis of these extracts detected proteins of three different molecular weights. In the testis lysate two proteins were detected, one with a molecular weight of approximately 230 kDa (band 1 in Fig. 7.2), which is consistant with the reported molecular weight of CRIK in the mouse testis (Madaule et al., 1998; Di Cunto et al. 1998), and another of about 55 kDa (band 2 in Fig. 7.2). The 55 kDa band does not correspond to the predicted molecular weight of CRIK or CIT-N, and may represent a degradation product of the protein or another sperm protein reacting

non-specifically with the antibody. In lysates generated from epididymal spermatozoa no protein of the expected molecular weight was detected. The 55 kDa band observed in the testis however, was again detected in the lysate derived from caput spermatozoa (band 3 in Fig. 7.2); while a protein of approximately 85 kDa was detected in spermatozoa taken from the corpus and cauda regions of the epididymis (bands 4 and 5 in Fig. 7.2).

The 85 kDa protein detected in sperm derived from the corpus and cauda epididymis was not detected in sperm isolated from the caput region. This suggests that the protein was acquired during epididymal transit or that a larger sperm protein, possibly CRIK is proteolytically processed as sperm mature in the epididymis. It is well documented that epididymal proteins are involved in the site-specific proteolytic cleavage of precursor proteins associated with the sperm plasma membrane (Petruszak et al., 1991; Tulsiani et al., 1995). Proteolytic processing of the sperm protein fertilin (PH-30) in the epididymis facilitates its redistribution to the posterior head (Hunnicutt et al., 1997) and exposure of the domain responsible for fusion with the oolemma (Lum and Blobel, 1997). A number of other sperm proteins have been demonstrated to be processed during epididymal transit, including Sp-10 (Foster et al., 1994), PH-20 (Primakoff et al., 1985; Deng et al., 2000) and β-galactosyltransferase (Scully et al., 1987). Other reports have suggested that a major class of enzymes secreted into the epididymal lumen are proteinases and proteinase inhibitors (Metayer, et al., 2002), which may facilitate such modifications.

Alternatively a replacement mechanism may be operating, whereby sperm proteins of testicular origin, CRIK in this case, are lost as sperm pass through the initial segment and are replaced by epididymal isoforms of the same protein exhibiting a unique pattern of post-translational modification, e.g. clusterin (Sylvester et al., 1991). At present we have no data to support either the cleavage or replacement of CRIK.

Indirect immunofluorescence (IIF) studies localised CRIK to the peri-acrosomal area in sperm taken from all regions of the epididymis (Fig. 7.6). This localisation pattern was unchanged by capacitation, but lost once sperm had undergone calcium ionophore A23187 induced acrosomal exocytosis (Fig.7.7). Flow cytometry demonstrated that the antibody bound a protein exhibited on the surface of mature

spermatozoa. In uncapacitated sperm populations, anti-CRIK antibody bound to the surface of 69.5% of viable cells (Fig. 7.4 & 7.5). After capacitation 76.0% of live cells bound the antibody. Control experiments where the primary anti-CRIK antibody was omitted displayed negligible levels of fluorescence.

The surface localisation of CRIK demonstrated by flow cytometry was supported by biotinylation experiments targeting proteins of the plasma membrane. Western analysis of biotin purified sperm surface proteins with an anti-CRIK antibody detected two proteins in the molecular weight range of 80-90kDa, which is consistent with the protein detected in lysates of corpus and caudal sperm (Fig. 7.2).

The acrosomal and cell-surface localisation of the molecule are consistent with the protein having a potential role in gamete interactions. Although there is no precedent for CRIK having an acrosomal or cell surface localization in spermatozoa, other Rho effector proteins have been described at these locations, as has Rho. de la Sancha et al. (2007) localized ROCK and Rho to the acrosomal region of sea urchin sperm.. Moreover, they demonstrated that ROCK antagonists inhited the acrosome reaction, suggesting that a Rho/ROCK-dependent signaling pathway may regulate the acrosome reaction in this species. Furthermore, it has been reported that bacterial toxin, C3, which specifically inhibits Rho function (Wilde et al., 2000) prevents the ionophore-induced acrosome reaction in several mammalian species (Ducummon and Berger, 2006; Brener et al., 2003).

Other literature reports similar localisations in mammalian spermatozoa. Ducummon and Berger localized the Rho GTPases RhoA, RhoB, Rac1 and Cdc42; as well as the effectors RhoGDI, PI(4)P5K and ROCK to the acrosomal region of the sperm head in boar, human, rat, ram, bull and elephant sperm (Ducummon and Berger, 2006); a localization consistent with the hypothesized role for a Rho-mediated signaling pathway culminating in acrosomal exocytosis (Castellano et al., 1997). It is possible that CRIK could play a role in such a signaling pathway.

Alternatively, CRIK in sperm may function as a scaffolding molecule, similar to the role of CIT-N which regulates actin polymerization (Camera et al., 2003). It has been demonstrated that actin polymerization plays an important role in the zona pellucida

induced acrosome reaction of mammalian sperm (Liu et al., 1999; Spungin et al., 1995; Howes et al., 2001b; Liu et al., 2002; Palecek et al., 1999; Vogl, 1989). The polymerization of globular actin to filamentous actin between the outer acrosomal membrane and the plasma membrane of sperm during capacitation provides a scaffold that immobilizes phospholipase Cγ, which has been implicated in the acrosome reaction at the cell surface (Breitbart and Spungin, 1997).

The mechanism by which CRIK might regulate sperm function remains open to question. It has been proposed that HSPD1-associated proteins form part of a macromolecular protein complex in the sperm plasma membrane and that tyrosine phosphorylation of chaperones during sperm capacitation may then signal the activation of the complex and stimulate a signaling cascade leading to the acquisition of zona binding ability. The data presented in this chapter lend support to the notion that CRIK, or a fragment there of, maybe a constituent of just such a macromolecular protein complex.

Although reciprocal co-immunoprecipitation was unable to confirm the association of CRIK with HSPD1 in capacitated sperm, western and indirect immunofluorescence analysis demonstrated that CRIK immunoreactive products are present in sperm isolated from all regions of the epididymis, localizing to the peri-acrosomal region of the head, a position consistent with the protein having a role in fertilisation-related events. Localisation of CRIK to the acrosomal region is suggestive of a role in interaction with the oocyte.

CHAPTER 8

LOCALISATION OF CHAPERONE INTERACTING PROTEINS DURING EPIDIDYMAL MATURATI ON, CAPACITATION AND THE ACROSOME REACTION: PROACROSIN BINDING PROTEIN ACRBP

8. Localisation of chaperone interacting proteins during epididymal maturati on, capacitation and the acrosome reaction: Proacrosin binding protein ACRBP.

8.1 Introduction

As discussed in chapter four, the proacrosin binding protein (ACRBP) was identified as a putative binding partner of the molecular chaperone HSPD1 on the surface of capacitated sperm using a co-IP approach with HSPD1-specific antibodies (chapter four).

Fertilisation is determined to a large extent by the compatibility between ligand and receptor molecules on the surface of the male and female gametes. On the zona pellucida (ZP), the sperm receptor activity is associated with glycoproteins ZP3 (primary receptor for acrosome-intact sperm) and ZP2 (secondary receptor for acrosome-reacted sperm) but the complementary binding proteins on the sperm surface are less well defined.

Acrosin is the major protease present in the acrosome of mature spermatozoa from a number of different species, including the pig (Müller-Esterl and Fritz, 1981; Polakoski and Parrish, 1977; Parrish and Polakoski, 1978), guinea pig (Adekunle et al., 1987; Hardy et al., 1987) and human (Siegel et al., 1986); and is liberated as a consequence of the acrosome reaction.

The majority of acrosomal acrosin however, exists as an enzymatically inactive zymogen or precursor, proacrosin (Noland et al., 1989; Yi et al., 1991; Hardy et al., 1991; Westbrook-Case et al., 1994). The release of mature active acrosin from sperm during the acrosome reaction is believed to be prompted by self catalytic conversion

of proacrosin into acrosin through a sequential activation process (Kennedy and Polakoski, 1981).

Acrosin is a multi-functional enzyme combining several functional properties within a single molecule. It contains the catalytic triad of the protease, hydrophobic domains responsible for the membrane-associating character of the enzyme, and a carbohydrate (fucose) binding domain by which the molecule binds ZP2 mediating secondary binding of sperm to the ZP. Thus, acrosin functions both in the limited proteolysis of the zona pellucida, and retaining acrosome-reacted sperm on the ZP surface long enough to enable ZP penetration to begin (Howes and Jones, 2002, Jones and Brown, 1987, Moreno *et al.*, 1998, Mori *et al.*, 1995, Richardson and O'Rand, 1996, Williams and Jones, 1991).

Acrosin is not absolutely essential for successful fertilization. Mice null for the proacrosin gene were fertile (Baba *et al.*, 1994b). The penetration of the ZP by sperm lacking proacrosin was impeded, apparently due to a delay in dispersing the acrosomal matrix (Adham *et al.*, 1997, Yamagata *et al.*, 1998). Acrosin is presumably involved in the limited proteolysis and/or processing of other proteins in the acrosome and on the membranes during the acrosome reaction at least in the mouse.

The proacrosin binding protein (ACRBP), also known as sp32 and OY-TES-1, has been reported to play a major role in the activation and localisation of proacrosin and acrosin within the acrosome (Baba *et al.*, 1994a). Initially synthesised as a 61 kDa precursor protein, the carboxyl terminal half of the molecule corresponds to the mature protein, which is produced by the post-translational cleavage of the precursor form of the molecule.

ACRBP has been identified in a number of species (Yi and Polakoski, 1992), and most extensively studied in porcine sperm where it significantly accelerates the autoactivation of proacrosin (Bailey et al., 2005). In addition, Dubé and colleagues demonstrated that the protein is tyrosine phosphorylated and translocates to the plasma membrane during sperm capacitation in the pig (Dubé et al., 2005; Bailey et al., 2005). Visconti and colleagues also identified ACRBP as a target of tyrosine phosphorylation during capacitation of mouse sperm (Arcelay et al., 2008), and

Ficarro et al. showed that sp32 is tyrosine phosphorylated in capacitated human sperm (Ficarro et al., 2003).

To date, the characterization of proteins that undergo tyrosine phosphorylation during capacitation has been limited. Furthermore, the implication and significance of the tyrosine phosphorylation of ACRBP during capacitation and acrosin maturation remains to be established. As such ACRBP was selected for further study.

As with the previous chapters, the present study sought to confirm the interaction of ACRBP with HSPD1. As an initial step toward characterising the role of ACRBP in sperm and fertilization, determine the localisation and relative level of the protein during epididymal maturation, capacitation and the acrosome reaction.

8.2 Experimental Rational

As no commercial antibody for ACRBP was available, anti-peptide antibodies were raised against synthetic peptides derived from the amino acid sequence of murine ACRBP (IPI accession IPI00622867.1). Peptide immunogens were selected based on their predicted antigenicity. For this purpose a bioinformatic analysis (Sec. 2.10.1.1) was performed to predict likely B-cell epitopes in the ACRBP protein. Three web-based servers were employed:

(1) Bcepred. The server predicts linear B-cell epitopes in a protein sequence based on physico-chemical properties of amino acids known to correlate with B-cell epitopes. Physico-chemical properties used were hydophilicity (Parker et al., 1986), flexibility (Karplus and Schulz, 1985), accessibility (Emini et al., 1985), polarity (Ponnuswamy et al., 1980) and exposed surface (Janin and Wodak, 1978). The server progressively evaluates each physico-chemical property of the protein along its amino acid sequence using a moving-segment approach that continuously determines the average within a segment of predetermined length as it advances through the sequence. Results are presented in graphical form with consecutive scores plotted from the amino to the carboxy terminus. The peak of the amino acid residue segment above a pre-defined threshold value is considered as a predicted B-cell epitope. The server is accessible at www.imtech.res.in/raghava/bcepred.

(2) BetaTPred2. The location of beta turns in proteins has also been shown to correlate with the location of B-cell epitopes (Ripoll, 1992)**.** The BetaTPred2 server predicts beta turns (ß-turns) in proteins from multiple sequence alignment using a neural network. For ß-turn prediction, a position specific score matrix (PSSM) generated by PSI-BLAST, and secondary structure predicted by the PSIPRED algorithm (http://www.psipred.net/psiform.html) are used. The network was trained and tested on a set of 426 non-homologous protein chains with 7-fold cross-validation. It predicts ß-turns in proteins with residue accuracy of 75.5% and Matthews correlation coefficient (MCC) value of 0.43 (Kaur and Raghava, 2002). The server is accessible at www.imtech.res.in/raghava/betatpred2/

(3) ABCpred. The ABCpred server was used as the ultimate validation to confirm the predictions based on physio-chemical properties (BCEpred server) and the location of ß-turns (BetaTPred2 server).The ABCpred server predicts linear B-cell epitopes in a protein sequence using a recurrent neural network and was trained and tested on a clean data set consisting of 700 non-redundant B-cell epitopes obtained from Bcipep database (www.imtech.res.in/raghava/bcipep/data.html), and an equal number of non-epitopes obtained randomly from the Swiss-Prot database (www.expasy.org/sprot/). The server is accessible at www.imtech.res.in/raghava/abcpred/

Selected peptides were synthesised by solid phase peptide synthesis (SPPS) utilizing Fmoc chemistry (Mimotopes, Clayton, VIC, Australia) and purified by HPLC to a minimum of 75% homogeneity as determined by high-performance liquid chromatography (HPLC) and mass spectrometry. Purified peptides were then conjugated individually to diphtheria toxoid (DT) via an N-terminal cysteine residue using the heterobifunctional reagent, 6'-maleimido-caproyl n-hydroxy succinimide (MCS).

Each DT-conjugated peptide was used to immunize three goats by Antibodies Australia (Werribee, Victoria, Australia) using their standard method. Briefly, each animal was injected with immunogen and received subsequent boost injections 63, 92, 127, 147, 174, 196, and 212 days after the primary immunization. Pre-immune serum was collected prior to immunization, and serum was collected following the third, sixth, seventh and eighth injections.

Using the antisera generated, the interaction of ACRBP with HSPD1 was confirmed. The reciprocal co-immunoprecipitation (Sec. 2.4.5) was performed and the presence of HSPD1 in the immunoprecipitated material was assayed for by western blot analysis (Sec. 2.6.4). The sera were then used to determine the localisation of ACRBP in spermatozoa at different stages of epididymal maturation (Sec. 2.6.5).

The cell surface localisation of ACRBP was investigated using a membrane impermeable biotinylation reagent (Sec. 2.4.3) in conjunction with western blotting (Sec. 2.6.4). The results of the biotinylation experiments were confirmed by flow cytometry (Sec. 2.6.6).

The effect of capacitation (Sec. 2.3.5) and the acrosome reaction (Sec. 2.3.6) on the localisation of ACRBP were investigated using an indirect immunofluorescence technique (Sec. 2.6.5).

8.3 Results

Due to the unavailability of a commercial antibody to the ACRBP protein, it was necessary to produce an anti-peptide antibody that would allow us to further characterize the ACRBP in murine spermatozoa.

8.3.1 Selection of peptide - design and synthesis of the antigen.

The first step in the production of anti-peptide antibodies is the design of the synthetic peptide antigen that will be used to generate the antibodies. This initial step of peptide design carries the most influence in determining whether the antibodies generated will react with the native protein. When selecting a sequence to synthesise for use as an immunogen, a number of important attributes need to be considered, including:

- Peptide length
- Ease of synthesis
- Antigenicity (B-cell epitopes)

Although antibodies are capable of recognising single amino acid residues, or moieties within single molecules, within proteins are usually greater than six residues in length (Mackrill, 1997). Dyson *et al*. (1988) propose that in order to generate protein reactive anti-peptide antibodies, peptides no less than ten residues in length be employed. Tanaka et al. explored the efficiency of peptides of different sizes for the preparation of anti-peptide antibodies reactive with native oncoproteins (Tanaka et al. 1985). They found that immunisation with a peptide of 15-25 residues, containing 3-5 possible epitopes, enhanced their success significantly. Above 25 residues, efficiency of synthesis must be considered.

Not all peptides can be assembled with equal ease by solid phase peptide synthesis (SPPS). Each peptide presents its own unique set of complications. Generally, problems involve consecutive low yield (85-98%) couplings and are observed between 5 and 15 residues into a synthesis. Amino acids frequently observed in low

yield couplings include arginine, glutamine, isoleucine, threonine, and valine (Kent, 1988).

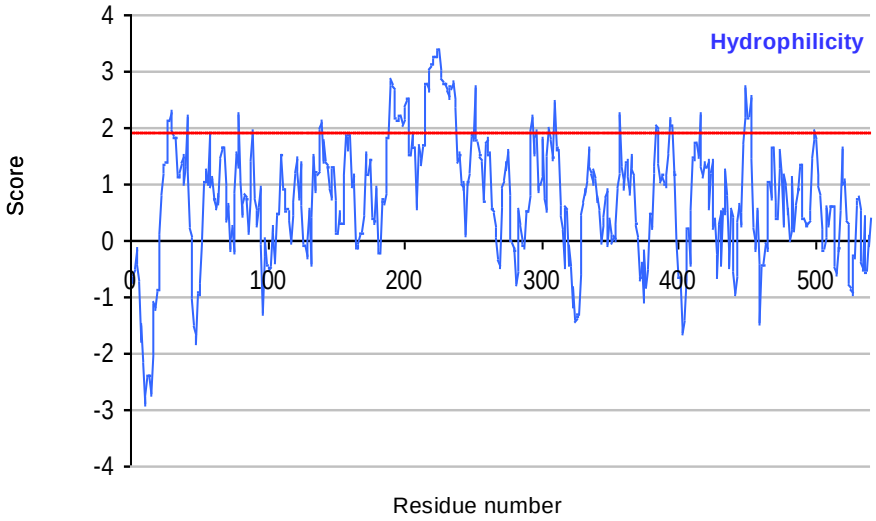
8.3.1.1 Bioinformatic analysis: B-cell epitope prediction I.

As most, if not all, B-cell epitopes or antigenic sites are located within surface-exposed regions of proteins (Emini et al., 1985), the presence of B-cell epitopes is often predicted by bioinformatic analysis based on measurement of different physio-chemical properties known to correlate with surface regions of proteins and the location of beta-turns.

The BcePred server was used to predict linear B-cell epitopes in the ACRBP protein based on different physico-chemical properties of amino acids. Physico-chemical properties used were **hydophilicity** (Parker et al., 1986), **flexibility** (Karplus and Schulz, 1985), **accessibility** (Emini et al., 1985), **polarity** (Ponnuswamy et al., 1980) and **exposed surface** (Janin and Wodak, 1978).

(a) Hydrophilicity

A



B

[1]MMNLAAGFLLMLLEVLLLPGTPLSAEESPASTPGSPLSSTEYERFFALLTPTWKAETTCRLR
ATHGCRNPTLVQLDQYENHGLVPDGAVCSDLPYASWFESFCQFAQYRCSNHVYYAKRVRC
SQPVSILSPNTLKEVESSAEVPPTSMTTPIVSHATATEHQAFQPWPERLNNNVEELLQSSLSL
GGKDQQSSRRPGQEQRKQEQIQEHKLEEAQEQEEQEEEEEEEEEAKQEEGQGTEAGLESVS
RLQSDSEPKFQSQSLSSNPSFFTPRVREVESAPLMMKNIQELIRSAQEMDEMNELYDDSWR
SQSTGSLQQLPHMETLMVLCYSIMENTCTMTPTAKAWSYMEEEILGFGDSVCDNLGRRHTA
ACPLCAFCSLKLEQCHSEASVLRQKCDASHKIPFISPLLSAQSISTGNQARIPDKGRFAGLEM
YGGLSSEFWCNRLAMKGCEDDRVSNWLKAEFLSFQEGDFPTKICDTNYIQYPNYCSFKSQQ
CLLRNQNRKMSRMRCMLNERYNVLSLAKSEEVILRWSQEFSTLAIGQFG[540]

Figure 8.1. Hydrophilicity plot of peptide segments for ACRBP (A) The hydrophilicity profile of the ACRBP protein was calculated by the method of Parker et al., 1986. Residues in the protein above the threshold value of 1.9 (——) are considered as putative B-cell epitopes. (B) Putative antigenic regions of ACRBP identified by the method (blue). Peptides ultimately used as immunogen are underlined.

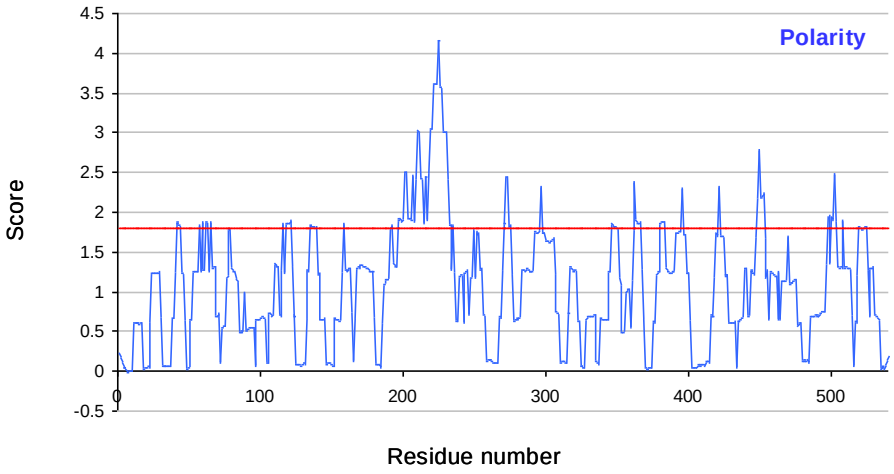
The hydrophilicity profile of the ACRBP protein was determined by the method of Parker et al. In this method, the hydrophilic scale is based on peptide retention times during reversed phase high-performance liquid chromatography (HPLC) (Parker et al., 1986). A sliding window of seven residues was used for analysis. The corresponding value of the scale was introduced for each of the seven residues and the arithmetical mean of the seven residue value was assigned to the fourth (i+3) residue in the segment. The default threshold value of 1.9 was used.

Using these parameters, positive values identify hydrophilic regions and negative values identify hydrophobic regions within the protein. Positive regions above the threshold value are considered as potentially antigenic or putative B-cell epitopes.

Figure 8.1 indicates that a hydrophilicity maximum of 3.373 was recorded at residue 225, and a minimum of -2.892 at residue 10. Moreover, a number of regions of ACRBP are above the threshold value. Those region of sequence above the critical threshold, and therefore considered as potential antigenic regions of the protein are highlighted in blue in figure 8.1 B. Also evident in figure 8.1B is that the two peptides used as immunogen for antibody production are considered as potentially antigenic.

(b) Polarity

A



B

[1]MMNLAAGFLLMLLEVLLLPGTPLSAEESPASTPGSPLSSTEYERFFALLTPTWKAETTCRLR
ATHGCRNPTLVQLDQYENHGLVPDGAVCSDLPYASWFESFCQFAQYRCSNHVYYAKRVRC
SQPVSILSPNTLKEVESSAEVPPTSMTTPIVSHATATEHQAFQPWPERLNNNVEELLQSSLSL
GGKDQQSSRRPGQEQRKQEQIQEHKLEEAQEQEEQEEEEEEEEEAKQEEGQGTEAGLESVS
RLQSDSEPKFQSQSLSSNPSFFTPRVREVESAPLMMKNIQELIRSAQEMDEMNELYDDSWR
SQSTGSLQQLPHMETLMVLCYSIMENTCTMTPTAKAWSYMEEEILGFGDSVCDNLGRRHTA
ACPLCAFCSLKLEQCHSEASVLRQKCDASHKIPFISPLLSAQSISTGNQARIPDKGRFAGLEM
YGGLSSEFWCNRLAMKGCEDDRVSNWLKAEFLSFQEGDFPTKICDTNYIQYPNYCSFKSQQ
CLLRNQNRKMSRMRCMLNERYNVLSLAKSEEVILRWSQEFSTLAIGQFG[540]

Figure 8.2. Polarity plot of peptide segments for ACRBP. (A) The polarity profile of the ACRBP protein was calculated by the method of Ponnuswamy et al., 1980. Residues in the protein above the threshold value of 1.8 (——) are considered as putative B-cell epitopes. (B) Putative antigenic regions of ACRBP identified by the method (blue). Peptides ultimately used as immunogen are underlined.

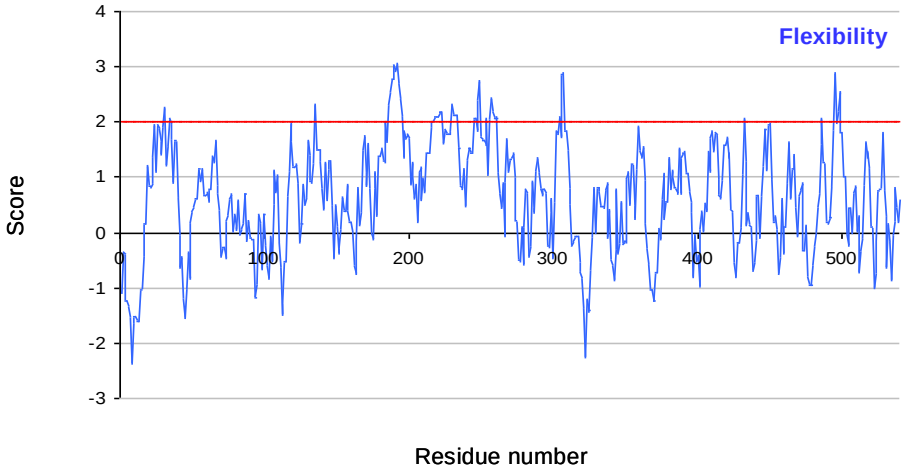
The polarity profile of the ACRBP protein was determined by the method of Ponnuswamy et al., 1980. A sliding window of seven residues was used for analysis. The corresponding value of the scale was introduced for each of the seven residues and the arithmetical mean of the seven residue value was assigned to the fourth (i+3) residue in the segment. The default threshold value of 1.8 was used.

Using these parameters, positive values identify polar regions and negative values identify non-polar regions within the protein. Polar regions above the threshold value are considered as potentially antigenic or putative B-cell epitopes.

Figure 8.2 indicates that a polarity maximum of 4.167 was recorded at residue 225, and a minimum of -0.01 at residue 10. Moreover, a number of regions of ACRBP are above the threshold value. Those regions of sequence above the critical threshold, and therefore considered as potential antigenic regions of the protein are highlighted in blue in figure 8.2, B. Also evident in figure 8.2B is that the two peptides used as immunogen for antibody production are considered as polar and potentially antigenic.

(c) Flexibility

A



B

[1]MMNLAAGFLLMLLEVLLLPGTPLSAEESPASTPGSPLSSTEYERFFALLTPTWKAETTCRLR
ATHGCRNPTLVQLDQYENHGLVPDGAVCSDLPYASWFESFCQFAQYRCSNHVYYAKRVRC
SQPVSILSPNTLKEVESSAEVPPTSMTTPIVSHATATEHQAFQPWPERLNNNVEELLQSSLSL
GGKDQQSSRRPGQEQRKQEQIQEHKLEEAQEQEEQEEEEEEEEAKQEEGQGTEAGLESVS
RLQSDSEPKFQSQSLSSNPSFFTPRVREVESAPLMMKNIQELIRSAQEMDEMNELYDDSWR
SQSTGSLQQLPHMETLMVLCYSIMENTCTMTPTAKAWSYMEEEILGFGDSVCDNLGRRHTA
ACPLCAFCSLKLEQCHSEASVLRQKCDASHKIPFISPLLSAQSISTGNQARIPDKGRFAGLEM
YGGLSSEFWCNRLAMKGCEDDRVSNWLKAEFLSFQEGDFPTKICDTNYIQYPNYCSFKSQQ
CLLRNQNRKMSRMRCMLNERYNVLSLAKSEEVILRWSQEFSTLAIGQFG[540]

Figure 8.3. Flexibility plot of peptide segments for ACRBP. (A) The flexibility profile of the ACRBP protein was calculated by the method of Karplus et al., 1985. Residues in the protein above the threshold value of 2 (——) are considered as putative B-cell epitopes. (B) Putative antigenic regions of ACRBP identified by the method (blue). Peptides ultimately used as immunogen are underlined.

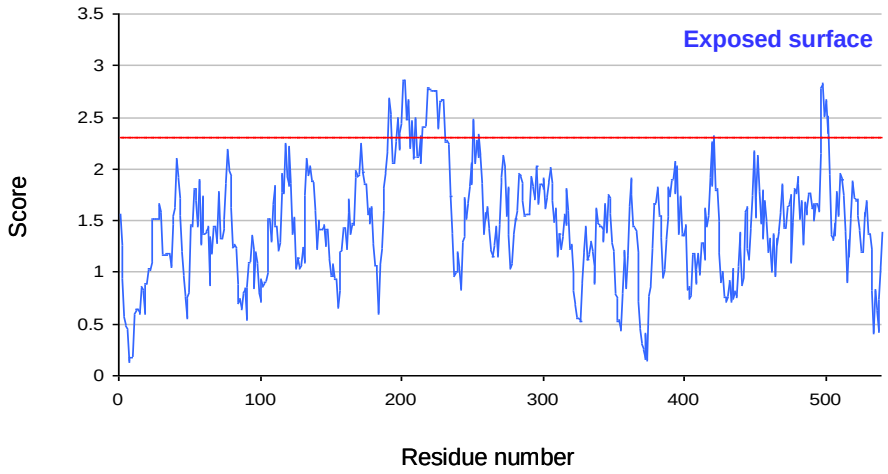
The flexibility profile of the ACRBP protein was determined by the method of Karplus et al., 1985. In this method, the flexibility scale is based on parameters derived from three-dimensional structures, the normalized temperature B-values of the alpha carbons in 31 protein structures. A sliding window of seven residues was used for analysis. The corresponding value of the scale was introduced for each of the seven residues and the arithmetical mean of the seven residue value was assigned to the fourth (i+3) residue in the segment. The default threshold value of 2 was used.

Using these parameters, positive values identify regions of increasing flexibility and negative values identify more rigid regions within the protein. Flexible regions above the threshold value are considered as potentially antigenic or putative B-cell epitopes.

Figure 8.3 indicates that a flexibility maximum of 3.05 was recorded at residue 192, and a minimum of -2.38 at residue 11. Moreover, several regions of ACRBP are above the threshold value. Those region of sequence above the critical threshold, and therefore considered as potential antigenic regions of the protein are highlighted in blue in figure 8.3, B. Also evident in figure 8.3B is that the two peptides used as immunogen for antibody production are considered as flexible and potentially antigenic

(d) Exposed surface

A



B

Figure 8.4. Exposed surface plot of peptide segments for ACRBP. (A) The exposed surface profile of the ACRBP protein was calculated by the method of Janin and Wodak, 1978. Residues in the protein above the threshold value of 2.3 (——) are considered as putative B-cell epitopes. (B) Putative antigenic regions of ACRBP identified by the method (blue). Peptides ultimately used as immunogen are underlined.

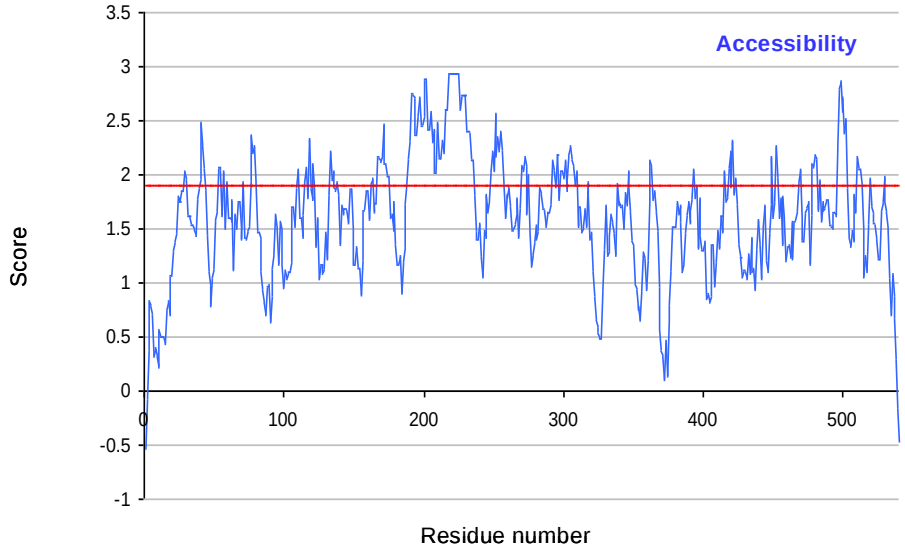
The exposed surface profile of the ACRBP protein was determined by the method of Janin and Wodak et al., 1978. This scale predicts the accessible and buried amino acid residues of proteins. A sliding window of seven residues was used for analysis. The corresponding value of the scale was introduced for each of the seven residues and the arithmetical mean of the seven residue value was assigned to the fourth (i+3) residue in the segment. The default threshold value of 2.3 was used.

Using these parameters, higher values identify regions predicted to be at the surface of the protein. Regions above the threshold value are considered as potentially antigenic or putative B-cell epitopes.

Figure 8.4 indicates that an exposed surface maximum of 2.862 was recorded at residue 202, and a minimum of 0.136 at residue 7. Five regions of ACRBP are above the threshold value. Those region of sequence above the critical threshold, and therefore considered as potential antigenic regions of the protein are highlighted in blue in figure 8.4, B. Also evident in figure 8.4 B is that both of the two peptides used as immunogen for antibody production are considered to be surface exposed and potentially antigenic.

(e) Accessibility

A



B

[1]MMNLAAGFLLMLLEVLLLPGTPLSAEESPASTPGSPLSSTEYERFFALLTPTWKAETTCRLR
ATHGCRNPTLVQLDQYENHGLVPDGAVCSDLPYASWFESFCQFAQYRCSNHVYYAKRVRC
SQPVSILSPNTLKEVESSAEVPPTSMTTPIVSHATATEHQAFQPWPERLNNNVEELLQSSLSL
GGKDQQSSRRPGQEQRKQEQIQEHKLEEAQEQEEQEEEEEEEEAKQEEGQGTEAGLESVS
RLQSDSEPKFQSQSLSSNPSFFTPRVREVESAPLMMKNIQELIRSAQEMDEMNELYDDSWR
SQSTGSLQQLPHMETLMVLCYSIMENTCTMTPTAKAWSYMEEEILGFGDSVCDNLGRRHTA
ACPLCAFCSLKLEQCHSEASVLRQKCDASHKIPFISPLLSAQSISTGNQARIPDKGRFAGLEM
YGGLSSEFWCNRLAMKGCEDDRVSNWLKAEFLSFQEGDFPTKICDTNYIQYPNYCSFKSQQ
CLLRNQNRKMSRMRCMLNERYNVLSLAKSEEVILRWSQEFSTLAIGQFG[540]

Figure 8.5 Accessibility plot of peptide segments for ACRBP. (A) The accessibility profile of the ACRBP protein was calculated by the method of Emini et al., 1985. Residues in the protein above the threshold value of 1.9 (——) are considered as putative B-cell epitopes. (B) Putative antigenic regions of ACRBP identified by the method (blue). Peptides ultimately used as immunogen are underlined.

The accessibility profile of the ACRBP protein was determined by the method of Emini et al., 1985. The accessibility scale is based on the surface accessibility of the amino acids (Emini et al., 1985).

A sliding window of seven residues was used for analysis. The corresponding value of the scale was introduced for each of the seven residues and the arithmetical mean of the seven residue value was assigned to the fourth (i+3) residue in the segment. The default threshold value of 1.9 was used.

Using these parameters, increasing values identify regions of the protein predicted to be at the surface. Regions above the threshold value are considered as potentially antigenic or putative B-cell epitopes.

Figure 8.5 indicates that an accessibility maximum of 2.935 was recorded at residue 221, and a minimum of 0.094 at residue 372. Moreover, a number of regions of ACRBP are above the threshold value. Those region of sequence above the critical threshold, and therefore considered as potential antigenic regions of the protein are highlighted in blue in figure 8.5, B. Also evident in figure 8.5B is that the two peptides used as immunogen for antibody production are considered at the surface of the protein and potentially antigenic.

(f) Prediction of protein secondary structure and Beta turns

A ß-turn is a region of a protein involving four consecutive residues where the polypeptide chain folds back on itself by nearly 180 degrees (Lewis et al. 1971, 1973; Kuntz,1972; Crawford et al.,1973; Chou & Fasman, 1974). It is these chain reversals which give a protein its globularity rather than linearity. Turns usually occur on the exposed surface of proteins and hence represent potential antigenic sites or sites involved in molecular recognition (Rose *et al*., 1985; Takano *et al*., 2000).

The BetaTPred2 server (www.imtech.res.in/raghava/betatpred2/) was used to predict ß turns ACRBP. The algorithm employed by the server uses a multiple alignment, a neural network, the position specific score matrices generated by PSI-BLAST, and secondary structure predicted by the PSIPRED algorithm.

In figure 8.6, the residues of the ACRBP protein (first row in each block) are predicted as either turn, '**t**'; or non-turn residues, '**n**' (bottom row in each block). The middle row in each block displays the secondary structure predicted by the PSIPRED algorithm. Figure 8.6 indicates that the two peptides (shaded) used for raising polyclonal antibodies are predicted to contain beta turn residues.

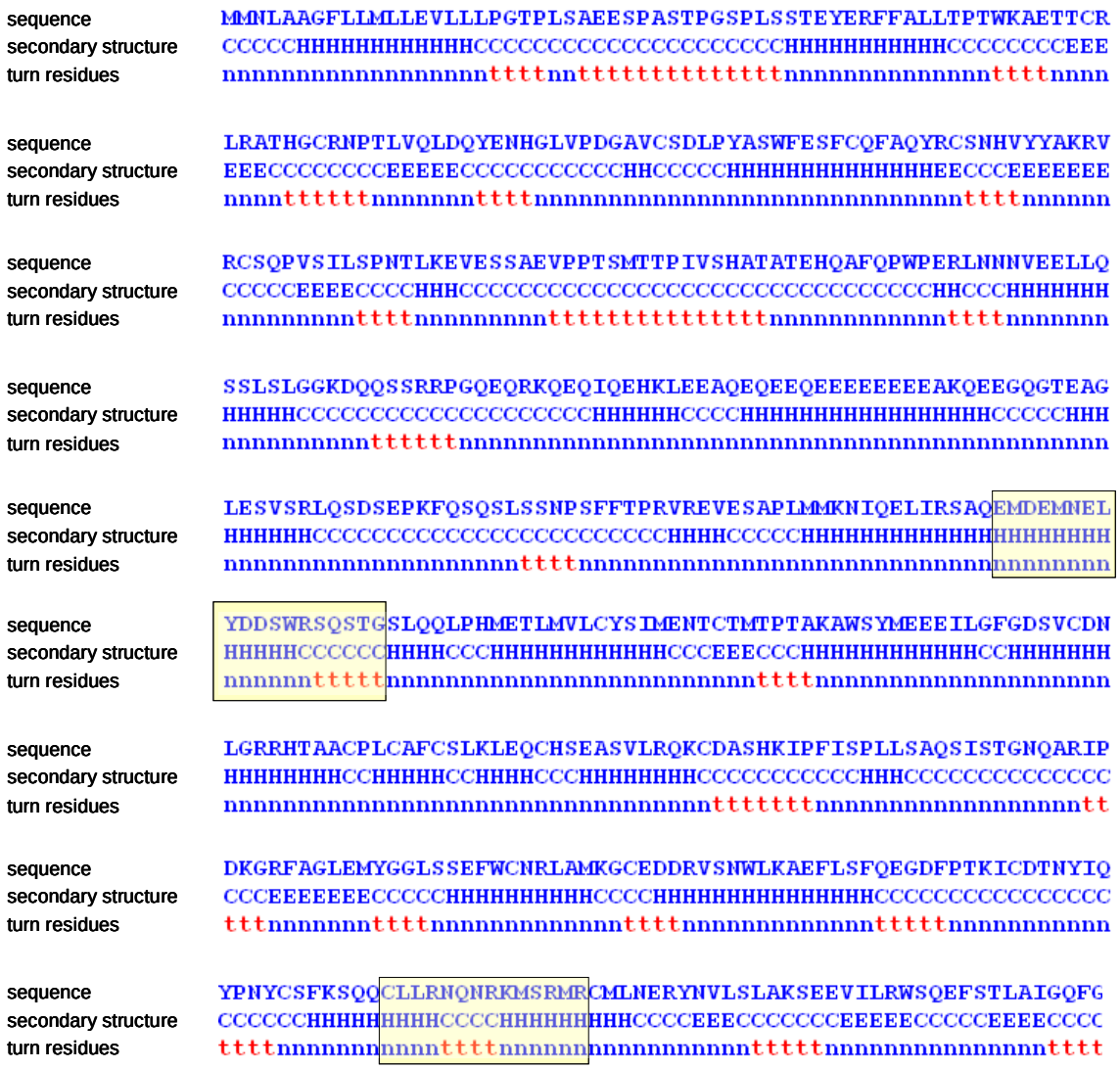


Figure 8.6. Prediction of Beta-turns in ACRBP by the BetaTPred2 server. The predictions are grouped in blocks of 60 residues. For each block: row 1 records the amino acid sequence; the second row has the secondary structure predicted by PSIPRED. PSIPRED predicts 3 states: Helix denoted by 'H', Strand denoted by 'E' and Coil denoted by 'C'. The third row has the predicted turns/non-turns. The turns are denoted as small 't' and non-turns are denoted as small 'n'. Peptides ultimately used as immunogen are shaded yellow.

8.3.1.2 Candidate selection and summary

To be selected for further consideration as a candidate for use as an immunogen to generate antibodies, peptides had to be predicted as putative antigenic regions in four out of the five forecasts based on physico-chemical properties (hydrophilic, accessibility, flexibility, and antigenicity). In addition, peptides had to contain a putative beta turn as predicted by the BetaTPred2 server.

Considering these criteria, four regions of the ACRBP protein became the focus for peptide antigen selection (Fig. 8.7). In figure 8.7, pink amino acid residues were predicted as antigenic in 4 out of 5 forecasts based on physico-chemical properties, predicted beta turn residues are in bold, while residues fulfilling both criteria are underlined.

[1]MMNLAAGFLLMLLEVLLL**PGTP**LS**AEESPASTPGSPL**SSTEYERFFALLTPT**WKAE**TTCRLR
AT**HGCRNP**TLVQLDQ**YENH**GLVPDGAVCSDLPYASWFESFCQFAQYRC**SNHV**YYAKRVRC
SQPVSIL**SPNT**LKEVESSAE**VPPTSMTTPIVSHAT**ATEHQAFQPWPE**RLNNN**VEELLQSSLSL
GGKDQ**QSSRRP**GQEQRKQEQIQEHKLEEAQEQEEQEEEEEEEEAKQEEGQGTEAGLESVS
RLQSDSEPKFQSQSL**SSNP**SFFTPRVREVESAPLMMKNIQELIRSAQEMDEMNELYDDSWR
SQSTGSLQQLPHMETLMVLCYSIMENTCTM**TPTA**KAWSYMEEEILGFGDSVCDNLGRRHTA
ACPLCAFCSLKLEQCHSEASVLRQKC**DASHKIP**FISPLLSAQSISTGNQAR**IPDKG**RFAGLEM
YGGLSSEFWCNRLAMKG**CEDD**RVSNWLKAEFLSF**QEGDF**PTKICDTNYIQ**YPNY**CSFKSQQ
CLLR**NQNR**KMSRMRCMLNERYNVLS**LAKSE**EVILRWSQEFSTLAI**GQFG**[540]

Figure 8.7 Predicted antigenic regions of the ACRBP protein. Regions predicted as antigenic in 4 out of 5 physio-chemical forecasts are in pink while beta turn residues are in bold. Regions of sequence fulfilling both criteria are underlined.

Region 1： GGKDQ**QSSRRP**GQEQRKQEQIQEHKLEEAQEQE

While predicted to be antigenic and contain a beta turn, region one was rejected due to its high content of residues known to be problematic in solid phase peptide synthesis protocols (R, Q, I ,T, V).

Region 2 : DEMNELYDDSWR**SQSTG**SLQQLPHMETL

Region 2 was selected for consideration as an immunizing peptide as it fulfilled both selection criteria and would facilitate the production of a peptide antigen 15-20 residues in length.

Region 3 : CNRLAMKG**CEDD**RVSNWLKAEF

While predicted to be antigenic and contain a beta turn, region three was rejected due to the presence of a cysteine (potential disulfide bond) in the area of the peptide fulfilling both selection criteria.

Region 4 : SFKSQQCLLR**NQNR**KMSRMRCML

Region 4 was selected for consideration as an immunizing peptide as it fulfilled both selection criteria and would facilitate the production of a peptide antigen 15-20 residues in length.

Focussing on regions 2 and 4 as outlined above, peptides A and B (Fig. 8.8) were selected for use as imunogen to raise antibodies capable of recognising ACRBP. Peptide A, derived from region 2, had a cysteine residue added to its amino terminus to facilitate subsequent coupling to a carrier protein. Peptide B was derived from region 4.

peptide A :	CEMNELYDDSWRSQSTG
peptide B :	CLLRNQNRKMSRMR

Figure 8.8. Peptides selected for use as imunogen to raise anti-ACRBP antibodies.

8.3.1.3 Confirmation of peptide sequences for use as immunogen. Bioinformatic analysis : B-cell epitope prediction II .

The ABCpred server (www.imtech.res.in/raghava/abcpred/) was used as the ultimate confirmation and used to verify the predictions made by both the BCEpred server and the BetaTpred2 server.

The ABCpred server was developed for predicting continuous or linear B-cell epitopes based on machine learning techniques and uses a more progressive recurrent neural network approach for predictions. When evaluated on protein sequences not used in the development of its algorithm, ABCpred was shown to produce better predictive performance (Saha and Raghava 2006) than that obtained using single-parameter forecasts based on physico-chemical properties (hydrophilic, accessibility, flexibility, and antigenicity). The server was trained on B-cell epitopes obtained from the Bcipep database. Users can select a window length of 10, 12, 14, 16, and 20 as predicted epitope length.

Table 8.1 shows the output of the ABCpred server when asked to predict likely B-cell epitopes in the ACRBP protein.

Sequence	Start position	Score
SVSRLQSDSEPKFQSQSLSS	243	0.91
EEEAKQEEGQGTEAGLESVS	226	0.91
RSQSTGSLQQLPHMETLMVL	306	0.9
NERYNVLSLAKSEEVILRWS	509	0.88
SFKSQQCLLRNQNRKMSRMR	486	0.88
HGCRNPTLVQLDQYENHGLV	65	0.86
MTPTAKAWSYMEEEILGFGD	336	0.85
MSRMRCMLNERYNVLSLAKS	501	0.84
CDNLGRRHTAACPLCAFCSL	358	0.84
HTAACPLCAFCSLKLEQCHS	365	0.83
SFCQFAQYRCSNHVYYAKRV	101	0.83
RLNNNVEELLQSSLSLGGKD	170	0.82
LKLEQCHSEASVLRQKCDAS	377	0.81
LMVLCYSIMENTCTMTPTAK	322	0.81
DTNYIQYPNYCSFKSQQCLL	475	0.8
EVESSAEVPPTSMTTPIVSH	136	0.8
VRCSQPVSILSPNTLKEVES	120	0.8
EFWCNRLAMKGCEDDRVSNW	437	0.79
NHVYYAKRVRCSQPVSILSP	112	0.79
PLSAEESPASTPGSPLSSTE	22	0.78
SLSLGGKDQQSSRRPGQEQR	182	0.78
PKFQSQSLSSNPSFFTPRVR	253	0.77
MNELYDDSWRSQSTGSLQQL	297	0.76
PGSPLSSTEYERFFALLTPT	33	0.75
LIRSAQEMDEMNELYDDSWR	287	0.75
VREVESAPLMMKNIQELIRS	271	0.74
QEEQEEEEEEEEAKQEEGQG	217	0.74
KQEQIQEHKLEEAQEQEEQE	202	0.74
PTSMTTPIVSHATATEHQAF	145	0.74
QYENHGLVPDGAVCSDLPYA	77	0.71
RIPDKGRFAGLEMYGGLSSE	418	0.71
CSDLPYASWFESFCQFAQYR	90	0.7
ILGFGDSVCDNLGRRHTAAC	350	0.7

Table 8.1. Prediction of Beta-turns in ACRBP by the BetaTPred2 server. The predicted B cell epitopes are ranked according to their score obtained by trained recurrent neural network. Higher score of the peptide means the higher probability to be as epitope. All the peptides shown here are above the threshold value chosen.

Potential epitopes were ranked based on the score obtained from the trained recurrent neural network. The data in Table 8.1 supports the predictions based on physio-chemical properties and beta turn location used to select peptides for immunization. Not only did four of the predicted epitopes contain residues from the two candidate peptides, all residues in both candidate peptides A and B were predicted to be in at least one potential epitope (red residues in table 8.1).

Peptides were synthesised by solid phase peptide synthesis (SPPS), conjugated individually to diphtheria toxoid (DT) and used to immunize goats as described in section 2.10. Antiserum generated to peptide B was used for subsequent investigations.

8.3.2 Confirmation of HSPD1 interaction with ACRBP by reciprocal co-immunoprecipitation and western blot analysis.

Co-immunoprecipitation experiments using an HSPD1 antibody identified the acrosomal, proacrosin binding protein (ACRBP), as a putative HSPD1 interacting protein in capacitated sperm (chapter four). In an attempt to substantiate the interaction between HSPD1 and ACRBP, the reciprocal co-immunoprecipitation experiment utilising an antiserum raised against a synthetic peptide corresponding to internal peptides of the ACRBP protein (Fig.8.11) were performed.

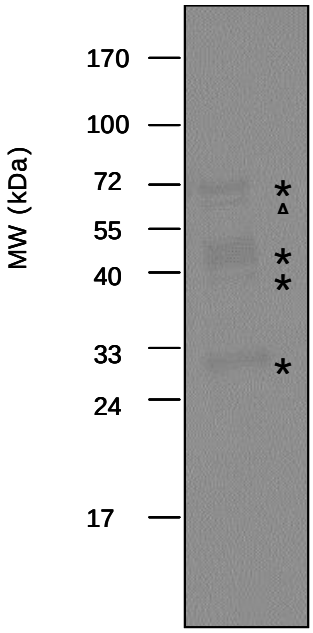


Figure 8.9. Confirmation of the HSPD1-ACRBP protein interaction by reciprocal co-immunoprecipitation. Caudal spermatozoa were incubated for 90 min in complete BWW supplemented with 1 mM pentoxifyline and 1 mM dibutyryl cAMP, washed and lysed in Buffer B. Clarified lysates were used for immunoprecipitation with an ACRBP-specific antibody. Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PVDF membrane for western analysis. The presence of both HSPD1 and ACRBP were detected with protein-specific antibodies. Proteins detected by the anti-peptide antiserum (∗), proteins detected an HSPD1-specific antibody (Δ).

As demonstrated in figure 8.9, western blotting detected the presence of HSPD1 in the precipitated material, adding further support for the association of the two proteins. Several proteins were detected by the anti-ACRBP antiserum (∗), and one protein of about 60 kDa was detected by an HSPD1 specific antibody (Δ).

8.3.3 ACRBP was localised to spermatozoa from the mouse epididymis.

In order to determine the origin of ACRBP in sperm, an adult testis extract and sperm isolated from different regions of the epididymis were assayed for the presence of the protein by western blot analysis. An antiserum raised against an internal sequence of the ACRBP protein detected several proteins in cell lysates. In the testis lysate five proteins were detected. Proteins with molecular weights of approximately 65-70 kDa (*), 50 kDa (Δ), 38 kDa (#) and two others in the order of 30 kDa (□) were detected (Fig. 8.10). Although the same proteins were observed in lysates derived from epididymal spermatozoa; all, save the lower of the two 30 kDa proteins, were fainter.

The use of β-tubulin as a loading control indicated that a comparable amount of protein was used in each sample. The predicted molecular mass of the ACRBP precursor is 61 kDa, while the mature molecule has a predicted mass of 32 kDa. However, a number of studies have detected a number of proteins in the 28-32 kDa range (Dube et al., 2005). In addition, immunoreactive products of 54 kDa have been detected in testis extracts with ACRBP-specific antibodies (Baba et al., 1994a).

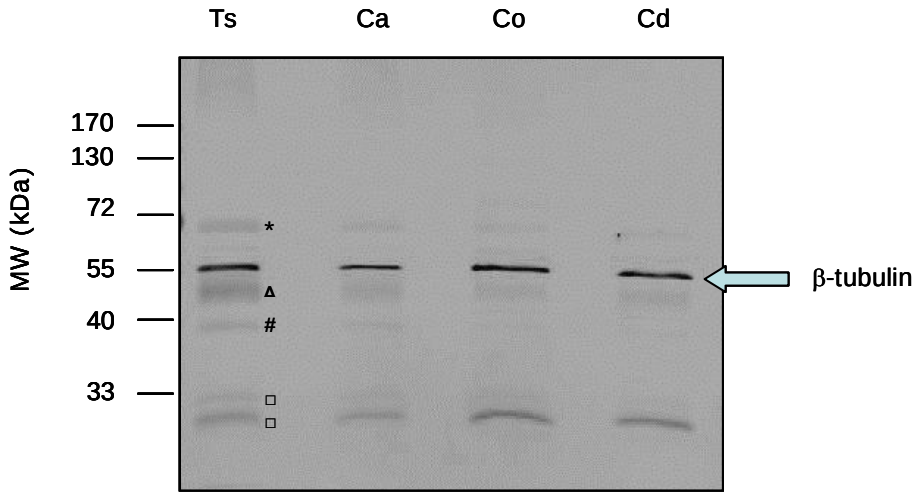


Figure 8.10. Western analysis of ACRBP in the mouse testis and epididymal spermatozoa. 5μg of cell extracts was resolved by SDS-PAGE and immunoblotted with a ACRBP-specific antibody. Blots were also probed with a β-tubulin antibody to confirm equal protein loading in each lane. Mouse testis total cell extract **(Ts)**, purified sperm from mouse caput **(Ca)**, corpus **(Co)**, and cauda **(Cd)** epididymides.

8.3.4 ACRBP localizes to the surface of capacitated mouse spermatozoa.

Since the co-IP experiments that pulled down ACRBP were designed to isolate chaperone-associated proteins from the surface of capacitated spermatozoa, experiments were performed to confirm the surface localisation of the ACRBP protein. Sperm surface proteins were labeled with a membrane impermeable biotinylation reagent (Sec. 2.4.3) and recovered from lysates with immobilised streptavidin. Putative surface proteins were separated by SDS-PAGE and the presence of ACRBP demonstrated by western analysis with an anti-ACRBP antiserum (Fig. 8.11).

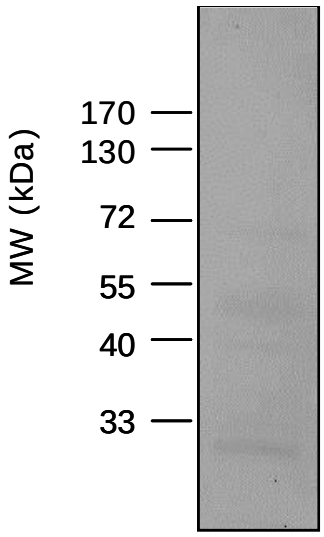


Figure 8.11. ACRBP protein was localised on the surface of capacitated spermatozoa. Caudal epididymal spermatozoa were capacitated for 90min in complete BWW. Surface proteins were biotinylated, affinity purified and resolved by SDS-PAGE. ACRBP was identified by immunoblotting with a ACRBP-specific antibody.

Several proteins were detected in the purified plasma membrane protein preparation (Fig. 8.11). These proteins were consistent with those detected in the reciprocal co-IP (Fig. 8.9) and cell extracts derived from the testis and epididymal spermatozoa (Fig. 8.10). Furthermore, the mass of the proteins is consistent with the predicted sizes for ACRBP reported in the literature (Dube et al., 2005; Baba et al., 1994a).

The localization of the ACRBP immunoreactive products on the sperm cell surface was subsequently substantiated by flow cytometry (Fig. 8.12 and 8.13). The remodeling of the plasma membrane, both in terms of protein complement and distribution, is a recognized correlate of capacitation (Lopez and Shur, 1987; Della Giovampaola et al., 2001; Focarelli et al., 1998). To assess whether the process of capacitation affected the proportion of sperm that exhibited surface ACRBP, both uncapacitated and capacitated spermatozoa were analysed by flow cytometry (Sec. 2.6.6).

Propidium iodide (PI) staining was used to facilitate the exclusion of dead and membrane compromised cells from the analysis. Since live (PI negative) sperm would

exclude antibody entry into the cytoplasm, any fluorescence would be indicative of ACRBP immunoreactive products at the cell surface. In figure 8.12, the ACRBP-FL channel measured fluorescein isothiocyanate (FITC) labeling of cells (ACRBP localisation), and the PI-FL channel measured propidium iodide staining (membrane compromised, dead cells).

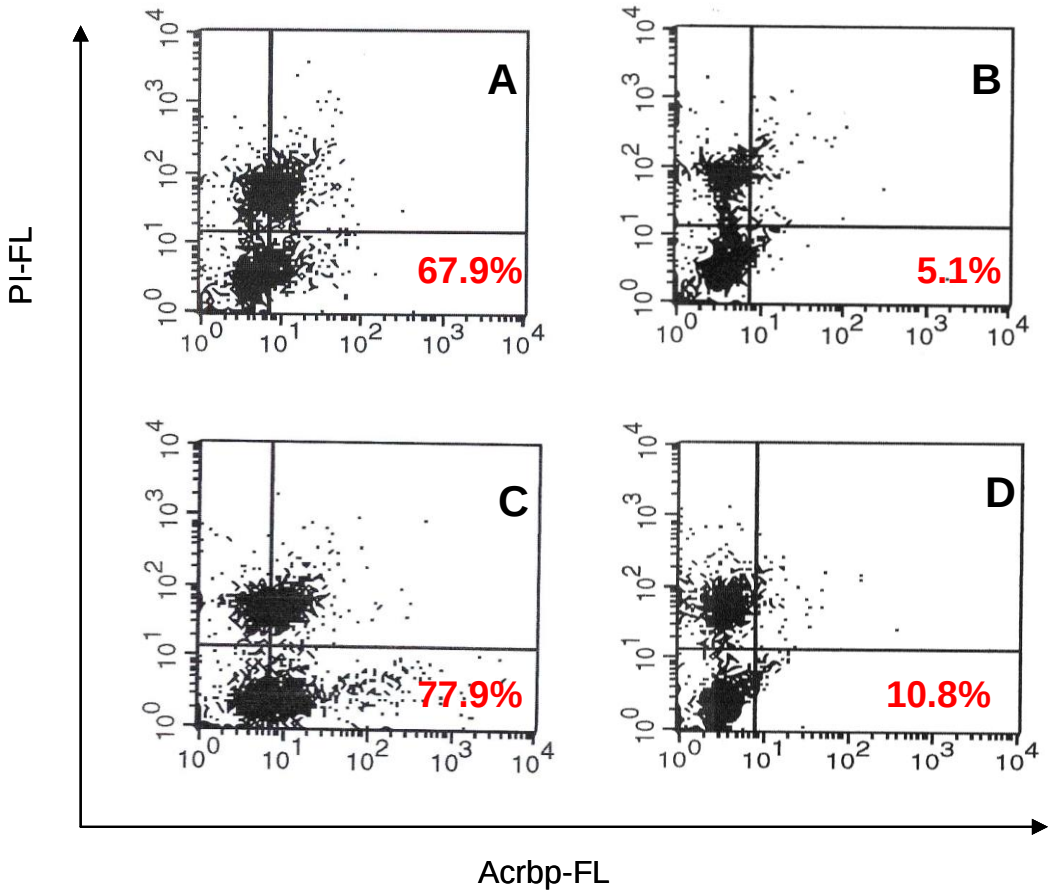


Figure 8.12. Flow cytometric analysis of ACRBP on the surface of mouse spermatozoa. Surface localisation of ACRBP on living sperm was demonstrated by cytofluorimetric analysis. Spermatozoa were recovered from the cauda epididymis and either labeled as described or capacitated in complete BWW media for 45min prior to labeling. Surface ACRBP was bound using a goat polyclonal ACRBP-specific antibody and visualized using FITC-labeled rabbit anti-goat antibody (ACRBP-FL). Cells were then treated with propidium iodide to label dead cells which were subsequently gated out of the analysis (PI-FL). The percentage of sperm with surface labeling is indicated in the bottom right hand quadrant. **A** and **B**) Uncapacitated spermatozoa. **C** and **D**) Capacitated spermatozoa. **B** and **D**) Control experiments omitting the primary ACRBP antibody.

As indicated in Fig's. 8.12 and 8.13, in uncapacitated sperm 67.9% of viable cells were positively labeled for ACRBP immunoreactive products (A, lower right quadrant). After capacitation, positive staining was observed in 77.9% of live cells; (C, lower right quadrant). In control experiments where the primary antiserum was omitted, both uncapacitated (5.1%) and capacitated (10.8 %) cells displayed only background levels of fluorescence (Fig. 8.12 B and D, lower right quadrants; Fig. 8.13). These data not only support the surface localization of ACRBP, but also indicate that the process of capacitation has no significant effect on the degree of surface localization.

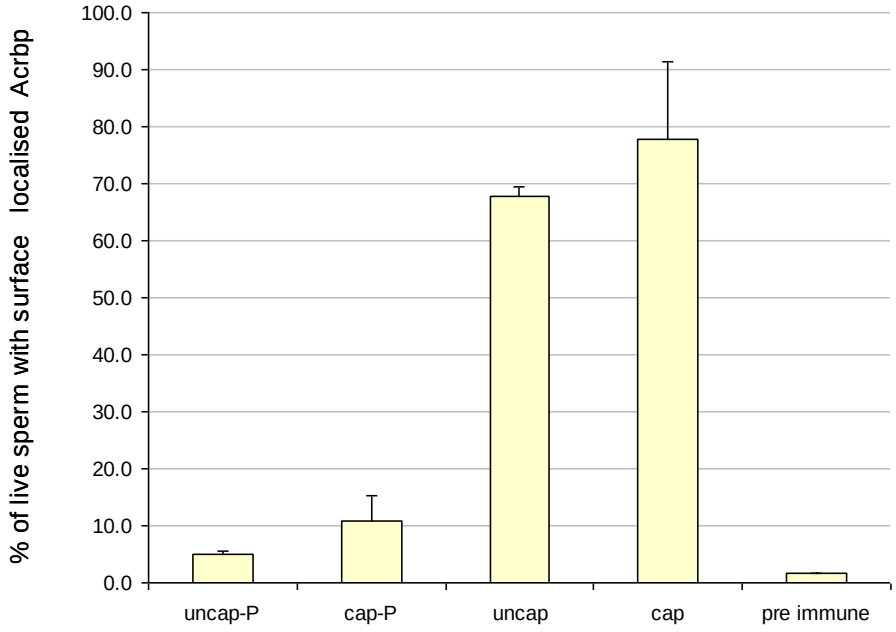


Figure 8.13. Flow cytometric analysis showing capacitation-dependent distribution of ACRBP on the surface of live mouse spermatozoa. Spermatozoa were recovered from the cauda epididymis and either labeled as described, or capacitated in complete BWW media for 45min prior to labeling. Surface localised ACRBP was bound using a goat polyclonal ACRBP-specific antibody and visualized using FITC-labeled rabbit anti-goat antibody. Cells were then treated with propidium iodide to label dead cells which were subsequently gated out of the analysis (n=3). uncap-P: uncapacitatd sperm control omitting the primary antibody, cap-P: capacitated sperm control omitting the primary antibody, uncap: uncapacitated sperm, cap: capacitated sperm, pre immune: capacitated sperm using pre-immune serum as the primary antibody.

8.3.5 ACRBP localises to the peri-acrosomal region of mouse spermatozoa.

Having confirmed the presence and surface exposure of ACRBP in epididymal sperm, we next sought to determine the localization of the protein in sperm. ACRBP immunreactive products were immunolocalised on fixed mouse spermatozoa taken from different regions of the epididymis, using indirect immunofluorescence (IIF) (Sec. 2.6.5).

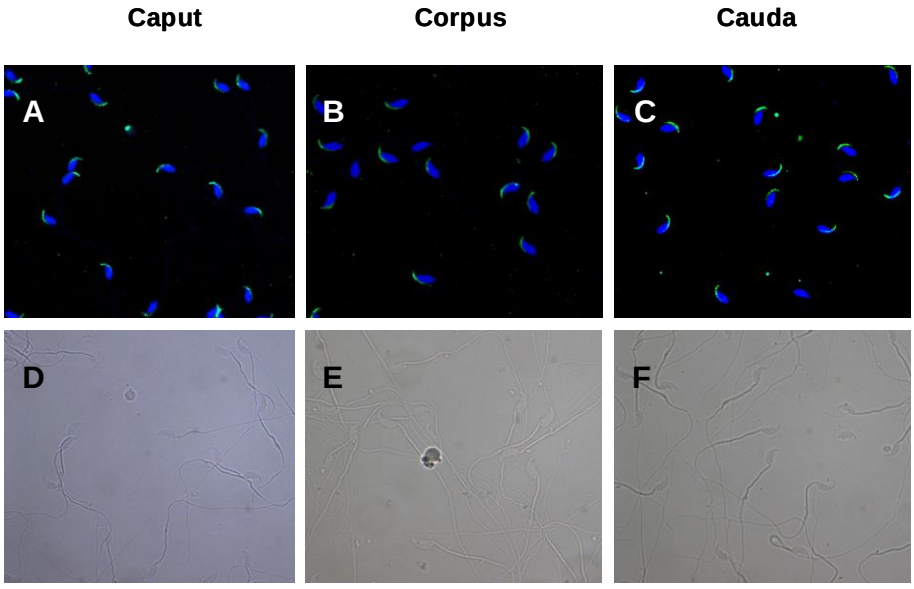


Figure 8.14. Indirect immunofluorescence localisation of ACRBP in epididymal spermatozoa. As described in section 2.6.5, epididymal sperm were incubated with anti-ACRBP serum followed by an FITC-conjugated secondary antibody. **A** and **D**) caput spermatozoa; **B** and **E**) corpus spermatozoa; **C** and **F**) caudal spermatozoa. **A**, **B**, and **C**) dual immunofluorescence and DAPI; **D**, **E**, and **F**) phase contrast. Control experiments omitting the primary antibody displayed negligible staining (not shown).

ACRBP was detected in the peri-acrosomal region of sperm taken from the caput, corpus and cauda epididymis (green labeling in Fig 8.14 A, B and C respectively). No

labeling was observed in controls in which the primary antibody was omitted (not shown). These data indicate that ACRBP immunoreactive products are present in the acrosomal region of spermatozoa taken from all regions of the mouse epididymis.

8.3.6 Acrosomal loss is correlated with loss of ACRBP from the sperm head.

Additional support for the peri-acrosomal localisation of ACRBP was achieved by following the fate of the protein subsequent to induction of the acrosome reaction. For these studies, capacitated caudal epididymal sperm were incubated with calcium ionophore, A23187 (Sec. 2.3.6). Both capacitated only, and capacitated and acrosome reacted spermatozoa were probed with ACRBP antiserum followed by FITC-conjugated secondary antibody (green labeling in Fig. 8.17). DNA was labeled with DAPI (blue labeling in Fig. 8.15). Figure 8.17 indicates that the process of capacitation has no effect on the localization of ACRBP in mouse caudal spermatozoa. The same localization pattern seen in uncapacitated cells (Fig 8.14 C) was observed with capacitated spermatozoa (Fig. 8.15 A). ACRBP immunoreactive products localised to the apical or periacrosomal region of the head in capacitated, acrosome intact sperm (Fig. 8.15 A). This acrosomal staining however, was absent in sperm that have undergone ionophore-induced acrosomal exocytosis (Fig. 8.15 B and E).

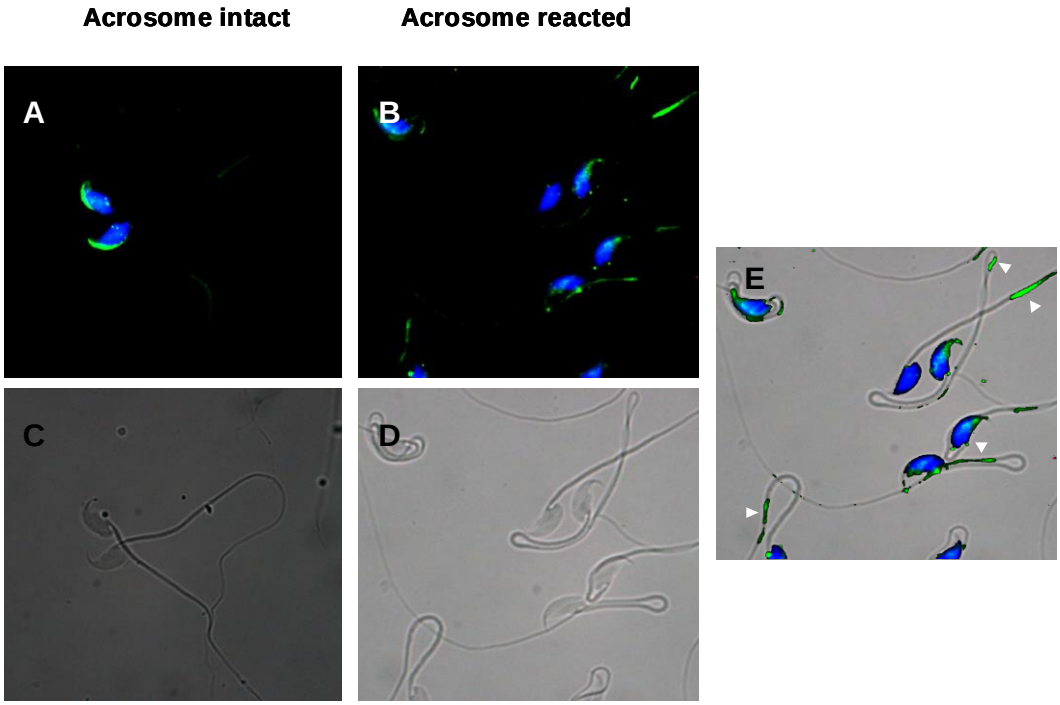


Figure 8.15. Indirect immunofluorescence demonstrating localisation of ACRBP in capacitated and acrosome reacted spermatozoa. Cauda epididymal sperm were capacitated (Sec 2.3.5), acrosome reacted (Sec 2.3.6) and incubated with an ACRBP serum followed by an FITC-conjugated secondary antibody (Sec 2.6.5). **A** and **C**) capacitated spermatozoa; **B** and **D**) acrosome reacted cells. **A** and **B**) dual immunofluorescence and DAPI; **C** and **D**) phase contrast; **E**) merged.

In addition, the protein localised to the principal piece of the sperm flagellum, but only in mature cells that have undergone the acrosome reaction (Fig. 8.15 B and E). Whether this represents a redistribution of the protein or the unmasking of an epitope remains to be established. These data further support the localisation of ACRBP immunoreactive products to the sperm plasma membrane overlying the acrosome.

8.4 Discussion

Reciprocal co-immunoprecipitation confirmed the association of ACRBP with HSPD1 in capacitated sperm. Western and indirect immunofluorescence analysis demonstrated that the protein is present in sperm throughout the epididymis and localises to the peri-acrosomal region, a position consistent with the protein having a potential role in the fertilization process. Surface biotinylation in conjunction with flow cytometry suggest that the protein is located in the plasma membrane. Taken together, these data indicate that ACRBP localizes to the plasma membrane overlying the acrosome. The process of capacitation had no discernable affect on either the protein localisation, or the proportion of sperm exhibiting the protein at the cell surface. Upon acrosomal exocytosis however, ACRBP was lost from the peri-acrosomal region of the sperm head and seemed to redistribute to the principal piece of the tail. The mechanism for this redistribution remains to be established.

The acrosome reaction of mammalian sperm is a calcium-dependent exocytotic process whereby the sperm acrosome is opened through numerous fenestrations created by focal point fusions between the plasma membrane and the outer acrosomal membrane. During this process, the inactive proacrosin zymogen is converted to acrosin, a serine-like proteinase, which appears to have multiple functions during the fertilization process (Hardy et al., 1991; Flaherty and Swann, 1993). Within the acrosome, ACRBP binds to (pro)acrosin (Baba et al., 1994a) and is believed to play a role in the activation and localization of proacrosin and acrosin.

Acrosin, is synthesised as an inactive precursor, proacrosin. It is reported that the acrosome reaction triggers a cascade of events during which proacrosin is converted into different active forms and the acrosomal granule is dispersed (Parrish and Polakoski, 1979) helping to release hydrolytic enzymes which aid sperm penetration through the zona pellucida. The acrosin activation cascade involves the sequential transformation of proacrosin into alpha-acrosin and then beta-acrosin followed by further cleavage into smaller, predominantly enzymatically inactive fragments (Moos et al., 1991).

Different roles for acrosin in the process of fertilisation have been proposed based on an accumulation of evidence. Acrosin, is believed to function in both sperm binding and penetration of the ZP (Meizel, 1984; Puigmulé et al., 2011; Słowińska et al., 2010; Veaute et al., 2010; Raterman and Springer, 2008; Gaboriau et al., 2007). Observations that acrosin binds non-enzymatically with high affinity to polysulfate groups on zona pellucida glycoproteins (ZPGPs) has led to the hypothesis that at fertilization it functions as a secondary ligand molecule to retain acrosome-reacted sperm on the surface of the egg (Jones and Brown., 1987; Topfer-Peterson et al., 1990). The mechanism of binding was shown to involve polysulfate groups on zona glycoproteins. Murine ZP2 (mZP2) binds to proacrosin null sperm less effectively than wild-type sperm (Howes et al., 2001a). The interaction between proacrosin and mZP2 is mediated by a strong ionic interaction between polysulfate groups on mZP2 and basic residues on the surface of the proacrosin protein.

Evidence has accumulated suggesting that acrosin may play a role in the interaction of the gametes prior to induction of the acrosome reaction (Tesarik et al., 1990). Participation of acrosin in the early steps of gamete recognition and binding however, is not compatible with the enzymes presumptive sequestration within the acrosome until a late phase of the acrosomal exocytosis. The classical dogma of the sequence of events during the acrosome reaction implies that membrane fusion leading to acrosomal permeabilization and exocytosis precedes the liberation and activation of the acrosomal enzymes including pro(acrosin).

Proacrosin is complexed with several non-proteolytic components in the acrosomal matrix. The first report of ACRBP was in 1977, when Polakoski and Parrish (1977) reported their proacrosin purifications were contaminated with a 30 kDa protein in pig sperm. Subsequently, several groups reported a proacrosin binding protein in the 28-32 kDa mass range (Baba et al., 1989; Yi et al., 1992; Moos et al, 1993; Kennedy and Polakoski, 1981). Also referred to as sp32, ACRBP is specifically expressed in the testis and is the product of the post-translational processing of a precursor protein whose predicted mass is 61 kDa (Baba et al., 1989). A 54 kDa variant of the precursor molecule has also been reported (Baba et al., 1994a). The presence of a proacrosin binding protein similar in molecular weight to that described here, has been

demonstrated in cattle (Yi and Polakoski, 1992), hamsters (Yi and Polakoski, 1992), humans (Yi and Polakoski, 1992), and rams (Yi and Polakoski, 1992; Harrison, 1982), guinea pig (Hardy et al., 1991).

Although ACRBP binds two proacrosins and an acrosin intermediate, the biological role of the protein and its precursor in sperm maturation and fertilization are unclear at present. A number of roles and functions for these proacrosin-associated proteins have been proposed. Hardy and colleagues postulated that the proteins are involved in the localization of proacrosin in the acrosomal matrix (Hardy et al., 1991). While others proposed that the proteins may regulate the release of proacrosin from acrosome reacted sperm (Moos et al., 1993). On the other hand it has been suggested that the proteins are involved in the activation/maturation process of proacrosin (Baba et al.,1989; Kennedy and Polakoski, 1981; Yi et al., 1992). These studies suggest that ACRBPs immobilize and stabilise proacrosin until the acrosome reaction.

However, the potential importance of ACRBP in fertilization was highlighted in 2001 when evidence was presented that proacrosin/acrosin are complementary binding proteins on sperm for ZP2, the secondary receptor that retains acrosome reacted sperm to the zona pellucida (Howes et al., 2001a; Howes and Jones, 2002). Further, it has been demonstrated that ACRBP is tyrosine phosphorylated during capacitation in the pig (Dube et al., 2005) and as a consequence of this phosphorylation appeared to redistribute to the plasma membrane of the sperm head (Bailey et al., 2005). It has also been demonstrated that ACRBP is tyrosine phosphorylated in capacitated human sperm (Ficarro et al., 2003).

Work presented in Chapter four of this thesis identified ACRBP, the sp32 precursor as a putative HSPD1-interacting protein on the surface of capacitated mouse spermatozoa (Chapter four). The data in figure 8.9 supports the physical association of the two proteins in capacitated mouse sperm. In the reciprocal co-immunoprecipitation experiment, HSPD1 was detected in material precipitated by a serum raised against peptides derived from the ACRBP protein. The identification of ACRBP as a binding partner of a cell surface chaperone has not been previously been reported.

Having confirmed the presence and interaction of HSPD1 and ACRBP in capacitated caudal sperm, we next sought to examine the localization of the ACRBP during epididymal maturation, capacitation and the acrosome reaction as an initial step to characterising the role of the protein in sperm function.

The relative levels of ACRBP in spermatozoa at different stages of epididymal maturation was determined. For this purpose lysates of sperm taken from different regions of the epididymis, together with a testis total cell extract were compared. Western blot analysis of these extracts detected several proteins with molecular weights consistent with reports of ACRBP in the literature (Dube et al., 2005; Baba et al., 1994a). With the exception of the smallest protein which became more prevalent, the relative level of the detected proteins appeared to decrease as sperm progressed through the epididymis. This may indicate the loss of existing and acquisition of additional protein during the epididymal maturation process, a well documented phenomenon. Alternatively, this may be a consequence of ACRBP precursor molecule maturation.

If HSPD1-associated proteins, including ACRBP, have a role in the molecular interactions that take place between the gametes, they should be localised on the surface of capacitated spermatozoa. Cell surface biotinylation experiments employing a membrane impermeable biotinylation reagent indicated that ACRBP immunoreactive products were surface localised in caudal spermatozoa (Fig 8.11). The results of these experiments were confirmed by flow cytometry which indicated both uncapacitated and capacitated sperm exhibit the protein at the cell surface. No significant difference between the percentage of capacitated and uncapacitated cells demonstrating surface exposure of ACRBP was evident (Fig. 8.12 & 8.13). More than 65% of both uncapacitated and capacitated sperm exhibit the protein. This is significantly more that the percentage of spermatozoa reported to exhibit HSPD1 at the cell surface, 6% and 25% for uncapacitated and capacitated cells respectively (Asquith et al., 2005).

The detection of ACRBP on the surface of sperm is at odds with its historical intra-acrosomal loction. The observance of acrosomal constituents on the surface of acrosome intact sperm however, is not without precedence. Sperm protein 56 (sp56),

a component of the acrosomal matrix (Kim et al., 2001) has been localized to the surface of capacitated sperm (Bookbinder et al., 1995) and been proposed as a potential receptor protein for the murine zona pellucida (Bleil and Wassarman, 1990). In addition, the cell surface exposure of intra-acrosomal glycohydrolases on acrosome-intact murine sperm has been reported (Abou-Haila and Tulsiani, 2003). Proacrosin has also been observed at the surface of acrosome intact cells (Tesarik et al., 1990). It was demonstrated that an anti-acrosin monoclonal antibody bound to the plasma memebrane overlying the acrosome of human sperm while the acrosome was still impermeable to intra-acrosomal-directed probes. Subsequently, it was confirmed that acrosin becomes partly exposed, in its proenzyme form, on the sperm plasma membrane before the beginning membrane fusion in the human sperm acrosome reaction. This externalised proacrosin is then preferentially converted into active forms of the enzyme (Tesarik et al., 1990).

How do intra-acrosomal proteins appear on the surface of capacitating sperm without permeablisation? It has been suggested that the expression of these molecules at the cell surface is the result of a membrane priming process occurring during capacitation (Tulsiani and Abou-Haila, 2004). While relatively little is known about mechanisms orchestrating this membrane priming, the end result of the process is responsiveness of capacitated sperm to undergo the acrosome reaction. Tulsiani proposed that the outer acrosomal membrane forms secretory vesicles that then fuse with the plasma membrane. Maybe there is transfer at focal points of membrane contact during the acrosome reaction (Tulsiani and Abou-Haila, 2004)

It has been proposed that the proacrosin/acrosin system may be instrumental not only in the secondary, consolidated phase of sperm-zona binding, but also in the initiation of the process. Inhibition of sperm binding to the zona by trypsin inhibitors has been described in the mouse (Saling, 1981). An important consequence of early proacrosin exposure on the sperm surface is its accessibility to the activators produced by the cumulus cells and secreted into the cumulus intercellular matrix (Tesarik et al., 1988). The proacrosin activator, a high molecular weight proteoglycan (Drahorad et al., 1991) acts selectively on the first step of the acrosin activation cascade, converting proacrosin into alpha-acrosin which is remarkably stable when associated with living sperm and is the main form of acrosin implied in fertilisation.

Interesingly, it has been reported that the proacrosin activator present in the cumulus matrix potentiates the effects of progesterone on sperm, calcium ion influx and acrosomal exocytosis (Mendoza et al., 1993). The effects of progesterone are known to be inhibited by synthetic protease inhibitors. It was suggested that the protease may be acrosin and its action may be involved in a process coupling the cross-linking of the surface progesterone receptor to the opening of calcium ion channels.

It is known that ACRBP binds proacrosin and alpha-acrosin. In addition, in the absence of ACRBP, alpha–acrosin is quickly converted to beta-acrosin. The role of ACRBP at the cell surface may be to present proacrosin and stabilize alpha-acrosin for subsequent participation in interaction with the zona pellucida and the acrosome reaction.

The role of acrosin has been challenged by experiments in which mice carrying a targeted mutation of the acrosin gene (Baba et al., 1994b), which were shown to be fertile. Nevertheless, although the spermatozoa of these mice were able to fertilise homologous oocytes *in vitro*, it took them 30 minutes longer than control mice. Such observations do not preclude the participation of the proacrosin/acrosin system in normal fertilization. Indeed it would be anticipated that fertilization, such a crucial phenomenon for living beings, would be ensured through multiple redundant mechanisms. Further, it has was shown that in *in vitro* fertilization assays in which wild type sperm compete with those lacking a functional acrosin gene, only the wild type sperm were able to fertilise oocytes (Adham et al., 1997). Taken together these observations suggest that during normal fertilization acrosin is required for fertilization.

Indirect immunofluorescence studies localised ACRBP to the peri-acrosomal area of the sperm head in spermatozoa taken from all regions of the epididymis (Figs. 8.14). Considering ACRBP appeared in the acrosomal region, it is possible that the protein would be lost or spatially redistributed as spermatozoa capacitated or underwent acrosomal exocytosis. The process of capacitation appeared to have no effect on the localisation of ACRBP which localised to the same region seen in uncapacitated cells (Fig. 8.15 A). This localisation however, was lost once sperm have undergone

calcium ionophore A23187-induced acrosomal exocytosis (Fig 8.15 B). The loss of the protein during the acrosome reaction is consistent with the observed peri-acrosomal localization. In addition, the protein appeared to redistribute to the principal piece of the tail in acrosome reacted cells. The mechanism for this redistribution remains to be established but may indicate a role for the protein in motility.

The mechanism by which ACRBP may regulate sperm function remains open to question. It has been proposed that HSPD1-associated proteins form part of a macromolecular protein complex in the sperm plasma membrane and that tyrosine phosphorylation of chaperones during sperm capacitation may then signal the activation of the complex and stimulate a signaling cascade leading to the acquisition of zona binding ability. The data presented in this chapter are in line with the notion that ACRBP maybe a constituent of just such a macromolecular protein complex. If ACRBP plays a role in the interaction of the gametes, as we have hypothesized for the chaperones HSPD1, then these proteins would be expected to colocalize on the apical surface of the head of live, capacitated sperm. Indirect immunofluorescence, surface biotinylation and flow cytometry demonstrated that ACRBP does indeed localise to the apical surface of the head of live, capacitated spermatozoa, where HSPD1 has been localised.

CHAPTER 9

CONCLUDING REMARKS

9. Final discussion and concluding remarks.

9.1 Fertilisation

Mammalian fertilization has been the subject of intensive research for many years now. Yet, despite the fundamental importance of this highly regulated and species-specific event, the molecular basis of how the gametes recognize and interact with each another remains unclear. One of the key issues that remains contentious is the identity of the molecules on the surface of sperm that recognize and bind the outer vestments of the ovulated oocyte complex, the zona pellucida.

Elucidation of the mechanisms that potentiate the ability of spermatozoa to recognize and bind the zona is highly relevant to the development of novel, safe, effective, and inexpensive contraceptives; the preservation of global reproductive health and the genetic conservation of endangered species as well as agriculturally valuable animals. Further, a stronger grasp of the molecular events underpinning mouse fertilization would support the breeding of transgenic mice for the preservation of important models for human disease (Evans and Florman, 2002).

The studies presented in this thesis contribute to our understanding of the biochemical and molecular foundations that underpin sperm capacitation and zona pellucida recognition and adhesion. The following text summarises the major findings of this work and its impact on our current understanding of reproductive biology. In addition, concepts that warrant further consideration are highlighted.

Fertilisation is one of the most important interactions between cells, the union of the male and female gametes, for sperm, represents the culmination of a complex series of post-testicular maturation events. Mammalian spermatozoa leave the testis morphologically differentiated but functionally incompetent. They neither possess the capacity for progressive motility nor the ability to engage and interact with the zona pellucida (Yanagimachi, 1994; Ecroyd *et al*., 2004; Gatti et al., 2004). Sperm

gradually acquire these properties during successive phases of post-testicular maturation in both the male (epididymal maturation) (Yanagimachi, 1994; Robaire et al., 2006) and female (capacitation) reproductive tracts.

9.2 Epididymal maturation

Sperm are transcriptionally inactive, and therefore unable to create new proteins by ribosomal protein synthesis. Thus, their molecular transformation into fertilisation-competent cells must be underpinned by post-translational modifications of existing proteins and remodeling of their cell surface. It is their sequential exposure to the *milieu* of the different microenvironments created along the epididymal lumen that is believed to facilitate the changes that ultimately render them competent for fertilisation; the ability to move, capacitate and interact with the zona pellucida. Although the characterization of this microenvironment has been the focus of a number of studies, the precise mechanisms underlying acquisition of the capacity for zona recognition and binding during epididymal maturation remain largely unresolved.

In mice, the ability of epididymal spermatozoa to bind the zona is first observed in the corpus and appears to coincide with the acquisition of the potential for movement. Futher, the acquisition of zona binding competense is temporally associated with the exposure of spermatozoa to chaperone-laden “dense bodies” in the epididymal lumen (Asquith et al., 2005). It has been suggested that the migration of molecular chaperones, and associated “cargo” proteins from these epididymal “dense bodies” to the sperm surface completes the repertoire of proteins required for zona binding (Nixon et al., 2007).

9.3 Capacitation

It is now over 50 years since Austin and Chang independently reported that ejaculated sperm still require a period of residence in the female reproductive tract before being capable of fertilisation. The changes that take place during this time and allow sperm

to gain the capacity for fertilisation have been collectively termed capacitation. The process of capacitation involves the activation of signalling pathways that allow these cells to express the abilities they gained in the epididymis and represents the ultimate phase of maturation during which sperm realize their full potential for fertilization.

One of the key correlates of the capacitated state is the dramatic increase in tyrosine phosphorylation via an unique cAMP-mediated pathway. These tyrosine phosphorylated proteins have traditionally been localized to the tails of sperm in most species. However, recent work has identified additional tyrosine phosphorylated proteins on the surface of capacitated spermatozoa (Asquith et al., 2005). Localising to the plasma membrane overlying the acrosome, these proteins are in an ideal position to participate in, or orchestrate, gamete interactions. An analysis of the surface phosphoproteome of capacitated sperm identified two molecular chaperones, heat shock protein 1 (HSPD1) and heat shock protein 90 beta (HSP90B1) (Asquith et al.., 2005). Both proteins have well characterised roles in protein folding and the assembly of multimeric protein complexes.

9.4 A role for chaperones in fertilisation

We hypothesized that the activation of HSPD1 and HSP90B1, by the tyrosine phosphorylation events associated with sperm capacitation triggers these proteins to assemble and present a functional zona pellucida recognition complex on the cell surface prior to fertilization. The studies presented in this thesis were targeted towards elucidation of the molecular basis of the initial recognition and adhesion events between the mammalian spermatozoon and oocyte. The aim of the current work was to further our understanding of the biological role played by molecular chaperones in fertilization by identifying proteins associated with these molecules during capacitation (chapter four). Although several putative HSP90B1–associated proteins were visualized on SDS-PAGE gels. Their identities could not be determined using conventional digestion and mass spectrometry techniques. In contrast, several potential HSPD1 binding proteins were identified from lysates of capacitated sperm. The characterization of four of these proteins became the focus of subsequent work.

9.5 Molecular Chaperones

Heat shock proteins (Hsps) were first observed by Ritossa in the 1960s as an increased expression of particular genes in *Drosophilia melanogaster* larvae following exposure to a sub-lethal heat shock. The first products of the genes observed by Ritossa were identified in a subsequent study in which they were referred to as heat shock proteins (Tissieres et al., 1974). Hsps are highly conserved cellular stress proteins present in every organism from bacteria to humans (Neuer et al., 2000). Although the phrase "molecular chaperone" was coined by Laskey in the late 1970s to describe nucleoplasmin (Laskey et al., 1978), from the late 1980s it came into general use to describe a range of different proteins that play roles in protein quality control by assisting in the assembly and folding of other proteins (Hightower, 1991; Hartl, 1996).

The presence Hsps has been demonstrated in a number of different tissues relevant to fertilization and reproduction including the endometrium (Tabibzadeh et al., 1996), decidua (Neuer et al., 1996), Fallopian tubes, and the placenta (Divers et al., 1995). In addition, a number of chaperone proteins including HSPD1 have been identified in the mammalian testis, epididymis and sperm (Neuer et al., 2000; Asquith et al., 2005; Ecroyd et al., 2003; Huszar et al., 2000; Miller et al., 1992).

Hsps were traditionally considered to be cytoplasmic proteins with functions restricted to the intracellular compartment. However there is a growing body of literature describing these proteins at the cell surface and in the extra cellular space. Hsps have been identified in serum in numerous studies, with levels changing in response to different factors. Both Hsp60 and Hsp70 are present at low levels in the serum of healthy individuals and increase in response to certain diseases. Increased serum Hsp60 levels are associated with arthrosclerosis (Pockley et el., 2000; Xu et al., 2000). Serum Hsp70 has been found to be elevated in individuals with chronic heart failure (Grenth-Zotz et al., 2004), pre-eclampsia (Jirecek et al., 2002), infection (Njemini et al., 2003) and peripheral and renal vascular disease (Wright et al., 2000). Surface expression of Hsps has been reported in a number of different mammalian

cell types. HSPD1 has been identified on macrophages, oligodendrocytes, and endothelial cells; while Hsp70 has been identified on B-cells and monocytes (Zugel and Kaufmann., 1999). It has been proposed that hsp70 expressed on the cell surface may also function as a target of natural killer (NK) cells (Malthoff et al., 1997).

9.6 HSPE1

The current study identified HSPE1 (formally known as HSP10) as a putative binding partner of HSPD1 (formally known as HSP60) on the surface of capacitated mouse sperm. This molecular association and localization of HSPE1 is not without precedent. HSPE1 and HSPD1 act together inside mitochondria and chloroplasts to mediate protein folding. The homologous molecules in *E. coli* are known as GroES and GroEL, respectively. GroEL forms a dual ringed tetradecamer structure, capped at either or both ends by a heptamer of GroES to form a symmetrical structure.

Consistent with the findings of the present study, recent studies suggest that chaperones have other roles at the cell surface (Barazi et al., 2002; Feng et al., 2001;Shin et al., 2003), or outside the cell (Lewthwaite et al., 2002; Xu, 2003) Calnexin, calreticulin, GP96, GRP74, HSP72, HSP70, HSPD1, HSP54, HSP27 and protein disulfide isomerase (PDI) have all been reported on the surface of a range of cell types including cancer cells, fibroblasts and blood cells (Akagi et al., 1988; Altmeyer et al., 1996; Essex et al., 1995; Ferrarini et al., 1992; Goicoechea et al., 2000; Okazaki et al., 2000; White et al., 1995; Wiest et al., 1997; Weist et al., 1995). A recent study has revealed the major proteins present on the surface of certain cancer cells are molecular chaperones (Shin et al., 2003).

Hsp10 localizes extracellularly during pregnancy and is hypothesized to be Early Pregnancy Factor (EPF), which is released during the first stages of gestation and is involved in the establishment of pregnancy (Morton et al., 1977). This extracellular form of HSPE1 has been proposed to be a major immunosuppressive agent during pregnancy (Morton et al, 1992). In addition, the protein has been shown to exhibit growth factor properties (Morton, 1998). Various reports show that extracellular

HSPE1/EPF modulate certain aspects of the immune response with anti-inflammatory effects in patients with autoimmune conditions improving clinically after treatment with recombinant HSPE1. Moreover, HSPE1/EPF are involved in embryonic development, acting as a growth factor, and in cell proliferation/differentiation mechanisms. Therefore, it becomes evident that Hsp10 is not only a co-chaperonin, but an active player in its own right in various cellular functions.

HSPE1 expression during post testicular maturation proved to be similar to that previously reported by us for both HSPD1 and HSP90B1 (Asquith et al., 2005). HSPE1 was detected in sperm isolated from all regions of the epididymis and localised to the peri-acrosomal region of the sperm head. However, in sperm isolated from the cauda epididymis, labeling was observed in the principal piece of the tail in addition to stronger labeling in the peri-acrosomal region of the sperm head. Whether this represents a redistribution of the protein or the unmasking of an epitope during epididymal maturation remains to be established. Capacitation had no effect on the localisation of HSPE1, the same localization pattern seen with uncapacitated cells is observed with capacitated spermatozoa. Although acrosomal exocytosis had no observable effect on the localization of HSPE1 in the sperm tail, the strong acrosomal staining observed in the acrosomal region of epididymal spermatozoa was absent in cells that hads undergone ionophore-induced acrosomal exocytosis. This is consistent with the concept that HSPE1 is expressed on the surface of capacitated sperm and may be involved in oocyte interactions.

If HSPE1 plays a role in the interaction of the gametes, it should be localised on the surface of functionally competent capacitated caudal spermatozoa. Biotinylation and flow cytometry experiments demonstrated that HSPE1 is indeed localized to the cell surface in mature mouse spermatozoa and following capacitation, there was a significant increase in the proportion of spermatozoa expressing HSPE1 at the cell surface. This documents for the first time the surface expression of the molecular chaperone HSPE1 in mouse spermatozoa. In addition, such findings not only implicate this chaperone in the capacitation-dependent acquisition of zona binding potential, but also suggest the possible use of this protein as a marker to monitor the capacitation status of spermatozoa.

Interestingly, the change in the level of surface expression of HSPE1 following capacitation is consistent with that reported HSPD1 where 6% of uncapacitated cells expressed the protein at the cell surface compared to 27% of capacitated cells (Asquith et al., 2005). The finding that the cell surface localisation of HSPE1 increases significantly during capacitation is unprecedented and has implications for the function of the protein in the male germ line. A number of possible roles for the protein at the cell surface may be proposed.

HSPE1 may be acting as an accessory molecule to HSPD1. This suggests a direct transposition of HSPD1-HSPE1 function to the plasma membrane where it could function to improve the efficiency of the folding and assembly of surface molecules. Similar levels of HSPD1 surface expression in uncapacitated and capacitated cells are consistent with this notion. Alternatively, HSPE1 may act as an accessory molecule to a protein other than HSPD1. At the cell surface, HSPE1 might functionally modify a receptor or adhesion molecule for the zona pellucida. Lastly, it is recognized that HSPE1 might function independently of HSPD1.

9.7 Aldose reductase

Although the AKR1B7 sequence has been identified in the mouse genome and localised to the male reproductive tract (Baumann et al., 2007), no literature regarding the role or activity of the enzyme in sperm function has been published.

The putative interaction between HSPD1 and the AKR1B7 was supported by reciprocal co-immunoprecipitation and represents the first identification of an aldose reductase as a binding partner of a cell surface chaperone .

It was demonstrated that sperm acquire the AKR1B7 protein during the process of epididymal maturation as they pass from the corpus to the cauda. The mechanism by which epididymal sperm acquire new proteins remains unclear, however recent reports have suggested that epididymosomes and what have been termed “dense bodies” in the epididymal lumen may be involved in the process (Sullivan et al., 2007;

Oh et al., 2009). Interestingly, recent literature (Frenette et al., 2003; Sullivan et al., 2007) has identified members of the AKR1B subfamily of aldose reductases as constituents of epididymosomes in other species, including humans and bulls. Epididymosomes are membranous secretory vesicles of epididymal origin implicated in the transfer of new proteins to sperm during epididymal transit (Sullivan et al., 2007).

AKR1B7 localizes to the plasma membrane overlying the acrosome, and capacitation has no effect on the relative expression level or localization of the protein, with little difference between the percentage of cells demonstrating surface localisation between the capacitated and uncapacitated samples. Approximately 80% of live cesll in both uncapacitated and capacitated samples exhibit the protein. This is significantly more that the percentage of spermatozoa reported to exhibit HSPD1 at the cell surface, 6% and 25% for uncapacitated and capacitated cells respectively (Asquith et al., 2005).

The fact that there is little difference in the level of surface localisation between uncapacitated and capacitated cells is inconsistent with the protein having a role in direct binding of the zona pellucida. Previous reports demonstrate an increased ability of sperm to bind the zona pellucida after capacitation (Asquith et al., 2005) suggesting that surface expression of the actual receptor for the zona pellucida is altered as a consequence of capacitation. Nevertheless,, surface exposure of this protein was lost upon induction of acrosomal exocytosis, which is consistent with a protein potentially involved in fertilization or the maintenance of cellular integrity.

The precise function of the AKR1B7 aldose reductase in the male reproductive tract remains to be elucidated, however, a number of roles may be proposed.

It has been proposed that the polyol pathway functions to modulate sperm motility during epididymal transit. In the polyol pathway, the rate determining step is the reduction of glucose to sorbitol by aldose reductase. Extracellular aldose reductase favours the accumulation of sorbitol within the epididymal fluid. Sorbitol being membrane impermeable; accumulates in the epididymal lumen, contributing to the relatively high osmotic pressure of the epididymal fluid, and deprives the sperm intracellular compartment of an energy source. Sorbitol is known to be an

osmotically active organic solute (Maneko et al., 1990), which suggests AKR1B7 may play an osmoregulatory role. Others have independently hypothesized that sorbitol in the epididymal lumen acts as an osmolyte required for volume regulation of the sperm cell (Pruneda et al., 2006). By its accumulation in the excurrent duct luminal compartment, aldose reductase activity, via the reduction of extracellular glucose concentration resulting from the generation of sorbitol and/or the latters ability to maintain a high extracellular osmotic pressure, could contribute to transient sperm immobilization within the epididymis.

Alternatively, AKR1B7 may fulfill other roles in the male reproductive tract. A detoxification role has been suggested for the protein. The role of AKR1B7 in the male reproductive tract may be related to its capacity to reduce isocaproaldehyde. Isocaproaldehyde is the residue left after the action of the cholesterol side chain cleavage enzyme cytochrome P450scc on the cholesterol molecule in the steroid biosynthesis pathway. It has been demonstrated that male gametes, *in vitro*, posses metabolizing enzymes capable of catalyzing the transformation of cholesterol to androgens (Gunasegaram et al., 1998). More importantly AKR1B7 might be involved in countering the cyotoxic aldehydes (such as 4-hydroxynonenal or acrolein) generated by spermatozoa under aerobic conditions, which are known to be highly damaging to these cells (Aitken et al., 2012).

The association of AKR1B7 with the plasma membrane of spermatozoa raises the question of a potential role for the protein in signaling pathways involved in fertilization. Mounting evidence suggests that aldose reductases interact in some way with signaling cascades involving protein kinase C (PKC). Additionally, studies indicate that the antioxidant and signaling roles of aldose reductase are interlinked and that aldose reductase regulates PKC via a redox sensitive mechanism. It has been proposed that the increase in NADH due to polyol pathway activity increases the production of diacylglycerol (DAG) from dihydroxyacetone phosphate (Thomas et al., 1994). DAG is the physiological activator of PKC whose presence and activity has been demonstrated in mammalian spermatozoa (Baldi et al., 2002; Breitbart et al., 1992; Breitbart and Naor, 1999) indicating a possible involvement in the acrosome reaction.

The mechanism by which AKR1B7 may regulate sperm function remains open to question. Particularly perplexing is determining how this enzyme manages to gain access to the NADPH it needs to be functionally active when located on the extracellular face of the plasma membrane. It has been proposed that HSPD1 associated proteins form part of a macromolecular protein complex in the sperm plasma membrane and that tyrosine phosphorylation of chaperones during sperm capacitation may then signal the activation of the complex and stimulate a signaling cascade leading to the acquisition of zona binding ability. The data presented in this chapter are in line with the notion that AKR1B7 may be a constituent of just such a macromolecular protein complex. However, it is difficult to envisage such a role for the protein based on its functions in other tissues and cell types where it is involved in glucose metabolism and the detoxification of cytotoxic carbonyl compounds. Further studies might therefore focus on the proposed role of aldose reductases in stimulating the protein kinase C pathway that mediates the the acrosome reaction.

9.8 Citron kinase

Data presented in this thesis has demonstrated for the first time the expression and localization of citron kinase, an effector protein of the GTPase Rho, in murine spermatozoa. Furthermore, Citron kinase was identified as a putative HSPD1-interacting protein in lysates of capacitated sperm. CRIK is ubiquitously expressed in most tissues and cell types with a cell-cycle dependent localization (Madaule *et al.*, 1998) and has been reported to play a key role in cytokinesis (Madaule *et al.*, 1998; Eda et al., 2001).

The Rho family of signaling molecules are small guanosine triphosphatases (GTPases). In mammalian cells the Rho family of GTPases have been characterized as having a role it the regulation and maintenance of the cytoskeleton (Kaibuchi et al., 1999).

Although the reciprocal co-ip strategy could not confirm the interaction of CRIK with HSPD1, the association of the two proteins cannot be ruled out and warrants further

investigation. Techniques not reliant on the availability of an epitope may be useful here. The fact that the CRIK-specific antibody could not pull down CRIK itself, let alone any associated proteins, indicates that either the relevant epitope in the CRIK protein was unavailable or that the antibody was not of sufficient affinity to be useful in an immunoprecipitation approach.

Notwithstanding, CRIK immunoreactive products were detected in both the testis and epididymal sperm lysates by western blotting. CRIK, or fragments thereof, localize to the peri-acrosomal region of the sperm head. Additionally, biotinylation and flow cytometry experiments indicate that the protein is expressed superficially at the cell surface. Although capacitation had no effect on the localization of CRIK, the protein could not be detected in sperm that had undergone acrosomal exocytosis which further supports the localisation of CRIK immunoreactive products to the plasma membrane overlying the acrosome.

The most extensively studied characterized Rho effector protein is the Rho-binding serine/threonine kinase, Rho kinase (ROCK) (Fujisawa et al., 1996). Citron kinase, resembles ROCK in its overall domain structure and organization (Leung et al., 1996; Matsui et al., 1996; Nakagawa et al., 1996) suggesting a similarity in function.

The acrosomal and cell-surface localisation of CRIK are consistent with the protein having a role in gamete interactions. Although there is no precedence for CRIK having an acrosomal or cell surface localization in spermatozoa, other Rho effector proteins have been described at these locations, as has Rho. Urióstegui et al. (2007) for example, localized ROCK and Rho to the acrosomal region of sea urchin spermatozoa. Moreover, they demonstrated that ROCK antagonists inhibited the acrosome reaction, suggesting that a Rho/ROCK-dependent signaling pathway may regulate the acrosome reaction in this species. Furthermore, it has been reported that bacterial toxin, C3, which specifically inhibits Rho function (Wilde et al., 2000) prevents the ionophore-induced acrosome reaction in several mammalian species.

Other recent literature reports similar localisations in mammalian spermatozoa. Ducummon and Berger (2006) localized the Rho GTPases RhoA, RhoB, Rac1 and Cdc42; as well as the effectors RhoGDI, PI(4)P5K and ROCK to the acrosomal

region of the sperm head in boar, human, rat, ram, bull and elephant sperm. Such a localization is consistent with the hypothesized role for a Rho-mediated signaling pathway culminating in acrosomal exocytosis (Castellano et al., 1997). These observations taken together suggest that the expression of Rho GTPases in sperm has been conserved throughout mammalian evolution, most likely due to the role of these GTPases in regulating acrosomal exocytosis. It is possible that CRIK could play a role in such a signaling pathway.

Alternatively, CRIK in sperm may function as a scaffolding molecule, similar to the role of CIT-N on Golgi membranes where it regulates actin polymerization (Camera et al., 2003). It has been demonstrated that actin polymerization plays an important role in the zona pellucida-induced acrosome reaction of mammalian sperm (Liu et al., 1999; Spungin et al., 1995; Howes et al., 2001; Liu et al., 2002; Palecek et al., 1999; Vogl, 1989). The polymerization of globular actin to filamentous actin between the outer acrosomal membrane and the plasma membrane of sperm during capacitation provides a scaffold that immobilizes phospholipase Cγ, which has been implicated in the acrosome reaction at the cell surface (Breitbart and Spungin, 1997).

9.9 ACRBP

During fertilization ACRBP appears to be the key regulator for autocatalytic conversion of proacrosin into intermediate forms, and ultimately to active acrosin (Baba et al., 1994a). How acrosin autoactivation is triggered by ACRBP is not clear, but phosphorylation of ACRBP during capacitation is likely to be an important preparatory event (Dube et al., 2005)

Work presented in Chapter four of this thesis identified ACRBP, as a putative HSPD1-interacting protein on the surface of capacitated mouse spermatozoa (Chapter four). The data in figure 8.9 supports the physical association of the two proteins in capacitated mouse sperm. The identification of ACRBP as a binding partner of a cell surface chaperone has not been previously been reported.

Western and indirect immunofluorescence analysis demonstrated that the protein is present in sperm throughout the epididymis and localises to the peri-acrosomal region, a position consistent with the protein having a potential role in the fertilization process.

Further, the data in chapter eight indicate that ACRBP localizes to the plasma membrane overlying the acrosome. And that capacitation had no discernable affect on either the protein localisation, or the proportion of sperm exhibiting the protein at the cell surface. Upon acrosomal exocytosis however, ACRBP was lost from the peri-acrosomal region of the sperm head and seemed to redistribute to the principal piece of the tail. The mechanism for this redistribution remains to be established.

The detection of ACRBP on the surface of sperm is at odds with its historical intra-acrosomal loction. However, it has been demonstrated that ACRBP is tyrosine phosphorylated during capacitation in the pig (Dube et al., 2005) and as a consequence of this phosphorylation appeared to redistribute to the plasma membrane of the sperm head (Bailey et al., 2005).

ACRBP binds proacrosin and alpha-acrosin. In addition, in the absence of ACRBP, alpha–acrosin is quickly converted to beta-acrosin. The role of ACRBP at the cell surface may be to present proacrosin and stabilize alpha-acrosin for subsequent participation in interaction with the zona pellucida and the acrosome reaction. Once acrosin is activated during acrosomal exocytosis the acrosomal contents are dispersed, but without acrosin activation the acrosomal contents disperse more slowly Thus, it is clear that ACRBP is an important regulator of proteolytic processing events during acrosomal matrix disassembly.

The mechanism by which ACRBP may regulate sperm function remains open to question. It has been proposed that HSPD1-associated proteins form part of a macromolecular protein complex in the sperm plasma membrane and that tyrosine phosphorylation of chaperones during sperm capacitation may then signal the activation of the complex and stimulate a signaling cascade leading to the acquisition of zona binding ability. The data presented in this thesis are in line with the notion that ACRBP maybe a constituent of just such a macromolecular protein complex.

If ACRBP plays a role in the interaction of the gametes, as we have hypothesized for the chaperones HSPD1, then these proteins would be expected to colocalize on the apical surface of the head of live, capacitated sperm. Indirect immunofluorescence, surface biotinylation and flow cytometry demonstrated that ACRBP does indeed localise to the apical surface of the head of live, capacitated spermatozoa, where HSPD1 has been localised.

Although proacrosin has been a major focus of research for a long time, ,The data presented in this thesis warrant futher investigation of the role of ACRBP,

9.10 The cell surface proteome

While the mechanisms that underpin maturation, capacitation, and sperm-egg interactions remain elusive it is known that these essential fertilisation events are driven by the protein complement of the sperm surface. Yet despite their importance in sperm physiology, the proteins of the plasma membrane have remained poorly characterized in the male gamete. The work detailed in chapter three identified cell surface proteins of murine sperm that may be important for sperm function, particularly in regards to sperm-oocyte interactions.

Our strategy of combining subcellular fractionation and an in-solution digestion protocol resulted in the identification of 186 proteins, 32 of which were identified with a higher confidence from higher stringency filtering.

In contrast to global proteomic profiling studies employing whole-cell lysates, this study has examined the plasma membrane fraction of fresh mouse sperm, which was demonstrated to be enrichment in plasma membrane-associated proteins. Bioinformatic analysis shows that 53% have been shown previously to be associated with the plasma membrane in sperm (Table 3.1). Twelve of these proteins have

previously been shown to be constituents of lipid rafts in sperm, while six others have been observed in lipid rafts from other cell types.

The data will be of interest in future studies and provide an important reference point for comparative or differential studies. The results of chapter three may be used in studies comparing sperm at different stages of epididymal maturation, or the surface proteomes of capacitated versus non-capacitated cells. Further, the results may provide candidates for the development of new approaches to fertility control.

9.11 Concluding remarks

Collectively these data invite speculation that molecular chaperones are involved in maturation of the sperm surface during capacitation to render these cells functionally competent to engage the process of fertilization. However it is hard to envisage a protein complex containing several chaperone proteins together with molecules with as diverse roles as aldose reductase and citron kinase, participating in zona recognition events. However, the proposal for a multimeric receptor complex is consistent with previous evidence that zona recognition is mediated by multiple molecules with different affinities (Thaler and Cardullo, 2002).

The future of this work rest with the identification and characterisation of further members of chaperone-containing protein complexes in spermatozoa and comparative proteomic studies. Investigations comparing proteome sets from sperm at different stages of epididymal maturation and capacitation may shed light on the molecules that are the key players when it comes to fertilization. In addition, further investigation of chaperone expression in the epididymis, and in particular the "dense bodies' and epididymosomes proposed to play a role in sperm maturation, will inform the mechanisms underlying sperm maturation in this organ.

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Appendix A

Reagents, Materials and Kits

DTSSP	Thermo Fisher Scientific, VIC
acetic acid	BDH Laboratory Supplies, Dorset, UK
acetone	Sigma Chemical Co. Ltd., St Louis, MO
acetonitrile	Merck Millipore, MA
acetonitrile	Merck Millipore, MA
alpha-cyano-4-hydroxycinnamic acid	Sigma Chemical Co. Ltd., St Louis, MO
ammonium bicarbonate	BDH Laboratory Supplies, Dorset, UK
b-mercaptoethanol	Research Organics, Clevland, OH
bovine serum albumin	Sigma Chemical Co. Ltd., St Louis, MO
bromophenol blue	Research Organics, Clevland, OH
calcium chloride	BDH Laboratory Supplies, Dorset, UK
calcium ionophore A23187	Calbiochem, San Diego, CA
CAS block	Invitrogen Life Technologies, NY
CHAPS	Research Organics, Clevland, OH
Coomassie brilliant blue R-250	Thermo Fisher Scientific, VIC
DAPI	Invitrogen Life Technologies, NY
dibutyryl cyclic andenosine monophosphate	Sigma Chemical Co. Ltd., St Louis, MO
dithiothreitol	Sigma Chemical Co. Ltd., St Louis, MO
donkey serum	Sigma Chemical Co. Ltd., St Louis, MO
Dynabeads M-280	Invitrogen Life Technologies, NY
ECL KIT	GE Healthcare Life Sciences, Buckinghamshire , UK
ethanol	AJAX Chemicals, Auburn, VIC
Ethylenediaminetetraacetic acid	Research Organics, Clevland, OH
FITC	Sigma Chemical Co. Ltd., St Louis, MO
FITC-PNA	Sigma Chemical Co. Ltd., St Louis, MO
formaldehyde	BDH Laboratory Supplies, Dorset, UK
Hoechst-3342	Sigma Chemical Co. Ltd., St Louis, MO
iodoacatamide	Sigma Chemical Co. Ltd., St Louis, MO
methanol	Merck, Whitehouse station, NJ
molecular weight standards	Sigma Chemical Co. Ltd., St Louis, MO
NeutrAvidin^{TM} gel	Thermo Fisher Scientific, VIC
Nonidet P-40	Sigma Chemical Co. Ltd., St Louis, MO
Odyssey blocking buffer	LI-COR Biotechnology, NE
pentoxifylline	Sigma Chemical Co. Ltd., St Louis, MO
percoll	GE Healthcare Life Sciences, Buckinghamshire , UK
polyvinyl alcohol	Sigma Chemical Co. Ltd., St Louis, MO
polyvinylidene fluoride	Invitrogen Life Technologies, NY
propidium iodide	Sigma Chemical Co. Ltd., St Louis, MO
protease inhibitors	Merck Millipore, MA

SDS	Sigma Chemical Co. Ltd., St Louis, MO
silver nitrate	Sigma Chemical Co. Ltd., St Louis, MO
sodium azide	BDH Laboratory Supplies, Dorset, UK
sodium carbonate	Sigma Chemical Co. Ltd., St Louis, MO
sodium chloride	Sigma Chemical Co. Ltd., St Louis, MO
sodium cyanoborohydride	Sigma Chemical Co. Ltd., St Louis, MO
Sodium deoxycholate	Thermo Fisher Scientific, VIC
sodium orthovanadate	Sigma Chemical Co. Ltd., St Louis, MO
sodium pyruvate	Sigma Chemical Co. Ltd., St Louis, MO
sodium thiosulfate	BDH Laboratory Supplies, Dorset, UK
strontium chloride	Sigma Chemical Co. Ltd., St Louis, MO
sulfo-NHS-SS-Biotin	Thermo Fisher Scientific, VIC
sypro ruby stain	Sigma Chemical Co. Ltd., St Louis, MO
TEMED	Sigma Chemical Co. Ltd., St Louis, MO
Trichloroacetic acid	Sigma Chemical Co. Ltd., St Louis, MO
trifluoroacetic acid	Sigma Chemical Co. Ltd., St Louis, MO
TCEP	Sigma Chemical Co. Ltd., St Louis, MO
Tris-HCl	Research Organics, Clevland, OH
Triton X-100	Sigma Chemical Co. Ltd., St Louis, MO
Trypsin modified sequencing grade	Promega Corporation, WI
Tween-20	Sigma Chemical Co. Ltd., St Louis, MO
Vectashield mounting media	Vector Laboratories, Inc., CA

Appendix B

Buffers and solutions

BWW	915 mM NaCl, 44 mM sodium lactate, 25 mM $NaHCO_3$, 20 mM HEPES, 5.6 mM d-glucose, 4.6 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 0.27 mM sodium pyruvate, 0.3% (w/v) BSA, 5 U/ml penicillin, and 5 ug/ml streptomycin, pH 7.4
BWW/PVA	as for BWW except the BSA was replaced with 1 mg/ml PVA
BWW-Ca^{2+}	as for BWW-Ca^{2+} but with the addition of Sr^{2+} to 1.7 mM
BWW-Ca^{2+} + Sr^{2+}	as for BWW except the $NaHCO_3$ was replaced with an additional 25 mM NaCl
BWW-HCO_3^-	as for BWW except the $CaCl_2$ was replaced with an additional 1.7 mM NaCl
CBB destain	50% (v/v) methanol in water, 8.75% (v/v) acetic acid
CBB stain	0.03% (w/v) CBB, 50% (v/v) methanol in water, 8.75% (v/v) acetic acid
lysis buffer A	150 mM NaCl, 20 mM Tris-HCl, 0.1 % Triton X-100, pH 7.4
lysis buffer B	150 mM NaCl, 20 mM Tris-HCl, 0.1 % Triton X-100, 0.5% NP40, pH 7.4
modified RIPA buffer	150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (v/v) Tween-20, 1 mM EDTA
PBS	4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH7.4
PBST	4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, 0.05% Tween 20, pH 7.4
percoll density gradient	30% (v/v) percoll in PBS
RIPA buffer	150 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 1 mM EDTA
SDS-PAGE resolving gel buffer	the appropriate amount of 40% acrylamide mix, 0.375 M Tris-HCl, 0.1% (w/v) SDS, 0.00025% ammonium persu;phate, 0.00025% (v/v) TEMED, pH 8.8
SDS-PAGE running buffer	25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS

SDS-PAGE sample buffer	0.5 M Tris-HCl, 20% Glycerol, 10% (w/v) SDS, 0.1% (w/v) Bromophenol Blue, pH 6.8
SDS-PAGE stacking gel buffer	3.6% (v/v) acrylamide, 0.2% (v/v) N'N'-bis-methylene-acrylamide, 0.3 M Tris-HCl, 0.1% (w/v) SDS, 0.003% (v/v) ammonium persulphate, 003% (v/v) TEMED, pH 8.8
Silver staining fixing solution	50% (v/v) methanol, 12% (v/v) acetic acid
TBS	20 mM Tris-HCl, 150 mM NaCl, pH 7.6
TBST	20 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween-20, pH 7.6
western transfer buffer	192 mM glycine, 25 mM Tris-HCl, 10% (v/v) Methanol
ziptip solutions - elute	70% (v/v) aqueous methanol containing 0.1% (v/v) formic acid
ziptip solutions - wash	5% (v/v) aqueous methanol containing 0.1 % (v/v) formic acid
ziptip solutions - wet	Methanol

Appendix C

Putative cell surface proteins (Group A)

Number	Unique ID	Protein name	Number of peptides	Seq cov
1	IPI00117857	Alpha-1-antitrypsin 1-3	2	9.95
2	IPI00276577	3-oxoacid CoA transferase 2B	8	20.00
3	IPI00379315	43 kda protein. 390	1	2.82
4	IPI00473734	45 kda protein. 419	1	4.53
5	IPI00752936	65 kda protein	1	2.13
6	IPI00153924	A disintegrin and metallopeptidase domain 1b	2	5.83
7	IPI00128144	A disintegrin and metallopeptidase domain 26A	2	4.16
8	IPI00381611	A disintegrin and metallopeptidase domain 6B	2	3.97
9	IPI00135189	Acetoacetyl-coa synthetase	1	2.83
10	IPI00154054	Acetyl-Coenzyme A acetyltransferase 1	2	8.25
11	IPI00110827	Actin, alpha skeletal muscle	1	4.77
12	IPI00261808	Activating molecule in beclin1-regulated autophagy isoform 1	1	0.69

13	IPI00221610	Activating transcription factor 7 interacting protein	1	13.71
14	IPI00133006	Acyl carrier protein, mitochondrial precursor	1	7.05
15	IPI00127260	Acyl-coenzyme a thioesterase 10, mitochondrial precursor	1	2.96
16	IPI00128144	Adam 26a precursor	1	2.87
17	IPI00331280	Adamts-2 precursor	1	1.15
18	IPI00269076	Adenylate kinase isoenzyme 2, mitochondrial	1	6.90
19	IPI00121490	Afadin- and alpha-actinin-binding protein	1	2.28
20	IPI00321308	Alanyl-trna synthetase, cytoplasmic	1	3.10
21	IPI00131695	Albumin	10	22.04
22	IPI00224181	Aldo-keto reductase family 1, member B7	2	9.49
23	IPI00462072	Alpha enolase	3	11.75
24	IPI00123927	Alpha-1-antitrypsin 1-5 precursor	1	4.60
25	IPI00403650	Anionic trypsin-2 precursor	1	8.13
26	IPI00354665	Apoptosis-stimulating of p53 protein 1	1	1.47
27	IPI00131460	Aspartyl aminopeptidase	2	8.88
28	IPI00468481	Atp synthase subunit beta, mitochondrial precursor	1	2.46
29	IPI00463589	ATPase, Ca++ transporting, plasma membrane 4	3	4.10

30	IPI00378485	ATPase, Na+/K+ transporting, alpha 4 polypeptide	6	8.53
31	IPI00648637	Carbonic anhydrase 4	1	6.75
32	IPI00113347	Carnitine acetyltransferase	2	4.14
33	IPI00319699	Centriolin	1	2.50
34	IPI00320217	Chaperonin containing Tcp1, subunit 2 (beta)	3	9.72
35	IPI00116283	Chaperonin containing Tcp1, subunit 3	2	5.87
36	IPI00116277	Chaperonin containing Tcp1, subunit 4	3	7.79
37	IPI00331174	Chaperonin containing Tcp1, subunit 7 (eta)	3	8.82
38	IPI00222419	Cytochrome c, somatic	1	13.33
39	IPI00230034	D-dopachrome decarboxylase	1	12.71
40	IPI00310669	Dihydroxyacetone kinase	1	3.63
41	IPI00125454	Dnaj homolog subfamily a member 4	1	3.27
42	IPI00474450	Dystrophin	1	0.57
43	IPI00553260	E2f8 transcription factor	1	1.40
44	IPI00461441	Ef-hand domain-containing family member c2	1	1.60
45	IPI00118342	Endothelin-converting enzyme 2a-1. 785	1	1.66
46	IPI00122684	Enolase 2	2	9.84

47	IPI00396812	Exosome complex exonuclease rrp40	1	6.57
48	IPI00340702	Ferm and pdz domain-containing protein 1	1	0.97
49	IPI00356888	Fibronectin type iii domain containing 3a	1	2.50
50	IPI00133580	Fructose-bisphosphate aldolase	2	9.89
51	IPI00221402	Fructose-bisphosphate aldolase a	1	6.32
52	IPI00228633	Glucose phosphate isomerase 1	3	7.35
53	IPI00123975	Glycerol kinase-like protein 2	1	3.61
54	IPI00828258	Gm732 predicted gene 732	2	17.31
55	IPI00129907	Golgi resident protein gcp60	1	3.05
56	IPI00283611	Hexokinase 1 (Hk1)	13	17.35
57	IPI00114342	Hexokinase 2	2	2.51
58	IPI00126513	Homeobox protein nkx-3.2	1	4.50
59	IPI00135803	Hypothetical protein loc321008	1	1.06
60	IPI00458896	Hypothetical protein loc434223 isoform 1	1	0.62
61	IPI00138892	Hypothetical protein loc666586	1	12.50
62	IPI00553429	Hypothetical protein loc70354	1	1.29
63	IPI00124412	Iq motif containing gtpase activating protein 3	1	1.35

64	IPI00135621	Iroquois-class homeodomain protein irx-4	1	3.11
65	IPI00128209	Isoform 1 of adenylate kinase isoenzyme 1	1	6.70
66	IPI00123886	Isoform 1 of dna-dependent protein kinase catalytic subunit	1	0.48
67	IPI00221530	Isoform 1 of f-box/lrr-repeat protein 21	1	3.46
68	IPI00117042	Isoform 1 of glial fibrillary acidic protein	1	2.56
69	IPI00336281	Isoform 1 of golgin subfamily a member 3	1	1.24
70	IPI00331091	Isoform 1 of integrator complex subunit 7	1	2.48
71	IPI00136056	Isoform 1 of keratin, type i cytoskeletal 13	1	2.52
72	IPI00604878	Isoform 1 of membrane-associated phosphatidylinositol transfer protein 3	1	1.44
73	IPI00108982	Isoform 1 of mhc class ii transactivator	1	1.21
74	IPI00330773	Isoform 1 of pleckstrin homology-like domain family b member 2	1	1.36
75	IPI00308446	Isoform 1 of putative adenosylhomocysteinase 3	1	2.61
76	IPI00465761	Isoform 1 of rho guanine nucleotide exchange factor 17	1	1.07
77	IPI00165850	Isoform 1 of structural maintenance of chromosomes protein 6	1	1.73
78	IPI00123783	Isoform 1 of teneurin-3	1	0.77
79	IPI00353237	Isoform 1 of tensin-3	1	1.18
80	IPI00228376	Isoform 2 of axin-1	1	1.45

81	IPI00113869	Isoform 2 of basigin precursor	1	6.23
82	IPI00828269	Isoform 2 of calpain-15	1	1.12
83	IPI00227900	Isoform 2 of camp-dependent protein kinase, alpha-catalytic subunit	1	4.96
84	IPI00228295	Isoform 2 of contactin-4 precursor	1	2.84
85	IPI00120981	Isoform 2 of dna-directed rna polymerase i subunit rpa49	1	3.69
86	IPI00224682	Isoform 2 of elongator complex protein 3	1	2.83
87	IPI00177080	Isoform 2 of enhancer of polycomb homolog 1	1	2.23
88	IPI00269481	Isoform 2 of f-actin-capping protein subunit beta	1	5.88
89	IPI00620674	Isoform 2 of myeloid/lymphoid or mixed-lineage leukemia protein 3 homolog	1	0.58
90	IPI00117839	Isoform 2 of nuclear receptor coactivator 1	1	1.00
91	IPI00404394	Isoform 3 of leucine-rich repeat-containing protein 33 precursor	1	2.64
92	IPI00128034	Isoform 3 of neuropathy target esterase. 1329	1	0.98
93	IPI00135892	Isoform 33-b of myeloid cell surface antigen cd33 precursor	1	3.97
94	IPI00112774	Izumo sperm-egg fusion protein 1 precursor	1	3.78
95	IPI00463282	Keratin (fragment)	1	3.33
96	IPI00347110	Keratin 73	1	2.23
97	IPI00346834	Keratin 76	1	1.52

98	IPI00755181	Keratin complex 1	3	6.06
99	IPI00348328	Keratin kb40	1	1.31
100	IPI00227140	Keratin, type i cytoskeletal 14	1	2.69
101	IPI00122621	Keratin, type i cytoskeletal 25	1	2.02
102	IPI00468696	Keratin, type i cytoskeletal 42	1	2.65
103	IPI00625729	Keratin, type II cytoskeletal 1	3	5.49
104	IPI00139301	Keratin, type ii cytoskeletal 5	1	2.07
105	IPI00131366	Keratin, type ii cytoskeletal 6b	1	2.14
106	IPI00130218	Kinesin-like protein kif11	1	1.33
107	IPI00225945	Lactate dehydrogenase A-like 6B (Ldhal6b)	6	20.42
108	IPI00467457	Lactate dehydrogenase C	7	29.82
109	IPI00378496	Lon peptidase n-terminal domain and ring finger 2	1	3.09
110	IPI00129253	Lymphocyte antigen 75 precursor	1	0.99
111	IPI00319973	Membrane-associated progesterone receptor component 1	1	7.69
112	IPI00113449	Mono-adp-ribosyltransferase sirtuin-6	1	5.09
113	IPI00395146	Myosin regulatory light chain 2, atrial isoform	1	9.14
114	IPI00349292	Nebulin. 1191	1	1.43

115	IPI00230310	Neuron-specific calcium-binding protein hippocalcin	1	8.81
116	IPI00309741	Novel protein	1	1.70
117	IPI00346042	Novel protein	1	6.52
118	IPI00116310	Novel protein similar to dynein. 4463	1	0.27
119	IPI00380737	Nuclear envelope pore membrane protein pom 121	1	1.17
120	IPI00117828	Occludin	1	1.73
121	IPI00353314	Olfactory receptor 1204	1	9.71
122	IPI00126116	Origin recognition complex subunit 4	1	3.93
123	IPI00605237	Oxysterol-binding protein	1	1.66
124	IPI00330252	Peroxisome proliferator-activated receptor gamma coactivator-related protein 1	1	1.28
125	IPI00137730	Phosphadtidylethanolamine-binding protein 1	3	17.65
126	IPI00555060	Phosphoglycerate kinase 2	1	3.60
127	IPI00230706	Phosphoglycerate mutase 2	1	6.32
128	IPI00661343	Phospholipase b1	1	1.08
129	IPI00225140	Piccolo	1	0.26
130	IPI00127713	Plasma membrane calcium-transporting atpase 2	1	1.42
131	IPI00137314	Plexin a3	1	0.75

132	IPI00230680	Potassium-transporting atpase alpha chain 1	1	1.84
133	IPI00312174	Prostaglandin e synthase 2	1	3.91
134	IPI00130391	Protease, serine, 1 (trypsin 1)	2	17.89
135	IPI00133066	Proteasome (Prosome, macropain) 26S subunit, non-ATPase, 12	1	4.17
136	IPI00109122	Proteasome subunit alpha type-7-like	1	5.60
137	IPI00128945	Proteasome subunit beta type-2	1	8.46
138	IPI00230108	Protein disulfide-isomerase a3 precursor	1	2.97
139	IPI00719851	Protein fam137a	1	4.99
140	IPI00223965	Protein fam180a	1	7.51
141	IPI00278624	Protein mcm10 homolog	1	1.47
142	IPI00420814	Putative gag-pol protein	1	1.78
143	IPI00127495	Putative hexokinase hkdc1	1	0.98
144	IPI00134476	Putative uncharacterized protein	4	15.56
145	IPI00134121	Putative uncharacterized protein	1	13.53
146	IPI00468037	Putative uncharacterized protein	1	10.56
147	IPI00474366	Putative uncharacterized protein	1	9.45
148	IPI00652938	Putative uncharacterized protein	1	3.55

149	IPI00283511	Pyridoxal kinase	1	6.73
150	IPI00137227	Ras-related protein rab-2a	1	7.55
151	IPI00108150	Rho-associated protein kinase 2	1	1.01
152	IPI00330472	Riken cdna 0710005i19 gene	1	7.67
153	IPI00461823	Saps domain family member 2	1	2.06
154	IPI00108725	Serpin b7	1	5.79
155	IPI00126181	Similar to guanine nucleotide exchange factor lbc isoform 11	1	0.65
156	IPI00831151	Similar to igh protein	1	9.45
157	IPI00848725	Similar to proteophosphoglycan ppg1	1	14.55
158	IPI00416117	similar to trypsinogen 15	1	8.13
159	IPI00330516	Snurportin-1	1	5.03
160	IPI00122048	Sodium/potassium-transporting atpase subunit alpha-3	1	1.09
161	IPI00134191	Solute carrier family 2 (facilitated glucose transporter), member 3	4	10.55
162	IPI00753038	Sorbitol dehydrogenase	1	4.20
163	IPI00230741	Sorcs1b. 1168	1	1.20
164	IPI00114402	Sperm adhesion molecule 1	2	8.01
165	IPI00224055	Sperm motility kinase w	1	4.21

166	IPI00117915	Sperm-associated antigen 4-like protein	1	7.18
167	IPI00351686	Spindlin-2	1	7.00
168	IPI00109109	Superoxide dismutase [mn], mitochondrial precursor	1	6.31
169	IPI00319814	Suprabasal-specific protein suprabasin	1	2.00
170	IPI00403993	Synaptic nuclear envelope 1 isoform 3	1	0.20
171	IPI00624098	Syntaxin-binding protein 3	1	2.20
172	IPI00125667	Telomerase protein component 1	1	0.76
173	IPI00123838	Transcription regulator protein bach2	1	1.68
174	IPI00223092	Trifunctional enzyme subunit alpha, mitochondrial	1	2.23
175	IPI00754607	Trub pseudouridine (psi) synthase homolog 2	1	9.52
176	IPI00395100	Try10-like trypsinogen	1	4.07
177	IPI00719897	Trypsin 10	1	10.57
178	IPI00131674	Trypsinogen 7	1	4.05
179	IPI00110753	Tubulin alpha-1a chain	1	3.10
180	IPI00109061	Tubulin beta-2b chain	1	5.84
181	IPI00330464	Tudor and kh domain-containing protein. 562	1	3.74
182	IPI00742341	Voltage gated channel like 1	1	0.92

183	IPI00453845	Zinc finger and scan domain-containing protein 12	1	2.59
184	IPI00463470	Zinc finger protein 31	1	2.23
185	IPI00380688	Zinc finger protein 406	1	1.72
186	IPI00120160	Zona pellucida sperm-binding protein 3 receptor precursor	1	2.76

Appendix D

Tissue expression of putative cell surface proteins (Group B)

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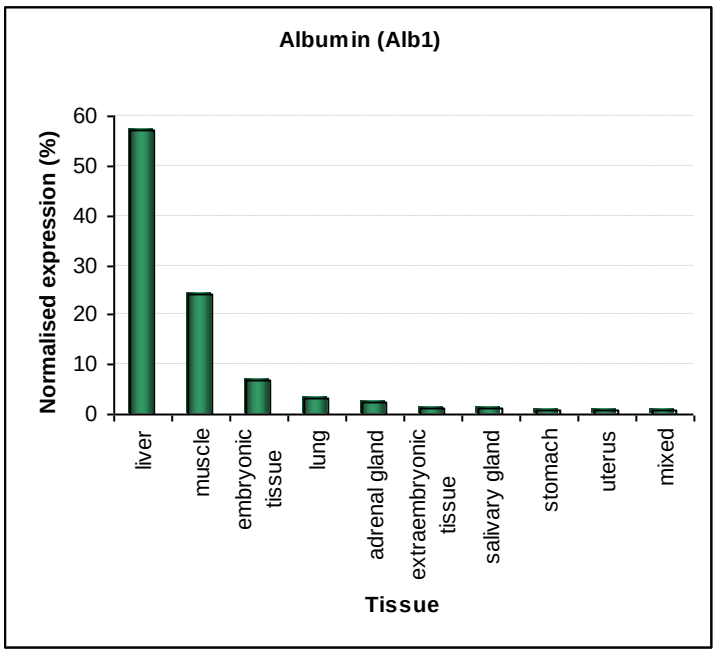
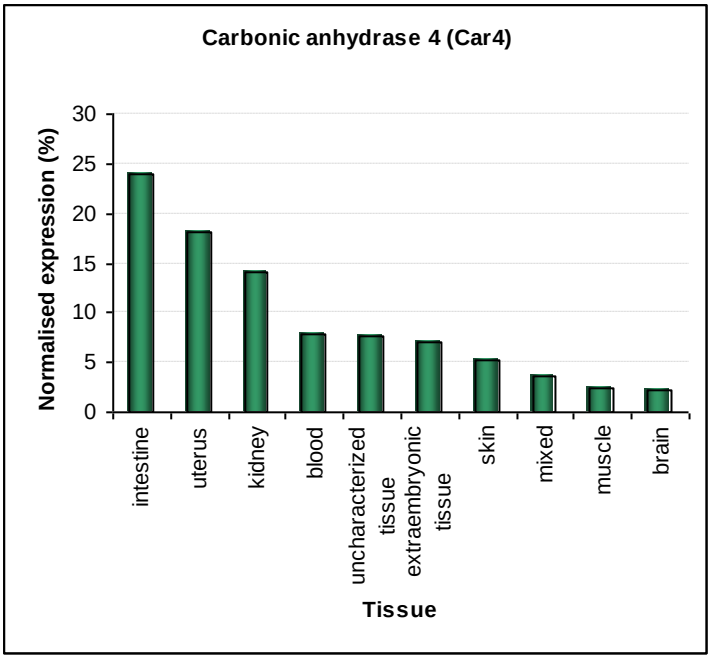
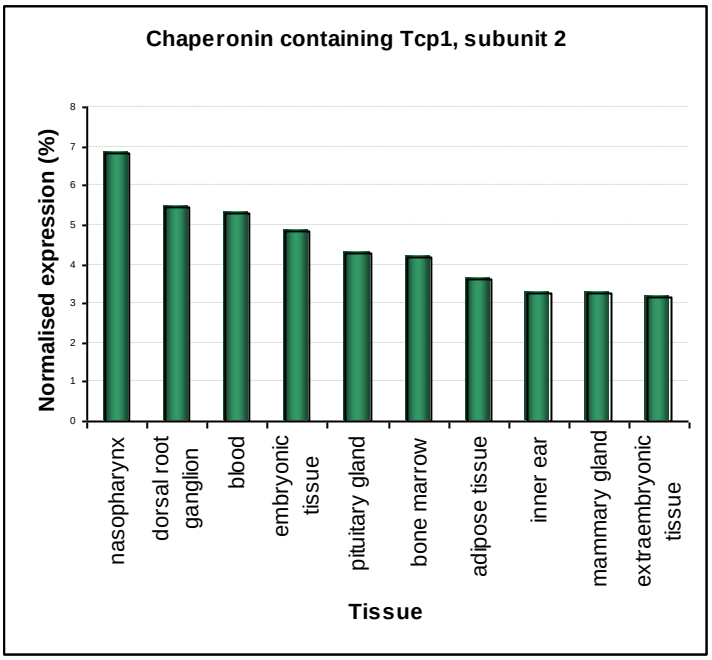
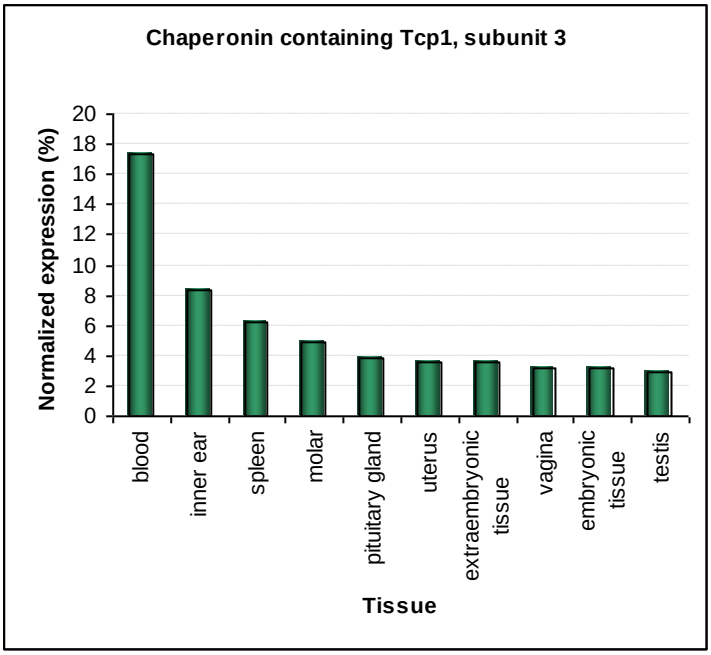
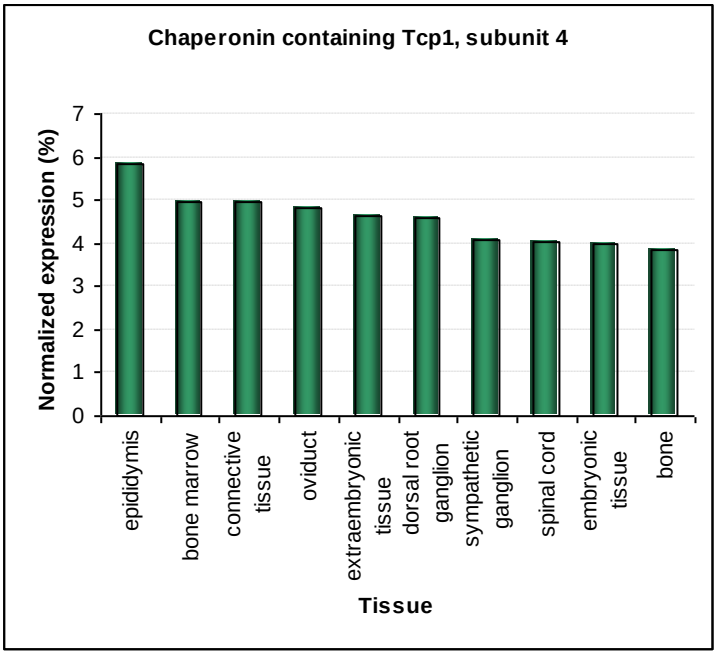
Error! Objects cannot be created from editing field codes.

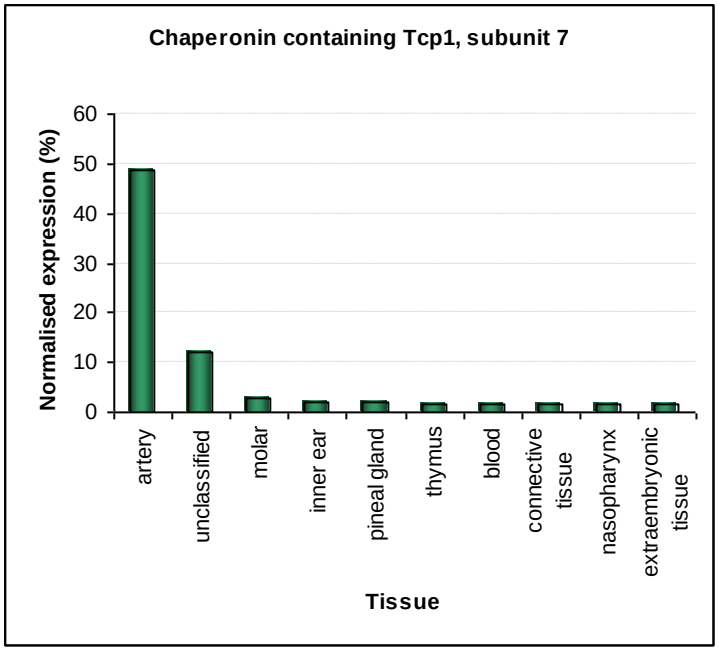


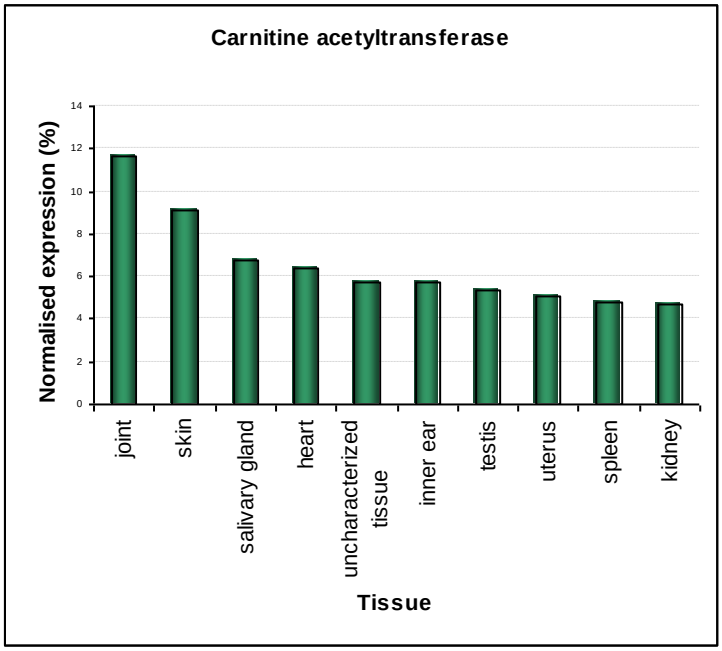

Carbonic anhydrase 4 (Car4)
Normalised expression (%)
30
25
20
15
10
5
0
intestine
uterus
kidney
blood
uncharacterized tissue
extraembryonic tissue
skin
mixed
muscle
brain
Tissue

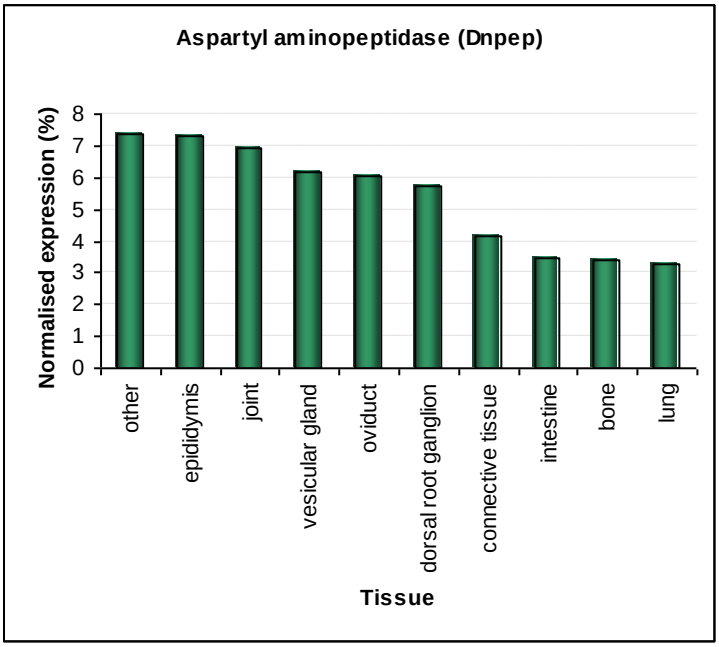

Chaperonin containing Tcp1, subunit 2
Normalised expression (%)
8
7
6
5
4
3
2
1
0
nasopharynx
dorsal root ganglion
blood
embryonic tissue
pituitary gland
bone marrow
adipose tissue
inner ear
mammary gland
extraembryonic tissue
Tissue

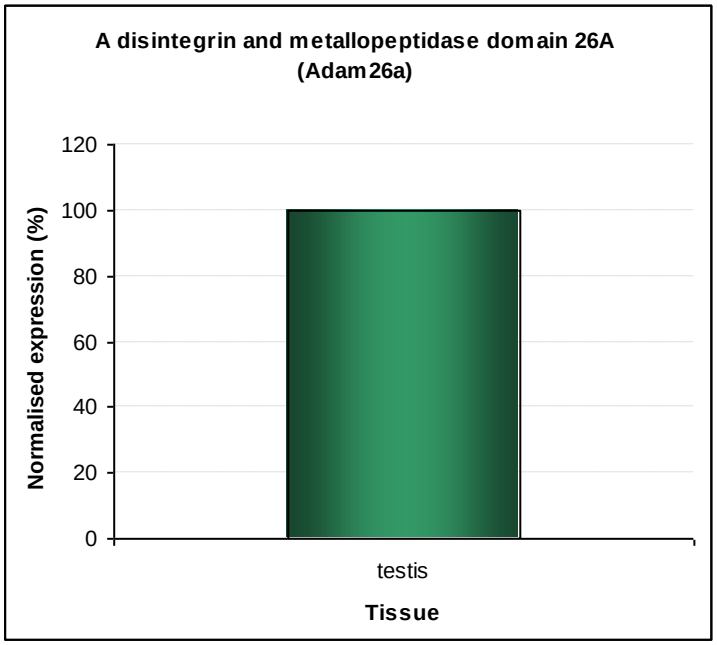

Chaperonin containing Tcp1, subunit 3
Normalized expression (%)
0
2
4
6
8
10
12
14
16
18
20
blood
inner ear
spleen
molar
pituitary gland
uterus
extraembryonic tissue
vagina
embryonic tissue
testis
Tissue

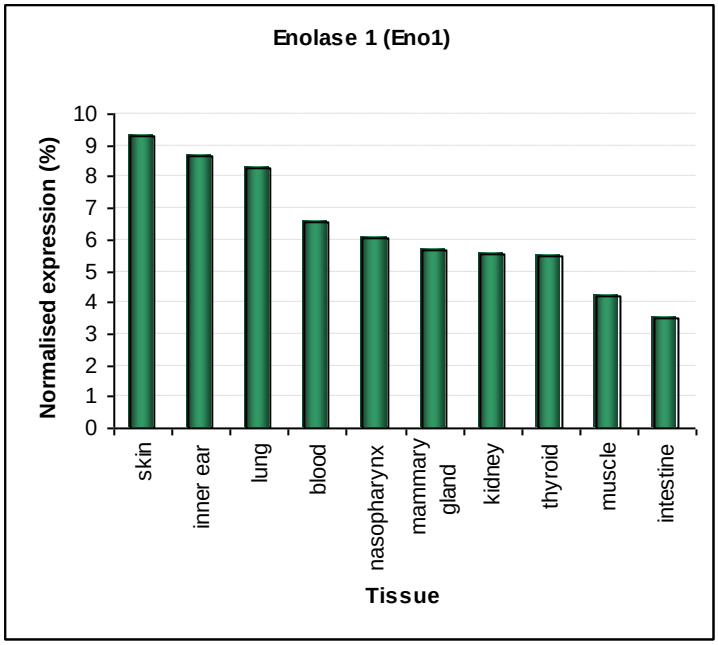

Chaperonin containing Tcp1, subunit 4
Normalized expression (%)
0
1
2
3
4
5
6
7
epididymis
bone marrow
connective tissue
oviduct
extraembryonic tissue
dorsal root ganglion
sympathetic ganglion
spinal cord
embryonic tissue
bone
Tissue

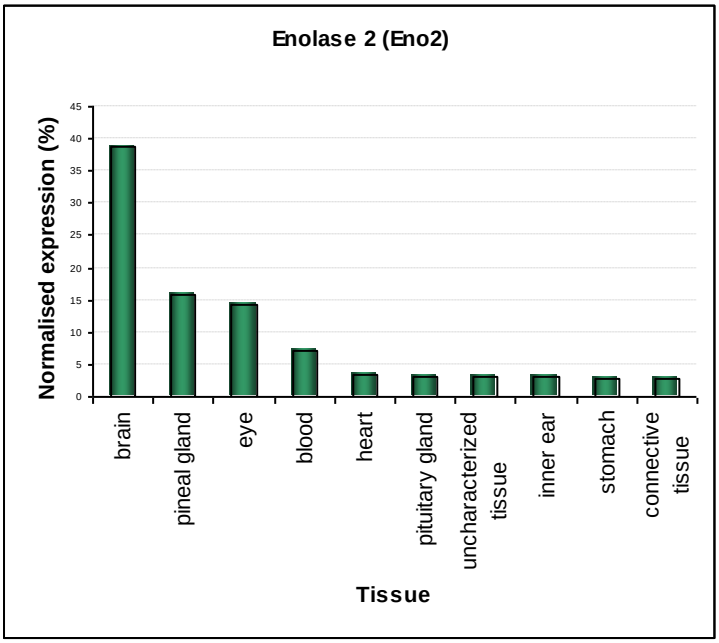

Chaperonin containing Tcp1, subunit 7
Normalised expression (%)
60
50
40
30
20
10
0
artery
unclassified
molar
inner ear
pineal gland
thymus
blood
connective tissue
nasopharynx
extraembryonic tissue
Tissue

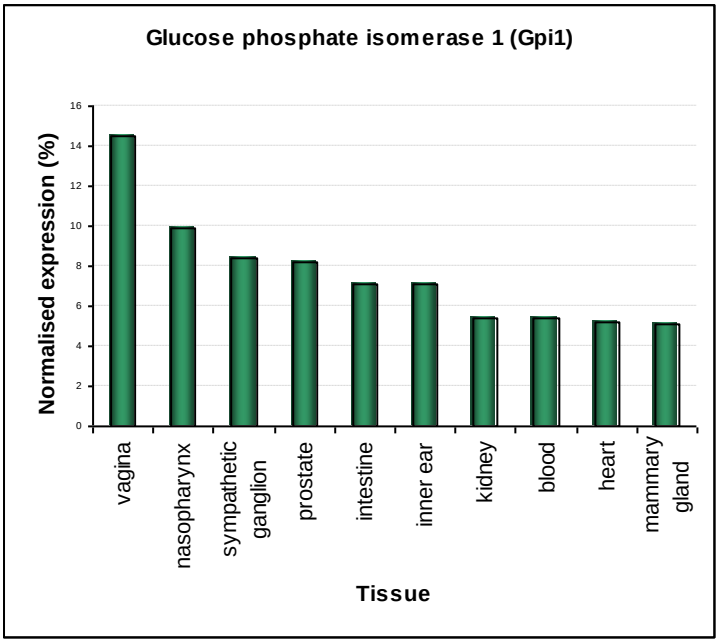

Carnitine acetyltransferase
Normalised expression (%)
14
12
10
8
6
4
2
0
joint
skin
salivary gland
heart
uncharacterized tissue
inner ear
testis
uterus
spleen
kidney
Tissue

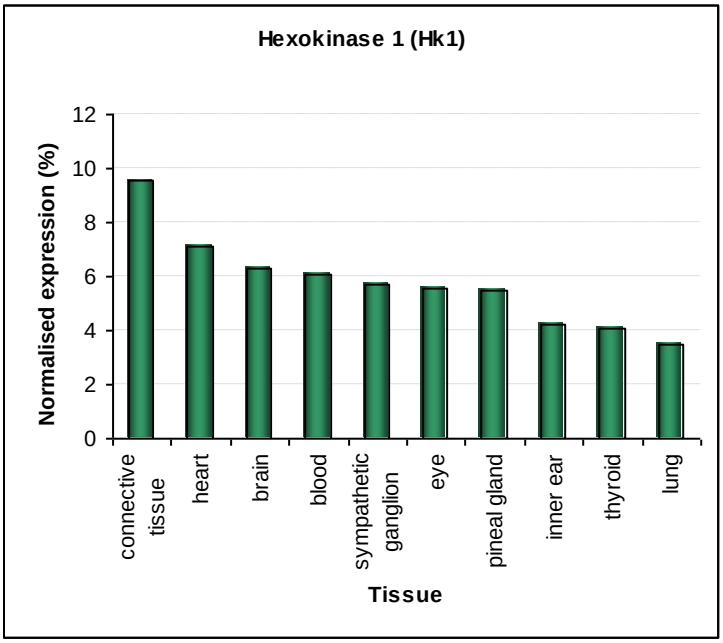

Aspartyl aminopeptidase (Dnpep)
Normalised expression (%)
0
1
2
3
4
5
6
7
8
other
epididymis
joint
vesicular gland
oviduct
dorsal root ganglion
connective tissue
intestine
bone
lung
Tissue

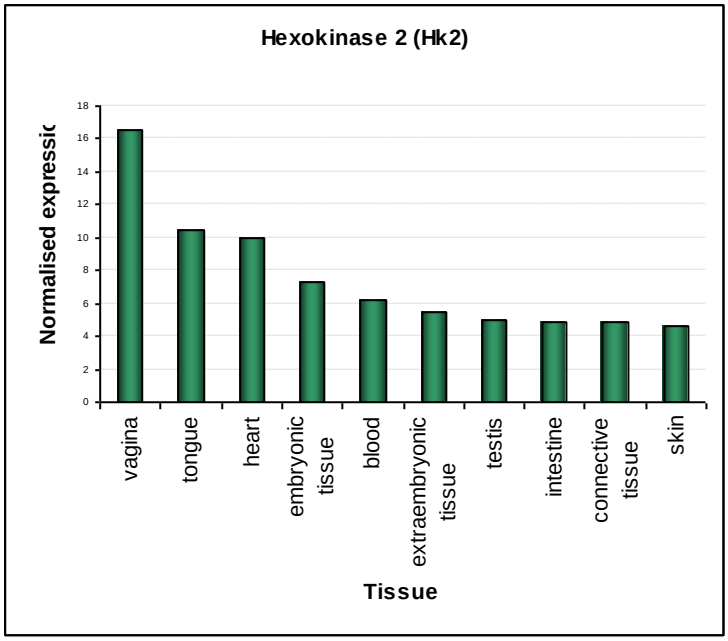

A disintegrin and metallopeptidase domain 26A (Adam26a)
Normalised expression (%)
0
20
40
60
80
100
120
testis
Tissue

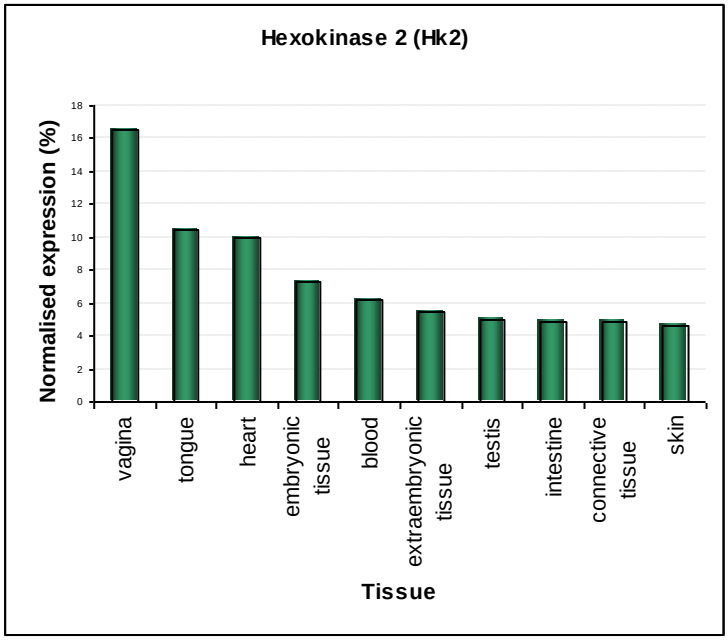

Enolase 1 (Eno1)
Normalised expression (%)
10
9
8
7
6
5
4
3
2
1
0
skin
inner ear
lung
blood
nasopharynx
mammary gland
kidney
thyroid
muscle
intestine
Tissue

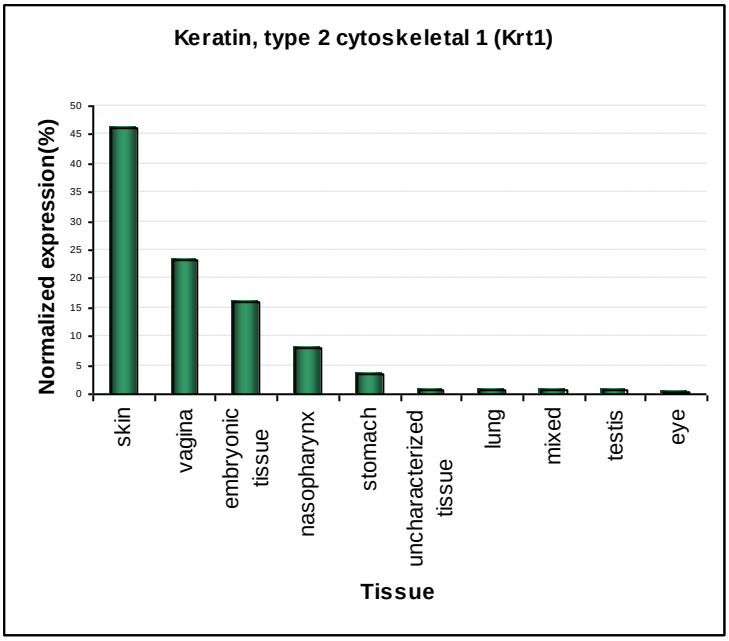

Enolase 2 (Eno2)
Normalised expression (%)
45
40
35
30
25
20
15
10
5
0
brain
pineal gland
eye
blood
heart
pituitary gland
uncharacterized tissue
inner ear
stomach
connective tissue
Tissue

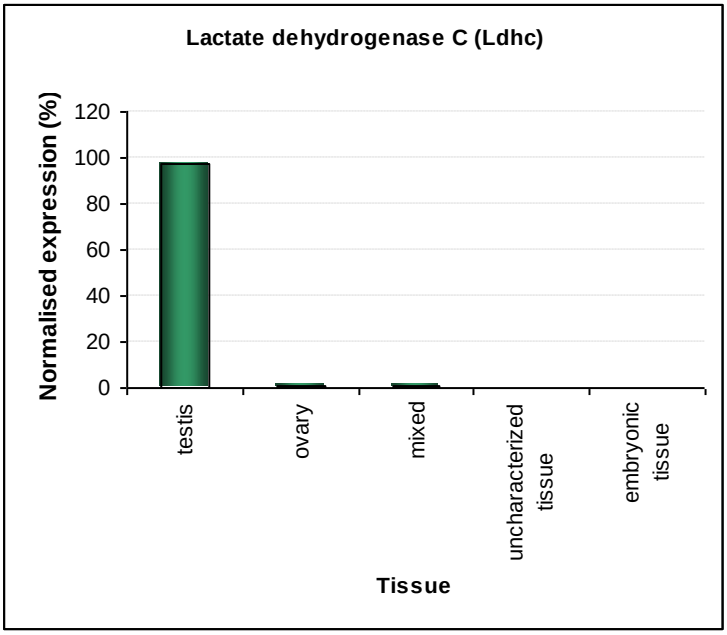

Glucose phosphate isomerase 1 (Gpi1)
Normalised expression (%)
0
2
4
6
8
10
12
14
16
vagina
nasopharynx
sympathetic ganglion
prostate
intestine
inner ear
kidney
blood
heart
mammary gland
Tissue


Hexokinase 1 (Hk1)
Normalised expression (%)
0
2
4
6
8
10
12
connective tissue
heart
brain
blood
sympathetic ganglion
eye
pineal gland
inner ear
thyroid
lung
Tissue

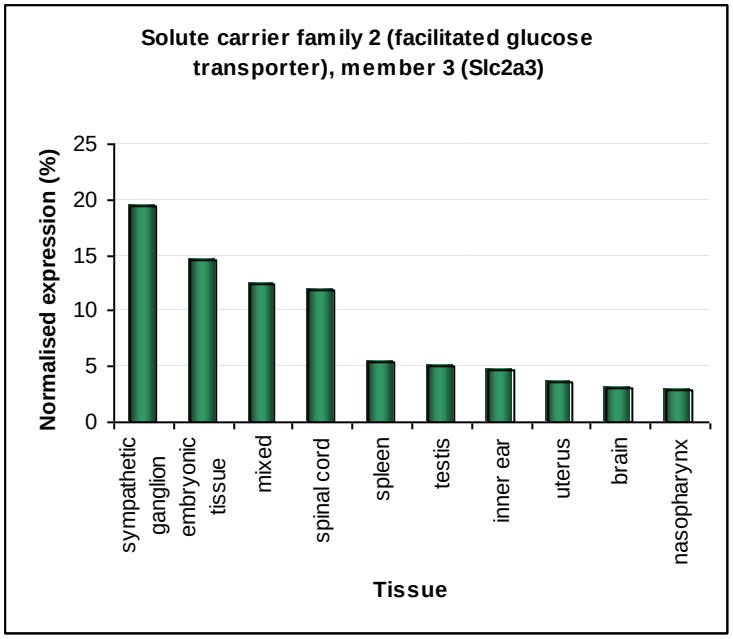

Hexokinase 2 (Hk2)
Normalised expressic
18
16
14
12
10
8
6
4
2
0
vagina
tongue
heart
embryonic tissue
blood
extraembryonic tissue
testis
intestine
connective tissue
skin
Tissue

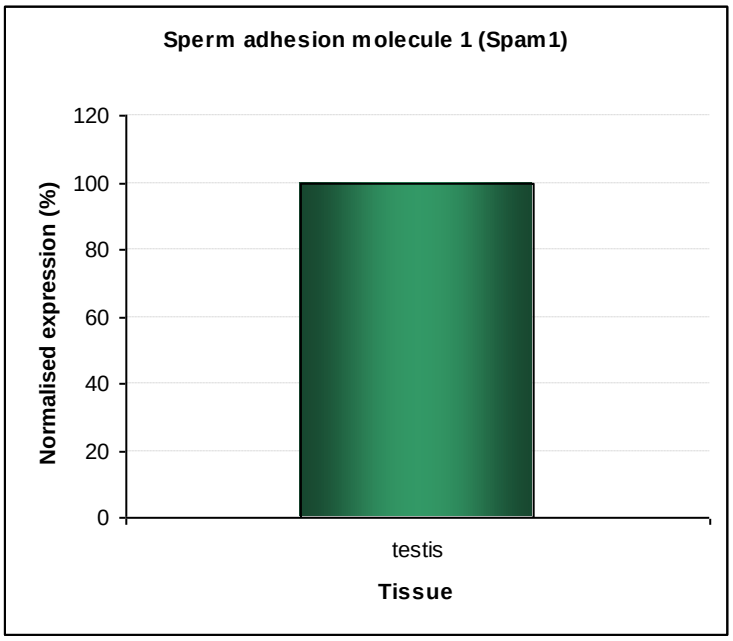

Hexokinase 2 (Hk2)
Normalised expression (%)
18
16
14
12
10
8
6
4
2
0
vagina
tongue
heart
embryonic tissue
blood
extraembryonic tissue
testis
intestine
connective tissue
skin
Tissue

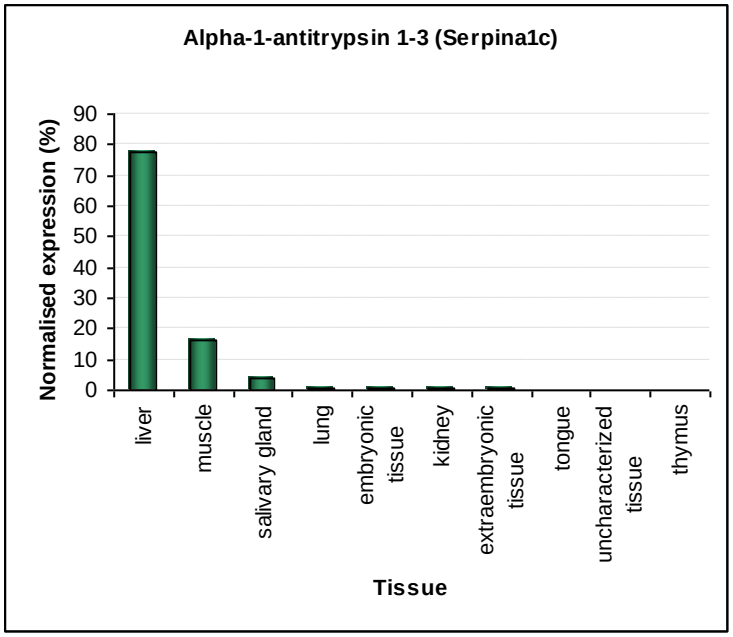

Keratin, type 2 cytoskeletal 1 (Krt1)
Normalized expression(%)
50
45
40
35
30
25
20
15
10
5
0
skin
vagina
embryonic tissue
nasopharynx
stomach
uncharacterized tissue
lung
mixed
testis
eye
Tissue

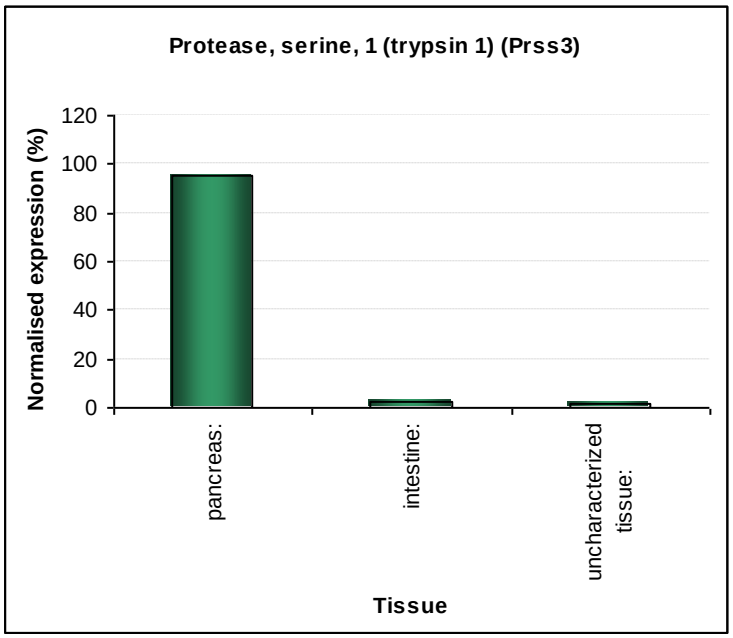

Lactate dehydrogenase C (Ldhc)
Normalised expression (%)
120
100
80
60
40
20
0
testis
ovary
mixed
uncharacterized tissue
embryonic tissue
Tissue

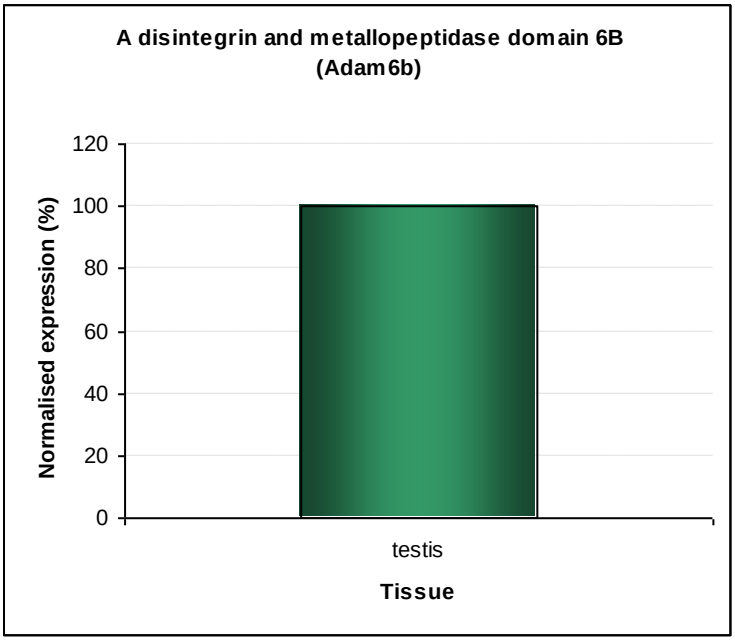
\

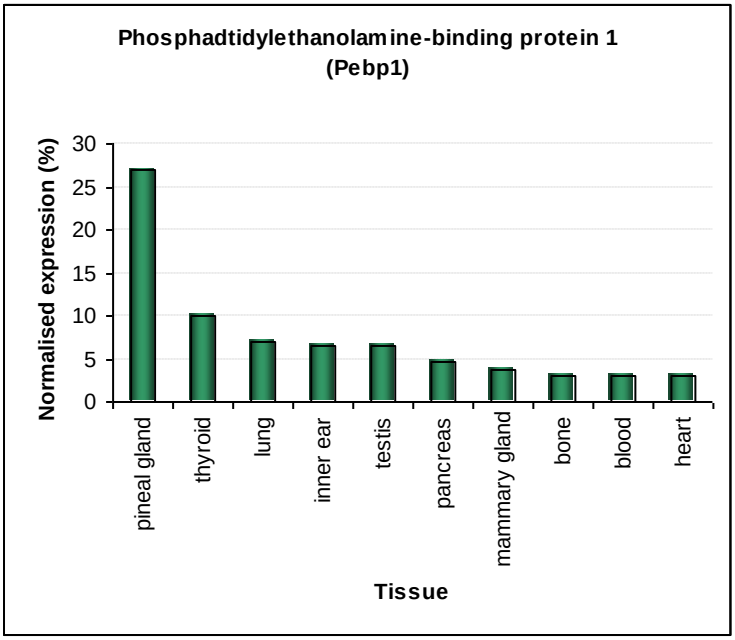

Solute carrier family 2 (facilitated glucose transporter), member 3 (Slc2a3)
Normalised expression (%)
25
20
15
10
5
0
sympathetic ganglion
embryonic tissue
mixed
spinal cord
spleen
testis
inner ear
uterus
brain
nasopharynx
Tissue

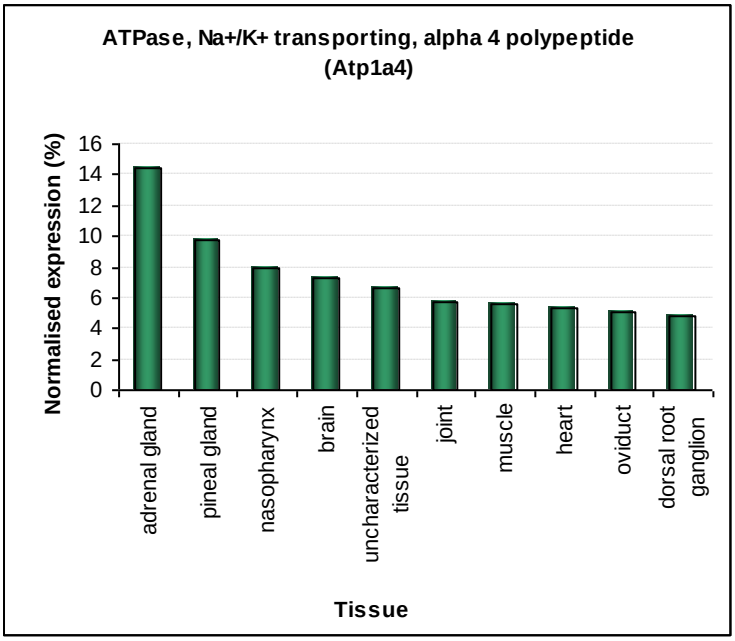

Sperm adhesion molecule 1 (Spam1)
Normalised expression (%)
120
100
80
60
40
20
0
testis
Tissue

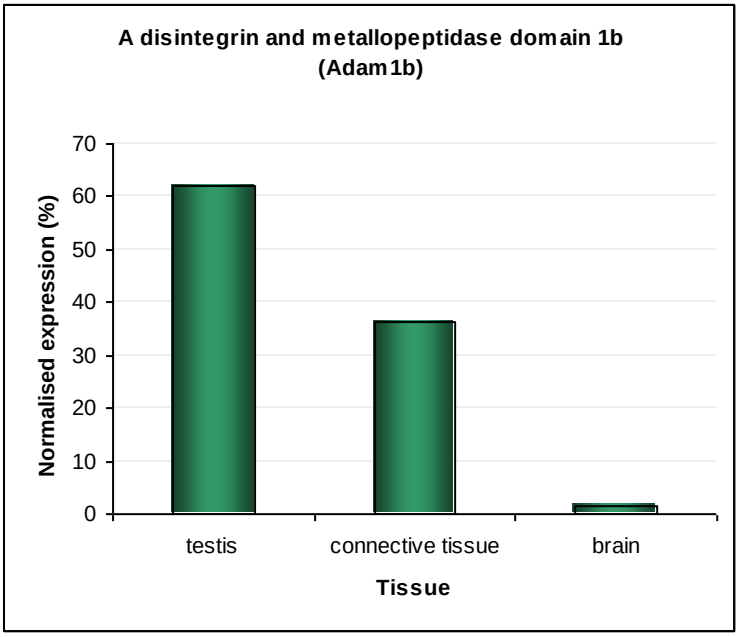

Alpha-1-antitrypsin 1-3 (Serpina1c)
Normalised expression (%)
90
80
70
60
50
40
30
20
10
0
liver
muscle
salivary gland
lung
embryonic tissue
kidney
extraembryonic tissue
tongue
uncharacterized tissue
thymus
Tissue

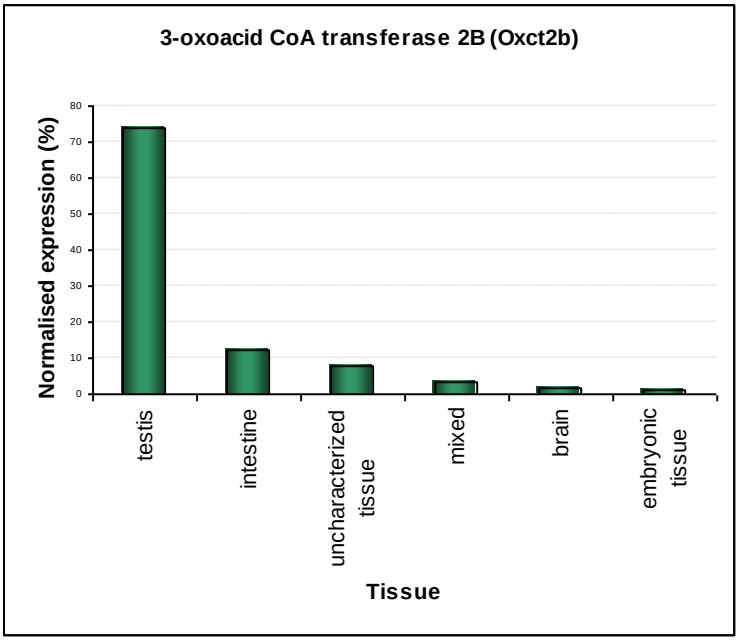

Protease, serine, 1 (trypsin 1) (Prss3)
Normalised expression (%)
120
100
80
60
40
20
0
pancreas:
intestine:
uncharacterized tissue:
Tissue

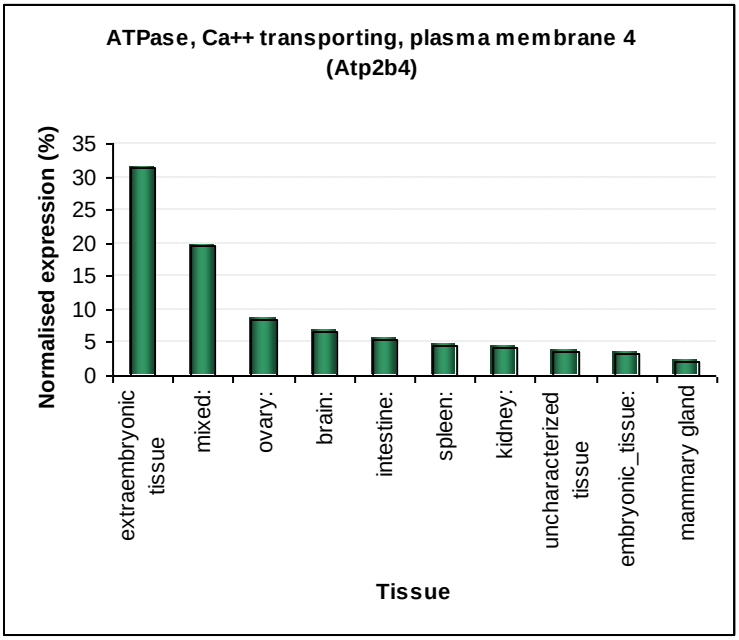

A disintegrin and metallopeptidase domain 6B (Adam6b)
Normalised expression (%)
120
100
80
60
40
20
0
testis
Tissue


Phosphadtidylethanolamine-binding protein 1 (Pebp1)
Normalised expression (%)
30
25
20
15
10
5
0
pineal gland
thyroid
lung
inner ear
testis
pancreas
mammary gland
bone
blood
heart
Tissue


ATPase, Na+/K+ transporting, alpha 4 polypeptide (Atp1a4)
Normalised expression (%)
16
14
12
10
8
6
4
2
0
adrenal gland
pineal gland
nasopharynx
brain
uncharacterized tissue
joint
muscle
heart
oviduct
dorsal root ganglion
Tissue


A disintegrin and metallopeptidase domain 1b (Adam1b)
Normalised expression (%)
70
60
50
40
30
20
10
0
testis
connective tissue
brain
Tissue


3-oxoacid CoA transferase 2B (Oxct2b)
Normalised expression (%)
80
70
60
50
40
30
20
10
0
testis
intestine
uncharacterized tissue
mixed
brain
embryonic tissue
Tissue


ATPase, Ca++ transporting, plasma membrane 4 (Atp2b4)
Normalised expression (%)
35
30
25
20
15
10
5
0
extraembryonic tissue
mixed:
ovary:
brain:
intestine:
spleen:
kidney:
uncharacterized tissue
embryonic_tissue:
mammary gland
Tissue

Appendix E

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Identification of the Molecular Chaperone, Heat Shock Protein 1 (Chaperonin 10), in the Reproductive Tract and in Capacitating Spermatozoa in the Male Mouse[1]

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ABSTRACT

Mammalian spermatozoa must undergo epididymal maturation in the male reproductive tract and capacitation in the female tract before acquiring the ability to fertilize an oocyte. Previous studies from our laboratory have demonstrated a causal relationship between capacitation-associated surface phosphotyrosine expres- sion and the ability of mouse spermatozoa to recognize the oocyte and engage in sperm-zona pellucida interaction. Our previous analyses of the surface phosphoproteome of capacitated murine spermatozoa identified two molecular chaperones, heat shock protein (HSP) D1 and HSP90B1, with well-characterized roles in protein folding and the assemblage of multimeric protein complexes. The expression of these chaperones was restricted to the rostral aspect of the sperm head, in an ideal position to mediate sperm-zona pellucida interaction. Herein, we report the characterization of an additional chaperone in this location, HSPE1 (chaperonin 10; HSP10). This chaperone was identified using a coimmunoprecipitation strategy employing HSPD1 as bait. The putative interaction between HSPE1 and HSPD1 was supported by reciprocal immunoprecipitation and colocalization studies, which demonstrated the coordinated appearance of both proteins on the surface of the sperm head during capacitation. However, the surface exposure of the protein was lost upon induction of acrosomal exocytosis, as would be expected of a protein potentially involved in sperm-zona pellucida interaction. Collectively, these data invite speculation that a number of molecular chaperones are involved in modification of the sperm surface during capacitation to render these cells functionally competent to engage the process of fertilization.

gamete biology, sperm, sperm capacitation, sperm maturation

INTRODUCTION

The development and maturation of spermatozoa is a complex, multifaceted process. Initially, spermatogenesis within the testes produces spermatozoa that, although structurally complete, are functionally deficient and incapable of progressive motility, zona pellucida recognition, or acrosomal exocytosis. Spermatozoa subsequently acquire these functional attributes after leaving the testes during their transit through the epididymal lumen. It has been well established that the epididymal maturation of spermatozoa is accompanied by significant changes in the composition and localization of sperm membrane proteins (reviewed in [1]). Even so, the exact mechanism by which spermatozoa gain their functional potential during epididymal maturation is yet to be elucidated.

After ejaculation, spermatozoa transit the female reproduc- tive tract to the site of fertilization in the fallopian tubes. Prior to engaging in the cascade of events associated with fertilization, including successive penetration of the cumulus oophorus and zona pellucida and subsequent fusion with the oocyte (reviewed in [2]), spermatozoa must undergo a process of postejaculatory maturation, termed capacitation [3, 4]. One of the most important properties acquired by spermatozoa during capacitation is the ability to recognize the zona pellucida. This exquisitely specific cell-cell recognition event signals the initiation of fertilization, and yet its molecular basis is still poorly understood. From the oocyte's perspective, ZP3 is acknowledged as the primary zona pellucida glycoprotein responsible for mediating mouse sperm-zona interaction [5-7]. In terms of the corresponding receptor on the surface of capacitated spermatozoa, a number of candidate proteins have been identified in previous studies including: SPAM1 (PH-20), ZP3R (SP56), B4GALT1 (b-1,4-galactosyltransferase), zonad- hesin, zona receptor kinase, arylsulfatase A, and MAN2B2 (a- D-mannosidase) (reviewed in [8]). This range of putative ZP3 receptors has lead to the proposal that zona recognition is coordinated by the sequential action of a variety of sperm proteins, each performing a specific role [9, 10].

More recently, tyrosine phosphorylation of proteins in the rostral region of the sperm head has been shown to be important for zona recognition [11]. Two of the major tyrosine-phosphorylated proteins were identified as the molecular chaperones, heat shock protein (HSP) 1 (HSPD1; formerly HSP60) and HSP90, beta (Grp94), member 1 (HSP90B1; formerly endoplasmin), both of which appear to be presented to the sperm surface during capacitation [11]. This has lead to the proposal that these chaperones may mediate the assembly of a protein receptor complex for the recognition of the zona pellucida [11]. Interestingly, this is not the first instance of chaperones acting in concert with other proteins on the surface of cells. For instance, HSP90a (HSP90AA1) has been shown to interact with matrix metalloproteinase 2 on the surface of fibrosarcoma cells and play an important role in promoting cancer invasiveness [12]. Furthermore, both HSP90 and HSP70

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form an integral part of the "activation complex" assembled in monocytic cell lines in response to bacterial lipopolysaccharide challenge [13]. Such findings highlight the emerging roles of chaperones as potential mediators of cell surface protein complex presentation and/or assembly.

To further our understanding of the role of molecular chaperones in the ability of mouse sperm to bind to the outer vestments of the oocyte, we have previously reported the patterns of HSPD1 and HSP90B1 expression in these cells throughout spermatogenesis and during their posttesticular development [11, 14]. As a continuation of these studies, we report here the characterization of an additional chaperone protein, HSPE1 (chaperonin 10; formerly known as HSP10), identified in capacitated mouse spermatozoa using an immunoprecipitation strategy with HSPD1 as the bait. Although eukaryotic HSPE1 has primarily been described as a mitochondrial chaperone [15-18], more recent reports suggest that it may also function in signal transduction, cell cycle regulation, nucleocytoplasmic transport, and metabolism [19]. This study extends the potential functions of HSPE1 by describing, for the first time, the progressive expression of this protein on the surface of murine spermatozoa during capacitation.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of molecular biology or research grade. Rabbit polyclonal anti-HSPE1 antibody (anti-Cpn10, ab13528) was obtained from Abcam (Cambridge, MA). Anti-rabbit immunoglobulin (Ig) G-HRP was from Upstate Biotechnology (Lake Placid, NY). Goat polyclonal anti-HSPD1 antibody (anti-HSP60 N-20), anti-goat IgG-fluorescein isothiocyanate (FITC), anti-rabbit IgG-FITC, and anti-goat IgG-tetramethylrhodamine isothiocyanate (TRITC) conjugates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-ZP3R (clone 5F12) and anti-OxPhos complex I were obtained from Biodesign International (Saco, ME) and Molecular Probes (Eugene, OR), respectively. Recombinant human HSPE1 was purchased from Abnova (Taipei City, Taiwan). Hepes, penicillin, and streptomycin were obtained from Gibco (Paisley, UK). BSA was obtained from Research Organics (Cleveland, OH). Minicomplete protease inhibitor tablets were from Roche (Mannheim, Germany). Nitrocellulose and percoll were from Amersham (Buckinghamshire, UK). Mowiol 4-88 was from Calbiochem (La Jolla, CA), paraformaldehyde was supplied by ProSciTech (Thuringowa, Australia), and protein G-coated Dynabeads were from Dynal (Oslo, Norway).

Animals

All experimental procedures were carried out with the approval of the University of Newcastle's Animal Care and Ethics Committee (ACEC) and the Monash Medical Centre animal ethics committee. Inbred Swiss mice were obtained from a breeding colony held at the institute's Central Animal House and maintained according to the recommendations prescribed by the ACEC. Mice were housed under a controlled lighting regime (16L:8D) at 21-228C and supplied with food and water ad libitum. Prior to dissection, animals were killed via CO_2 inhalation or cervical dislocation.

Collection and Preparation of Spermatozoa

Immediately after adult male mice (.8 wk old) were killed, their epididymides and testes were removed and carefully dissected free of fat and overlying connective tissue. The caudal region was isolated, blotted free of blood, and immersed under prewarmed, water-saturated mineral oil. Caudal spermatozoa were collected by back-flushing with water-saturated paraffin oil, after which the perfusate was deposited into a droplet of modified Biggers, Whitten, and Whittingham media (BWW; [20]) composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM $CaCl_2$Á2H_2O, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$Á7H_2O, 25 mM $NaHCO_3$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 lg/ml streptomycin, 20 mM Hepes buffer, and 3 mg/ml BSA, then allowed to disperse into the medium for 15 min. Where indicated, negative control (uncapacitated) incubations were conducted using medium prepared without $NaHCO_3$, while positive control (capacitated) incubations were conducted in media supplemented with 1 mM pentoxifylline and 1 mM dibutyryl cyclic adenosine monophosphate. These treatments have been demonstrated to both suppress and promote sperm capacitation, respectively [11]. An osmolarity of 300 mOsm/kg was maintained.

Following collection, sperm concentration was determined and the cells diluted as required. Sperm were then assessed for motility and the uncapacitated samples used immediately. Alternatively, populations of capacitated sperma- tozoa were prepared by incubation for 45 min at 378C under an atmosphere of 5% CO_2/95% air. At regular intervals throughout the incubation, sperm suspensions were gently mixed to prevent settling of the cells and, at the end of the incubation, sperm vitality and motility were again assessed. Neither parameter was affected by any of the treatments reported in this study.

To prepare caput and corpus spermatozoa, the appropriate region of the epididymis was dissected out and placed in a 500-ll droplet of BWW medium. Multiple incisions were then made in the tissue with a razor blade and spermatozoa gently washed into the medium with mild agitation. The resultant cell suspension was then layered over a discontinuous 25/45/65/80% percoll gradient and centrifuged (1300 3 g for 15 min). The 65/80 interface, consisting of .95% pure caput spermatozoa, was washed by gentle centrifugation (400 3 g for 2 min) to remove excess percoll and then resuspended in fresh BWW medium and counted as described above. Similarly, testicular spermatozoa were prepared by decapsulating the isolated testes, making multiple incisions in the tissue with a razor blade, and allowing the cells to gently disperse into the medium with mild agitation. Although the purity of the spermatozoa isolated by this technique was not suitable for immunoblotting, it was appropriate for immunofluorescent labeling of HSPE1.

Coimmunoprecipitation Strategy

Coimmunoprecipitation (co-IP) was used to identify HSPD1-associated proteins in sperm lysates using the ProFound Mammalian Co-Immunoprecipitation kit (Pierce, Rockford, IL). A 50-ll aliquot of the supplied antibody- coupling gel slurry (AminoLink Plus gel) was washed three times with PBS followed by conjugation with 50 lg of primary antibody. The primary antibody was covalently linked to the coupling gel by the addition of sodium cyanoborohydride. Following a 4-h incubation at room temperature with constant agitation, unreacted sites on the coupling gel were quenched with 1 M Tris-HCl, pH 7.4. The antibody-conjugated gel was then washed twice with 1 M NaCl, followed by two washes with PBS, pH 7.4. The antibody-coupled gel was stored at 48C in PBS containing 0.02% w/v sodium azide.

To generate lystates for co-IP, spermatozoa were capacitated as described above and cells were washed three times with protein-free media to remove any loosely associated proteins. Sperm viability and motility were assessed to ensure that neither parameter was compromised, and the cells were then resuspended in co-IP lysis buffer (M-PER, mammalian protein extraction reagent, Pierce) supplemented with a Complete Protease Inhibitor Cocktail tablet (Roche Applied Science, Indianapolis, IN) and lysed for 1 h at 48C with constant agitation. Insoluble material was removed by centrifugation at 20 000 3 g for 10 min at 48C.

For coexperiments, lysates were precleared against unconjugated gel slurry for 1 h prior to being diluted 1:1 with PBS and added to the prepared antibody- coupled gel. After incubation overnight at 48C with constant agitation, unbound proteins were removed by centrifugation at 4000 3 g for 1 min. The co-IP gel was washed five times with PBS before eluting the captured co-IP complexes in ImmunoPure elution buffer at room temperature for 5 min. Similarly, control incubations were included where nonconjugated gel slurry was incubated with the sperm lysate. Eluates were methanol precipitated and stored at À208C prior to analysis by SDS-PAGE or until being digested with trypsin in preparation for sequencing.

Proteomic Analysis of Coprecipitated Proteins

Proteins coimmunoprecipitated with HSPD1 were sequenced using an liquid chromatography tandem mass spectrometry (LC/MS/MS) interface at the Australian Proteome Analysis Facility. Briefly, precipitated samples were directly aliquoted into Vivaspin 500 centrifugal filter units (5000 MWCO; Viva Science Ltd, Gloucestershire, UK) and centrifuged at 12 000 3 g for 15 min. The pellets were resuspended in 50 mM ammonium bicarbonate before being reduced with dithiothreitol (DTT) and alkylated with iodoacetamide. The samples were then digested with trypsin overnight at 378C, dried, and redissolved in reverse-phase nano LC/MS/MS sample loading solution (0.1% formic acid, 2% acetonitrile, 97.9% water). The sample was injected onto a Michrome Peptide Captrap precolumn for concentration and desalting with 0.1% formic acid at 10 ll/min. The precolumn was then switched into line with the analytical column containing C18 RP silica (150 lm 3 100 mm, Protocol C18 3 micro; Scientific Glass Engineering, Austin, TX). Peptides were eluted

from the column using a solvent gradient from H_2O:CH_3CN (90:10, þ 0.1% formic acid) to H_2O:CH_3CN (60:40, þ 0.1% formic acid) at 600 nl/min over a 120-min period. The LC eluent was subjected to positive ion nanoflow electrospray analysis on an Applied Biosystems QSTAR mass spectrometer (ABI, Foster City, CA) in an information-dependant acquisition mode (IDA). In IDA mode, a time-of-flight mass spectrometry (TOFMS) survey scan was acquired (m/z 370-2000, 1.0 s), with the four largest multiply charged ions (counts .50) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 s (m/z 100-1600).

The LC/MS/MS data were searched through the MASCOT search engine using the National Center for Biotechnology Information (Bethesda, MD) nonredundant protein database. The following search parameters were used in all Mascot searches: Mus musculus taxonomy, maximum of one missed trypsin cleavage, cysteine carbamidomethylation, methionine oxidation, and a maximum 0.2-Da error tolerance in both the MS and MS/MS data. High- confidence positive identifications of HSPE1 was based on three matching peptides and the fact that significant peaks in the MS spectra were those used for the analysis.

SDS-PAGE and Western Blotting

Proteins were extracted in a modified SDS-PAGE sample buffer (2% w/v SDS, 10% w/v sucrose in 0.1875 M Tris, pH 6.8) with protease inhibitor tablets by incubation at 1008C for 5 min. Insoluble matter was removed by centrifugation at 20 000 3 g for 10 min and protein estimations were performed using the DC Protein Assay kit (Bio-Rad, Hercules, CA). Proteins were boiled in SDS-PAGE sample buffer (2% v/v mercaptoethanol, 2% w/v SDS, and 10% w/v sucrose in 0.1875 M Tris, pH 6.8, with bromophenol blue) and resolved by SDS-PAGE on polyacrylamide gels [21] followed by transfer onto nitrocel- lulose membranes [22]. Membranes were blocked with 3% w/v BSA in Tris- buffered saline (TBS; pH 7.4) for 1 h before being probed with 1:1000 dilutions of primary antibody in TBS containing 1% w/v BSA and 0.1% v/v polyoxyethylenesorbitan monolaurate (Tween-20; TBS-T) for 2 h at room temperature. Blots were washed three times in TBS-T followed by incubation with 1:1000 horseradish peroxidase-conjugated secondary antibody in 1% w/v BSA/TBS-T for 1 h. Following three washes in TBS-T, proteins were detected using an enhanced chemiluminescence kit (Amersham). Western blots were stripped in 100 mM mercaptoethanol, 2% w/v SDS, and 62.5 mM Tris (pH 6.7) at 608C for 1 h, followed by several washes in TBS-T before reprobing.

Tissue Immunofluorescence

Tissue was collected from mouse testes and epididymides, fixed in formalin, embedded in paraffin, and cut into 5-lm sections. Following dewaxing and rehydration, antigen retrieval was performed by subjecting the slides to microwaves (500 W) for 20 min in citrate buffer (10 mM trisodium citrate, 4.4 mM HCl, pH 6.0). All subsequent incubations were performed at 378C in a humid chamber, and all antibody dilutions and washes were conducted in PBS. Sections were blocked at 378C for 1 h in 10% v/v whole goat serum supplemented with 3% w/v BSA in PBS. Slides were rinsed and incubated at 48C overnight in 1:50 primary antibody. Following three washes, 1:100 dilutions of the corresponding FITC-conjugated secondary antibody were applied. Sections were washed three times and counterstained with 2 mg/ml propidium iodide, a nuclear dye included to aid visualization of the tissue structure. No nuclear stain was used for colocalization of HSPE1 with OxPhos complex I. Slides were mounted in antifade medium (10% w/v Mowiol 4-88 with 30% v/v glycerol in 0.2 M Tris [pH 8.5] with 2.5% w/v 1,4-diazobicyclo- [2.2.2]-octane) and viewed using an LSM510 laser scanning confocal microscope equipped with argon and helium/neon lasers (Carl Zeiss Pty, Sydney, Australia). Excitation wavelengths of 488 and 545 nm and emission spectra of 500-530 and .560 nm were used for detection of FITC conjugates and propidium iodide, respectively.

Immunolocalization of Chaperones on Fixed Spermatozoa

Following incubation, spermatozoa were fixed in 4% w/v paraformaldehyde, washed three times with PBS, plated onto glass slides coated with 0.1% v/v poly-L-lysine, and air-dried. All subsequent incubations were performed at 378C in a humid chamber, and all dilutions and washes were performed in PBS. Spermatozoa were permeabilized with 0.2% v/v Triton X-100 for 15 min, rinsed, and blocked in 10% v/v serum in 3% w/v BSA at 378C for 1 h. Slides were rinsed and incubated at 48C overnight in a 1:50 dilution of primary antibody. After three washes, cells were incubated with 1:100 FITC-conjugated secondary antibody at 378C for 1 h, washed again, and mounted in antifade medium. Images were captured using a confocal microscope, as described above.

Immunodetection of Chaperones on Live Sperm Using a Fluorescence-Activated Cell Sorter

Following preparation, spermatozoa were diluted to 1 3 10^6 cells/ml and incubated in either noncapacitating or capacitating medium. The sperm suspension was then incubated with primary antibody at a 1:100 dilution for 10 min at 378C. The cells were subsequently washed twice with BWW and incubated with FITC-conjugated secondary antibody at 1:200 for a further 10 min at 378C. Following two additional washes with BWW, the cells were incubated with propidium iodide (20 mg/ml) and analyzed using a fluorescence-activated cell sorting (FACS) Calibur FACS (Becton Dickinson, Franklin Lakes, NJ) with an FL4 530/30-nm band-pass filter, allowing the collection of fluorescence data in logarithmic mode and light-scatter data in linear mode. Ten thousand cells were counted in each sample at a rate of 50- 500 events per second. Data were analyzed using the CellQuest software (BD Biosciences, San Jose, CA).

Acrosome Reaction

Caudal epididymal spermatozoa were capacitated as described above, followed by 15 min of incubation in 1.25 lM calcium ionophore A23187 as described previously [14]. Vehicle (dimethyl sulfoxide) controls were included. Samples were then diluted 1:10 in prewarmed hypo-osmotic swelling medium (25 mM sodium citrate, 75 mM fructose) and incubated at 378C for 1 h. Spermatozoa were fixed and stained for HSPE1 using a TRITC-conjugated secondary antibody, as described above. The cells were then colabeled with FITC-conjugated Arachis hypogaea lectin (0.5 mg/ml in PBS) at room temperature for 15 min, washed, and mounted in antifade medium, as described above. At least 200 cells were scored on the basis of their viability, acrosomal status, and chaperone staining pattern.

Sperm-Zona Pellucida Binding Assay

Mouse oocytes were recovered from the oviduct of superovulated females as previously described [11]. Capacitated spermatozoa were incubated (30 min at 378C) with either rabbit sera (control) or anti-HSPE1 antibodies (10 lg or 100 lg/ml) in BWW before being washed to remove unbound antibody. Washed spermatozoa (5×10^4) were then coincubated with oocytes in a droplet of BWW under oil in 5% CO_2 for 30 min at 378C. A separate treatment incorporating oocytes that had been preincubated with recombinant HSPE1 (5 lg/12 oocytes) was included in each experiment. Following incubation with spermatozoa, oocytes were washed by gentle pipetting through a fine-bore pipette in three changes of BWW to remove any loosely bound sperm. The number of sperm remaining bound to the zona pellucida was then recorded.

Statistics

Experiments were replicated with material collected from at least three different animals and the graphical data presented represent means 6 SEM, the SEMs being calculated from the variance between samples. Statistical significance was determined using an ANOVA. The differences between group means were assessed using Fisher protected least-squares difference test.

RESULTS

HSPE1 Coimmunoprecipitated with HSPD1 in Capacitated Mouse Spermatozoa

A co-IP strategy was employed in order to identify proteins that associate with HSPD1 in capacitated mouse spermatozoa. For this purpose, soluble sperm lysates were incubated in the presence of polyclonal anti-HSPD1 serum covalently bound to agarose beads and associated proteins resolved on 1D SDS- PAGE gels. Although some minor differences were observed between replicates, a number of interacting proteins were consistently detected using this approach (Fig. 1). The specificity of the co-IP strategy was demonstrated by the absence of these proteins in unconjugated bead controls (results not shown). Sequencing of the predominant proteins by LC/ MS/MS analysis identified the HSPD1 cochaperone, HSPE1. The specificity of this putative interaction was confirmed by a reciprocal co-IP experiment with anti-HSPE1, which, as shown

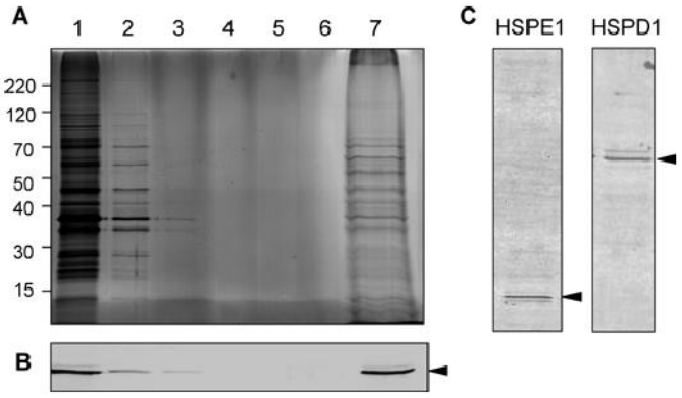

FIG. 1. Co-IP of HSPD1 and associated proteins using an immobilized anti-HSPD1 antibody. Caudal spermatozoa were incubated for 90 min in complete BWW, washed, and lysed. Clarified lysates were immunopre- cipitated with immobilized anti-HSPD1 antibody. Immunoprecipitated proteins were resolved by SDS-PAGE. A) Total protein was visualized by silver staining in capacitated sperm lysate (lane 1), successive washes of the captured antigen complex (lanes 2-6), and finally the immunoprecip- itated proteins (lane 7). B) A duplicate gel was prepared for immunoblot- ting with anti-HSPD1 (arrowhead) to confirm that the precipitation had been efficacious. The immunoprecipitated proteins were subjected to LC/ MS/MS sequencing analysis and HSPE1 was identified as a major HSPD1 binding partner. C) The interaction of HSPE1 and HSPD1 was confirmed by a reciprocal immunoprecipitation using anti-HSPE1 antibodies. An immunoblot of the proteins was probed successively with anti-HSPE1 (HSPE1) and anti-HSPD1 (HSPD1). The proteins corresponding to HSPE1 and HSPD1 are indicated by arrowheads.

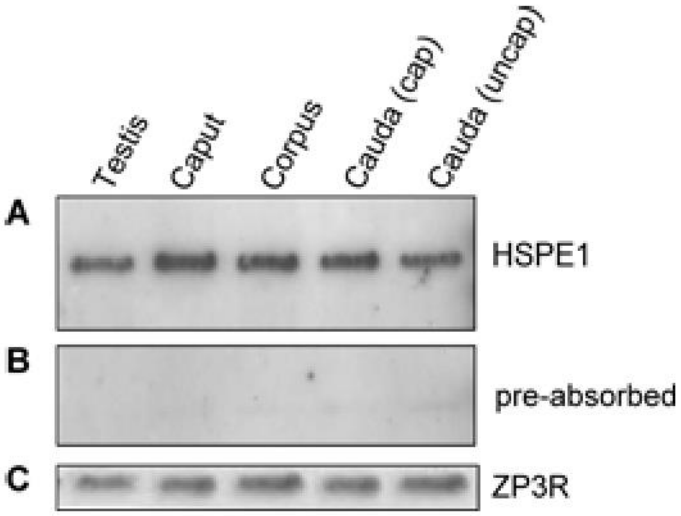

FIG. 2. Western blot analysis of HSPE1 expression in mouse testes and in epididymal spermatozoa. A) Cell extracts (5 lg) of whole testis and epididymal sperm were resolved by SDS-PAGE and immunoblotted with anti-HSPE1. Blots were stripped and sequentially reprobed with (B) anti- HSPE1 preabsorbed against recombinant HSPE1 to confirm the specificity of labeling, and (C) anti-ZP3R (ZP3R), an antibody that detects a constitutively expressed sperm protein and is thus used to confirm equal protein loading in each lane.

in Figure 1C, pulled down a protein of approximately 60 kDa that cross-reacted with anti-HSPD1 serum.

HSPE1 Is Expressed in Spermatozoa from the Mouse Epididymis

Given the novelty of the finding that HSPE1 is present in mouse spermatozoa, studies were undertaken to confirm this result and characterize the ontogeny of its expression during sperm development. For this purpose, soluble sperm lysates were prepared from populations of cells extracted from the caput, corpus, and cauda epididymis and isolated over percoll gradients to a purity that exceeded 95%. Western blot analysis of these samples revealed that a single, predominant, cross- reactive band of the appropriate size for HSPE1 was expressed in caput, corpus, caudal, and capacitated caudal spermatozoa (Fig. 2A). The specificity of this binding was confirmed by stripping the blot and reprobing with HSPE1 antibodies that had been preabsorbed against recombinant HSPE1 protein. This treatment eliminated the cross-reactivity of the antibody with the band of 10 kDa (Fig. 2B). Furthermore, reprobing of the blot with an antibody that recognized the constitutively expressed sperm protein, ZP3R (formerly Sp56) (Fig. 2C), revealed that the relative level of HSPE1 expression did not differ significantly at any stage of sperm maturation within the epididymis. Similarly, the molecular weight of the cross-reactive protein did not change, indicating that no overt processing of HSPE1 occurred during epididymal transit.

Although similar isolation strategies were adopted in an attempt to purify testicular sperm for Western blot analysis, these proved unsuccessful. Nonetheless, Western blot analysis of soluble protein lysates prepared from whole mouse testes did reveal HSPE1 protein expression within this tissue (Fig. 2).

HSPE1 Is Localized to the Periacrosomal Region And Colocalizes with Mitochondria in the Cytoplasm of Precursor Germ Cells in the Testes

Having confirmed the presence of HSPE1 in testicular tissue, we next sought to examine the localization pattern of the target protein in mouse testicular sections. This was achieved using laser confocal microscopy and indirect immunofluorescence. Sections were counterstained with propidium iodide, a nuclear stain, to assist in cell structure visualization and target protein localization. Anti-HSPE1 showed relatively weak, punctate labeling of the cytoplasm of spermatogonia (Fig. 3A). HSPE1 has traditionally been described as a mitochondrial protein (reviewed in [23]) and, in the testis, this protein was indeed colocalized with mitochondria in precursor germ cells (Fig. 4, B-D). However, in round spermatids, particularly intense labeling was observed in a discrete crescent-shaped pattern characteristic of that displayed by the developing acrosomal vesicle (Fig. 3A and Fig. 4D, arrows). This is consistent with the fact that, in other seminiferous tubule sections, possessing more advanced stages of spermatogenesis, HSPE1 was also localized in the crescent-shaped acrosomal region of late- elongating spermatids (Fig. 3B, arrows). However, in these tissue sections, this protein was not detectable in fully differentiated spermatozoa (Fig. 3A, red arrow). The localiza- tion pattern of HSPE1 in round and elongating spermatids in mouse testes suggests that it is acquired by spermatozoa during spermatogenesis, and fulfils a role other than that associated with its traditional mitochondrial function. Importantly, the specificity of this HSPE1 labeling was confirmed by the fact that preabsorption of the antibody with recombinant HSPE1 protein eliminated this labeling (results not shown).

HSPE1 Localizes to the Epididymal Epithelium and Dense Bodies in the Epididymal Lumen

Localization of HSPE1 in mouse epididymal sections was achieved as described above for mouse testes sections.

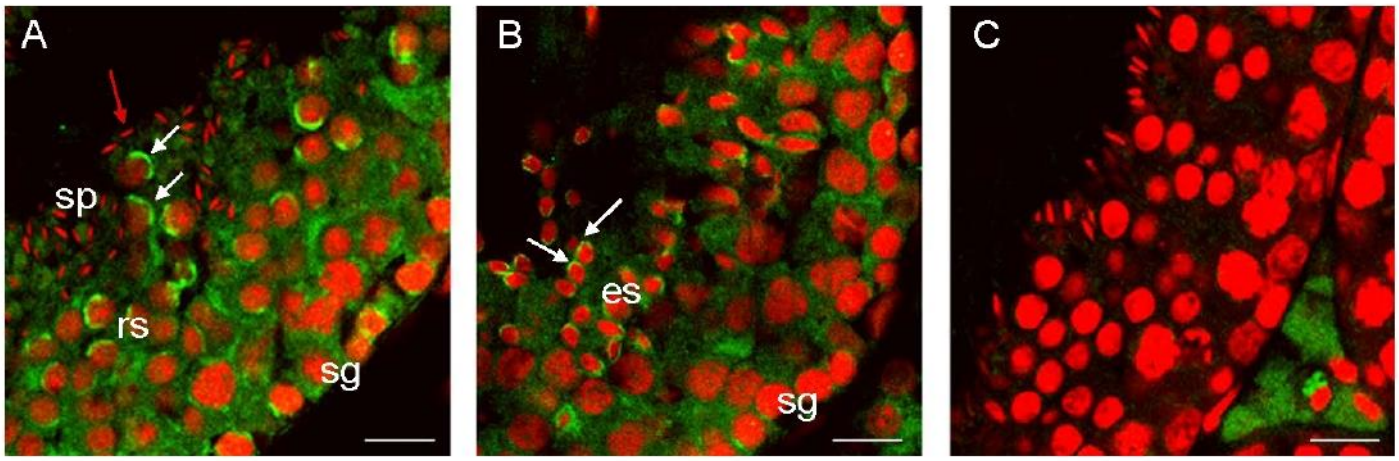

FIG. 3. Immunofluorescent localization of HSPE1 expression in mouse testis. Mouse testis sections were stained with anti-HSPE1 followed by FITC-conjugated secondary antibody (green) and counterstained with propidium iodide (red). HSPE1 labeling appeared in spermatogonia (sg), round spermatids (rs), and elongating spermatids (es), but not in sperm (sp, red arrow). A and B) HSPE1 is present in developing acrosomes of round and elongating spermatids (white arrows). C) Control treatment prepared in the absence of primary antibody failed to show any labeling other than that of surrounding the interstitial tissue. Bar ¼ 20 lm.

Polyclonal anti-HSPE1 labeling within caput, corpus, and caudal epididymal tissue sections was consistently seen in a diffuse pattern throughout the principal epithelial cells (Fig. 5, A-C). Interestingly, this antibody also strongly labeled discrete structures within the lumen of the duct that we have previously termed "dense bodies" [14] (Fig. 5, A-C). Consistent with our previous reports, the dense bodies observed in the epididymal lumen were distinct from spermatozoa, as shown by their failure to colocalize with the propidium iodide-labeled sperm nuclei (Fig. 5, A-C, arrowheads). The pattern of HSPE1 labeling in the epididymal lumen also precluded localization to the sperm flagellum, including the mitochondrial gyres. Interestingly, these unique structures have also been shown to contain HSPD1 and HSP90B1 [14].

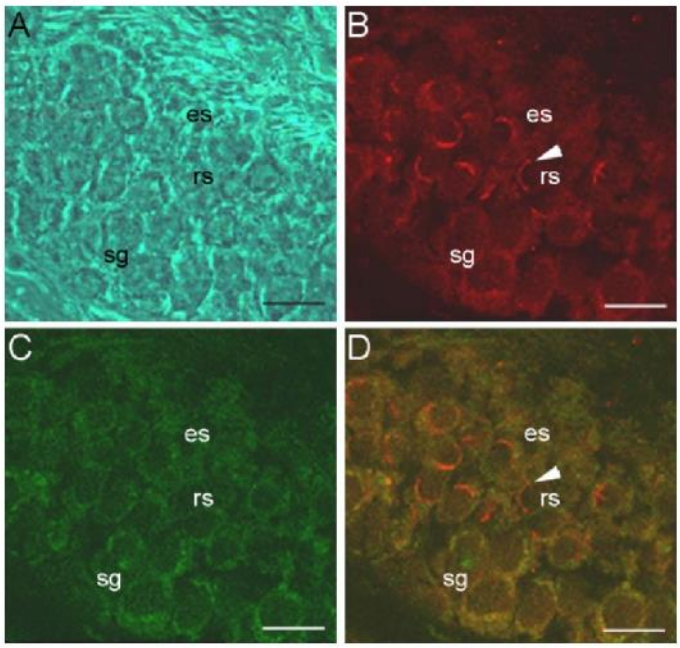

FIG. 4. Immunofluorescent colocalization of HSPE1 with mitochondria in mouse testis. Mouse testis sections (A, light microscopic image) were stained with anti-HSPE1 followed by TRITC-conjugated secondary antibody (B, red) and counterstained with anti-ox phos complex 1 followed by FITC-conjugated secondary antibody (C, green). HSPE1 colocalizes with mitochondria in the cytoplasm of spermatogonia (sg), round (rs), and elongating spermatids (es) (D, yellow), but only HSPE1 is present in the developing acrosome (arrows). Bar ¼ 20 lm.

HSPE1 Is Expressed in Spermatozoa Isolated from the Testis and Epididymis and Colocalizes with HSPD1 in Capacitated Spermatozoa

In light of our inability to detect HSPE1 in spermatozoa within testicular or epididymal tissue sections, populations of spermatozoa were isolated from the testis and various sites along the epididymis. They were then fixed, immunostained with anti- HSPE1 polyclonal antibody, and the localization patterns viewed with laser confocal microscopy. This approach revealed distinct patterns of HSPE1 expression coinciding with different stages of sperm maturation. In testicular spermatozoa, anti- HSPE1 strongly labeled the acrosomal region of the sperm head, the midpiece, and cytoplasmic droplet, and was also detected, albeit more weakly, in the principal piece of the tail (Fig. 6, testis). A very similar HSPE1 labeling pattern was observed in sperm isolated from the caput epididymis (Fig. 6, caput), although the head labeling appeared to be restricted to a discrete crescent overlying the acrosomal cap and posterior head, while the cytoplasmic droplet was still strongly labeled. During epididymal transit, the cytoplasmic droplet was lost and HSPE1 labeling of spermatozoa from the corpus epididymis was characterized by a similar crescent-shaped area of fluorescence over the acrosomal cap and strong labeling of the principal piece of the tail (Fig. 6, corpus). Finally, mature spermatozoa isolated from the caudal epididymis show anti-HSPE1 labeling over the apical crescent of the head, weak midpiece labeling, and strong labeling of the principal piece (Fig. 6, cauda).

To gain further insight into the putative association between HSPE1 and HSPD1, the two chaperones were colocalized in capacitated mouse spermatozoa. As shown in Figure 7, both antigens localized to the apical domain overlying the acrosome, the site of sperm-ZP interaction. However, while HSPD1 was also localized to the midpiece of the tail, HSPE1 was predominantly localized in the principal piece of the tail. The co-IP of HSPE1 with HSPD1 and their colocalization over the apical acrosomal domain support a role in capacitation-

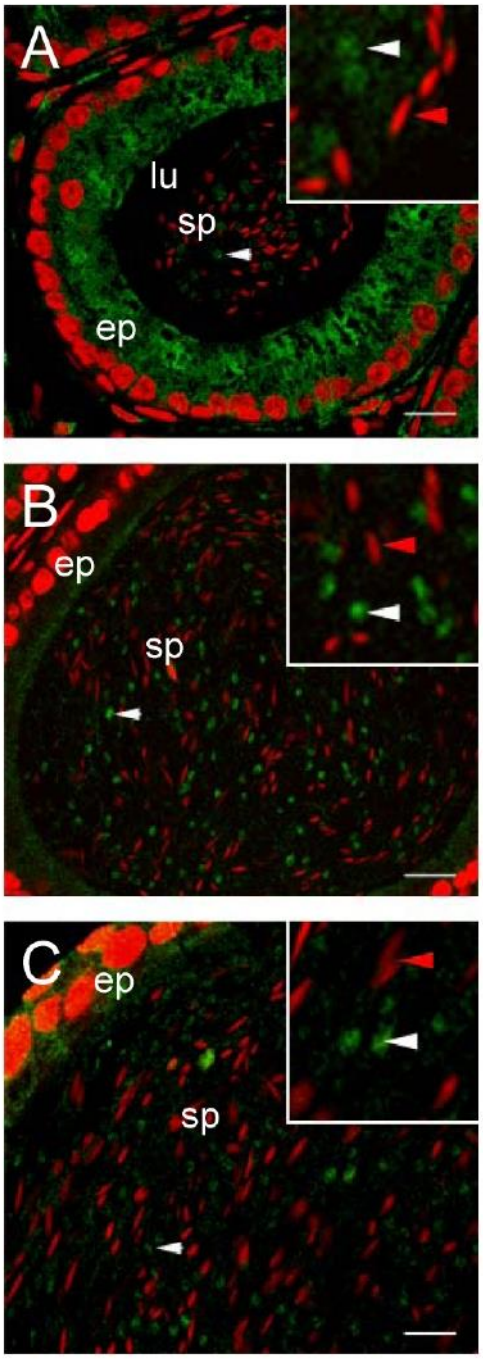

FIG. 5. Immunofluorescent localization of HSPE1 expression in the mouse epididymis. Mouse epididymal sections were stained with anti-HSPE1 followed by FITC-conjugated secondary antibody (green) and then counterstained with the nuclear stain, propidium iodide (red). HSPE1 labeling appeared in a diffuse pattern throughout the principal cells of the epithelium (ep) and in dense bodies (white arrows) in the lumen (lu). Sperm heads (sp) labeled red are distinct from HSPE1 staining (red arrows). A) Distal caput. B) Corpus. C) Cauda. Bar ¼ 20 lm (A and B); bar ¼ 10 lm (C).

dependent acquisition of zona binding ability, as previously suggested for HSPD1 [11].

HSPE1 Appears on the Surface of Spermatozoa During Capacitation

Expression of HSPE1 over the apical domain of the sperm acrosome is suggestive of a role in the interaction between the

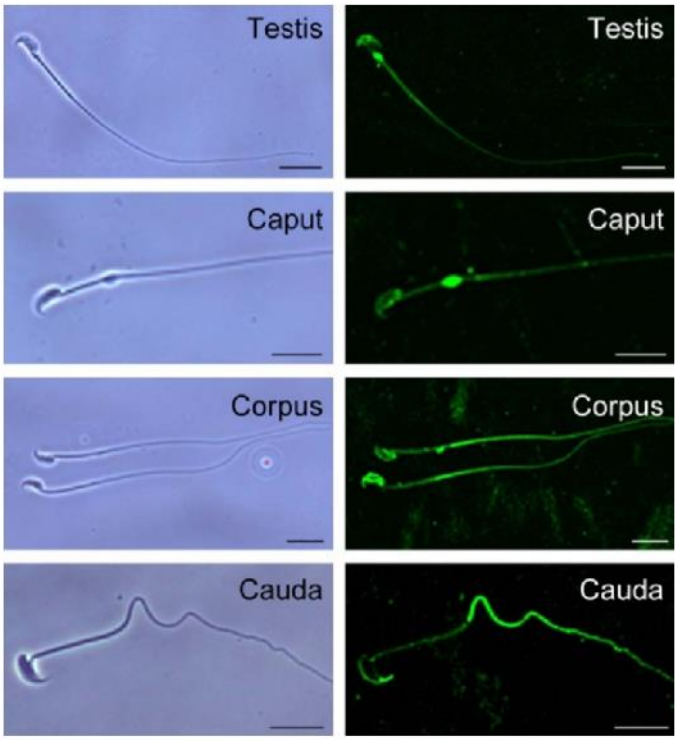

FIG. 6. Immunofluorescent localization of HSPE1 in mouse sperm from the testis and the epididymis. Sperm were isolated from mouse testis and each region of the epididymis, fixed, and stained with anti-HSPE1, followed by FITC-conjugated secondary antibody. Anti-HSPE1 labeling appears in the sperm tail from testis through to the cauda. Labeling is strong in the cytoplasmic droplet in testis and caput before being lost during epididymal transit. It was also present in the sperm head, initially appearing in a diffuse pattern, becoming more discretely localized to the periacrosomal head region during epididymal transit. Bar ¼ 10 lm.

sperm and the oocyte during fertilization. In order to confirm that HSPE1 is surface localized and, therefore, available to participate in this interaction, antigen localization studies were performed and surface labeling detected by flow cytometry. In these studies, nonviable cells were excluded from the analysis through the use of propidium iodide as a viability stain. In uncapacitated sperm populations, anti-HSPE1 was exposed on the surface of around 25% of the live cells (Fig. 8). However, after capacitation, there was a significant increase (P , 0.001) in sperm surface expression of HSPE1 (94 6 1.2%) (Fig. 8). As anticipated, spermatozoa in the control population, prepared in the absence of primary antibody, displayed only background
levels of fluorescence.

Loss of Acrosome Correlates with Loss of HSPE1 from the Sperm Head

The acrosomal localization of HSPE1 was further confirmed by analysis of its fate subsequent to loss of the apical sperm membrane through induction of acrosomal exocytosis. To perform these studies, mouse caudal sperm were capacitated and acrosomal exocytosis induced by addition of the calcium ionophore, A23187. Cells were then incubated in prewarmed hypo-osmotic swelling medium, allowing identification of the live sperm and, after fixation, they were successively immunostained with anti-HSPE1 and a TRITC-conjugated secondary antibody (Fig. 9, red labeling). The spermatozoa were then counterstained with FITC-conjugated Arachis hypogaea, a lectin that selectively stains acrosome-intact, but

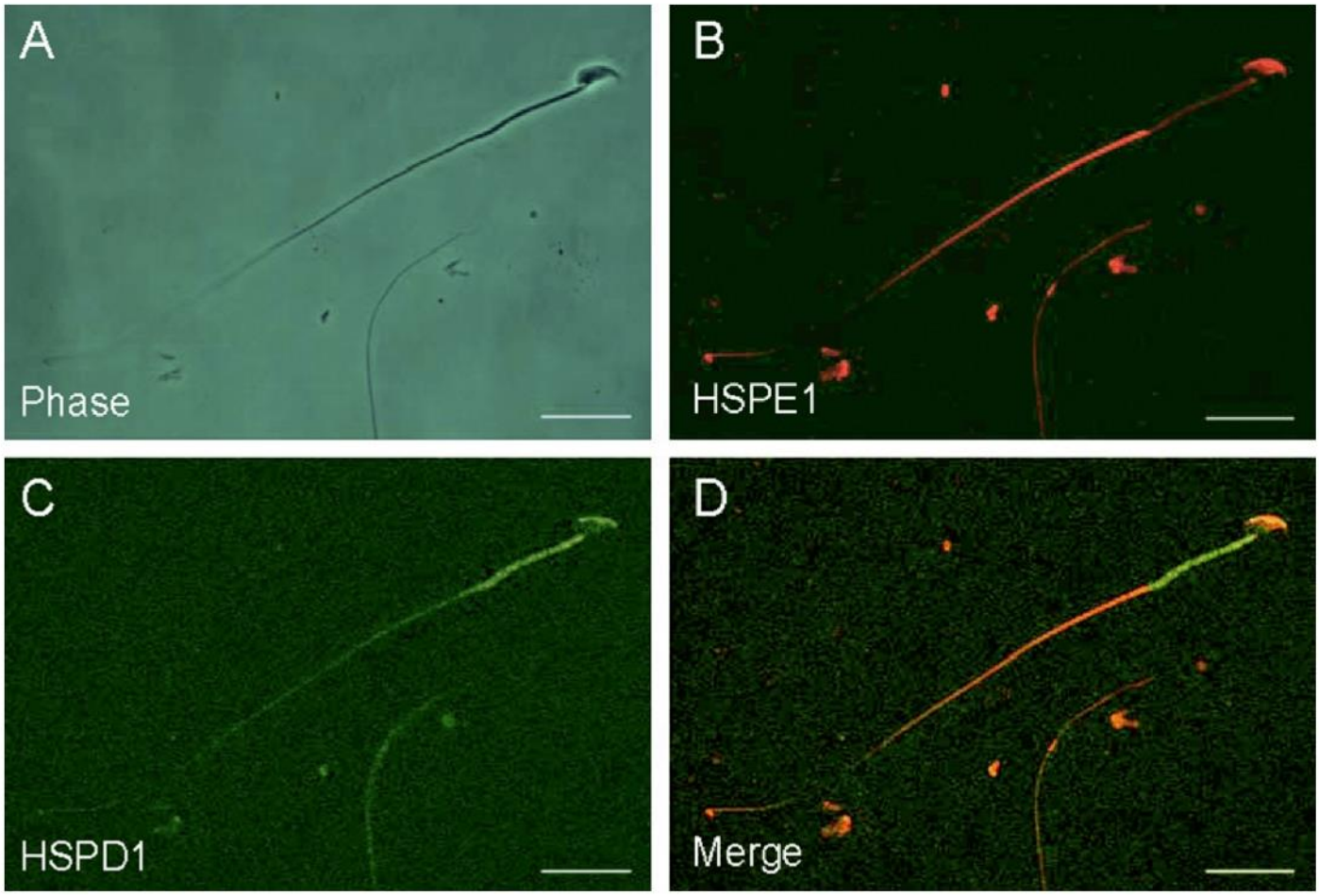

FIG. 7. Immunofluorescent colocalization of HSPE1 and HSPD1 in mouse spermatozoa. A) Sperm were isolated from the cauda epididymis, fixed, and successively stained with (B) anti-HSPE1 and a TRITC-conjugated secondary antibody (red) and then anti-HSPD1 followed by a FITC-conjugated secondary antibody (green). HSPE1 colocalized with HSPD1 in the periacrosomal region of sperm head (D), while only HSPE1 appeared in the principal piece (B), and only HSPD1 appeared in the midpiece (C). Bar ¼ 10 lm.

not acrosome-reacted, sperm (Fig. 9, green labeling). Analysis of the merged images revealed that sperm stained with anti- HSPE1 were also acrosome intact (Fig. 9, A-D). In contrast, those sperm that had undergone acrosomal exocytosis failed to bind anti-HSPE1 (Fig. 9, E-H).

HSPE1 Antibodies Inhibit Sperm-Zona Pellucida Interaction

In order to begin to assess the functional significance of HSPE1 in relation to fertilization, capacitated spermatozoa were preincubated with anti-HSPE1 antibodies and subse- quently examined for their ability to adhere to the zona pellucida of homologous mouse oocytes. As illustrated in Figure 10, anti-HSPE1 suppressed sperm-zona pellucida interaction in a significant dose-dependent manner without compromising either sperm viability or motility. At the highest concentrations of antibody used (100 lg), anti-HSPE1 reduced sperm binding to around 30% of that observed in the control populations. In contrast, preincubation of oocytes with recombinant HSPE1 failed to compromise sperm-zona pellu- cida interaction. From these data, we infer that HSPE1 does not, in itself, represent a receptor for the zona pellucida. Rather, it appears that the HSPE1 antibodies prevent sperm-zona interaction through mechanisms associated with steric hin- drance of the cognate receptors or, alternatively, by modifying the sperm surface architecture by promoting the movement of antigens within the plasma membrane.

DISCUSSION

Mammalian sperm-oocyte interaction is preceded by capacitation, a critical phase of cellular maturation during which immature spermatozoa are transformed into functionally competent gametes. Although numerous correlates of capaci- tation have been established, the molecular mechanisms that underpin many aspects of this process remain poorly understood. Recent studies from a number of independent laboratories have drawn attention to the importance of capacitation-associated increases in tyrosine phosphorylation in the functional maturation of mammalian spermatozoa [24- 27]. In the mouse, phosphotyrosine expression on the sperm surface is causally related to the acquisition of zona binding potential [11]. Studies from our laboratory have previously identified two of the major tyrosine-phosphorylated proteins as the molecular chaperones, HSPD1 and HSP90B1. In view of their recognized roles in facilitating protein folding and the assembly of multiple peptide subunits into mature protein complexes, these findings led us to hypothesize that, upon activation, these chaperones direct the assembly of a functional zona pellucida-receptor complex on the sperm surface (reviewed in [28]). In light of these data, we sought to further

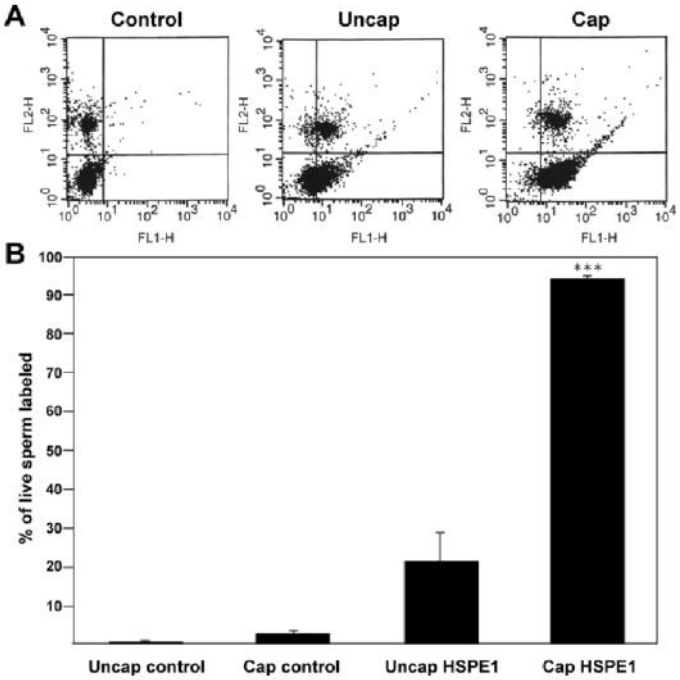

FIG. 8. Capacitation-dependent expression of HSPE1 on the surface of live mouse spermatozoa. Cauda epididymal sperm were incubated in either BWW prepared in the absence of HCO_3^{A} (Uncap) or BWW supplemented with pentoxifylline and dibutyryl cAMP (Cap), and the surface expression of HSPE1 was determined using FACS. Control incubations contained secondary antibody only. A) HSPE1 scatter plot showing populations of uncapacitated vs. capacitated sperm surface expression of HSPE1 (lower right quadrant). B) Graph of cell counts showing that, with capacitation, the proportion of sperm showing surface expression of HSPE1 significantly increased (***P , 0.001). Error bars indicate 6 SEM.

our investigation of the biological role played by chaperones in fertilization by identifying proteins that associated with these molecules during capacitation. Herein, we describe the characterization of an additional chaperone protein, HSPE1, isolated from capacitating mouse spermatozoa using an HSPD1 co-IP strategy.

Our discovery of a putative molecular association between HSPD1 and HSPE1 is not without precedent. Classically, in Escherichia coli, the GroEL (HSPD1) and GroES (HSPE1) families of molecular chaperones interact to fulfill a well-characterized role in mediating the correct folding of a variety of protein substrates (reviewed in [29, 30]). GroEL forms a dual ringed tetradecamer structure, capped at either or both ends by a heptamer of GroES to form a symmetrical structure [31-34]. Although eukaryotic HSPD1 and HSPE1 also interact to form an oligomeric structure that participates in protein folding, this differs from that of the GroES and GroEL complex [35]. In eukaryotic cells, HSPE1 and HSPD1 were originally described as mitochondrial proteins. However, consistent with the findings of the present study, an emerging body of literature suggests these proteins are also present on the cell surface [36-42] and in the extracellular fluid [43, 44].

In the current study, anti-HSPE1 staining revealed two distinct patterns of labeling within testicular sections. In spermatogonia and spermatocytes, a punctate staining pattern in the cytoplasm probably reflected the mitochondrial localization of this chaperone, given the colocalization of HSPE1 with an antibody targeting complex 1 of the inner mitochon- drial membrane (Fig. 4). However, as these precursor germ cells differentiated into haploid spermatids, this mitochondrial staining was lost and replaced by an intense crescent-shaped staining pattern corresponding to the developing acrosomal vesicle. From these data, we infer that HSPE1 may have a dual role in the development and/or function of both the mitochondria and acrosomal vesicle. In this context, it is

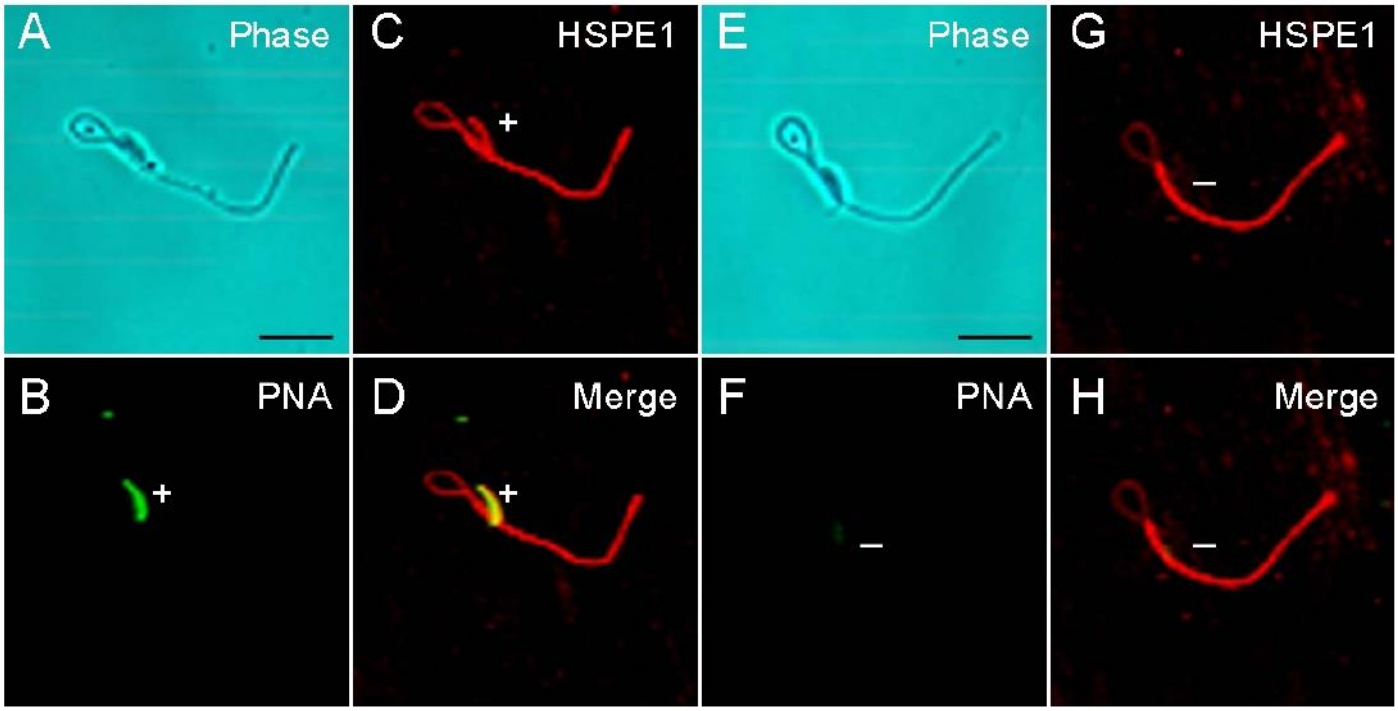

FIG. 9. Correlation between acrosome status and HSPE1 labeling of spermatozoa. Caudal epididymal sperm were capacitated, acrosome reacted, and stained with anti-HSPE1, followed by TRITC-conjugated secondary antibody, and counterstained with FITC-conjugated Arachis hypogaea lectin (PNA). Representative images of HSPE1/PNA dual labeling are shown. All cells that were positive for HSPE1 labeling over the sperm head (A-D) were also positive for PNA (þ), whereas cells negative for HSPE1 head labeling (E-H) were also negative for PNA (À). Bar ¼ 10 lm.

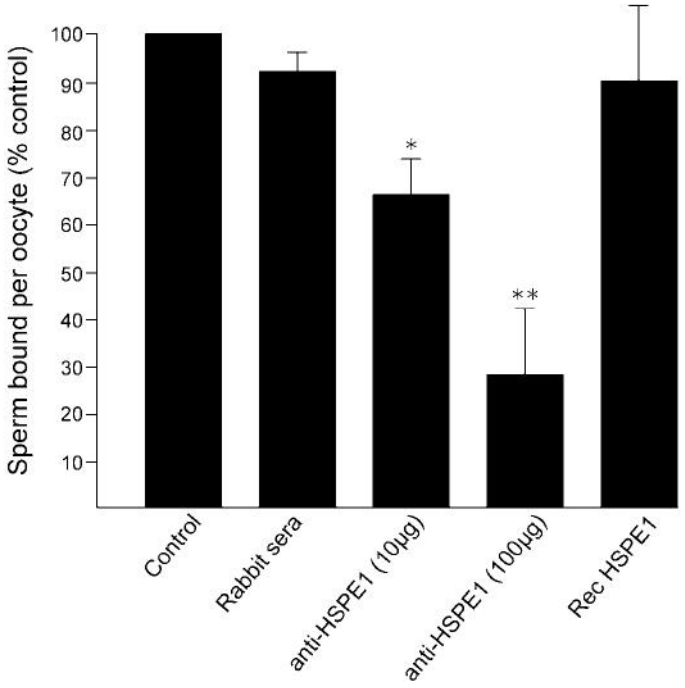

FIG. 10. HSPE1 inhibition of sperm-zona pellucida interaction. Capacitated spermatozoa (5 3 10 4) were incubated in either BWW (control), rabbit sera (100 lg/ml) or anti-HSPE1 antibody (10 or 100lg/ml) for 30 min at 378C. After washing, an aliquot of this suspension was added to a droplet of BWW containing oocytes and coincubated for an additional 30 min before scoring for the number of sperm tightly bound to the zona pellucida. An additional treatment in which oocytes were preincubated with recombinant HSPE1 (Rec HSPE1) prior to the addition of untreated, capacitated spermatozoa was also included. This experiment was replicated three times using a minimum of 12 oocytes per treatment. The data were normalized against the control treatment and are presented as the mean number of sperm bound to the zona pellucida 6 SEM. *P , 0.05; **P , 0.01.

possible that HSPE1 is integral to the mitochondrial protein import and assembly machinery in early, mitotically active germ cells, as has been proposed for HSPD1, based on similar patterns of expression in the testes of rats, humans, monkeys, and mice [14, 45-47]. Similarly, the staining of the residual bodies with anti-HSPE1 may be accounted for by the fact that only a limited number of mitochondria are retained as sperm mature and the rest are expelled within these structures (reviewed in [48]). Although the precise role of HSPD1 and HSPE1 during spermatogenic development awaits further investigation, the importance of chaperone proteins in this process is highlighted by the demonstration that male mice lacking HSPA2 (formerly HSP70-2) are infertile (reviewed in [49]). Removal of HSPA2 causes the arrest of spermatogenesis during prophase I at the pachytene stage of development, and germ cells are lost by apoptosis.

In addition to their role in spermatogenesis, a number of chaperones have also been identified in posttesticular spermatozoa, including HSPA2, HSP90AA1, and HSPD1 [11, 14, 50- 53], although their role in these cells remains less clear. Our studies of the ontogeny of HSPE1 expression during posttesticular sperm maturation revealed a similar pattern to that previously reported by us for both HSPD1 and HSP90B1 [14]. In purified populations of epididymal spermatozoa, HSPE1 was found to be predominantly localized to the apical (periacrosomal) region of the sperm head. Although this pattern was not altered at different stages of sperm development, the tail localization of HSPE1 appeared to undergo a dramatic reorganization from predominantly mid-piece in testicular spermatozoa to predominantly principal piece in cells from the cauda epididymis. This temporal pattern of HSPE1 redistribution is similar to that previously documented for a number of additional sperm proteins, including SPAM1 and ADAM2 [54]. However, in contrast to these proteins, we found no evidence to indicate that HSPE1 was processed during epididymal transit, and, thus, the mechanism responsible for its redistribution remains to be established.

Interestingly, HSPE1 antibodies consistently failed to label sperm within the lumen of testicular or epididymal tissue sections. They did, however, reveal diffuse labeling of the principal cells of the epididymal epithelium in addition to a large number of discrete entities within the lumen of the duct that appeared in the distal caput:proximal corpus region and beyond. Consistent with our previous account of these chaperone-laden dense bodies [14], they appeared to be closely apposed to the sperm heads, but failed to colocalize with these structures. Our previous ultrastructural analysis of dense bodies revealed that they are distinct from epididymosomes, mem- brane-bound vesicles secreted from the epididymal epithelium in an apocrine manner (reviewed in [55]). However, there are only a limited number of independent studies in which similar epididymal structures have been described. Among these are reports of electron-dense, carbohydrate-rich aggregates that are apparently involved in the formation of sperm rosettes [56-58]. At present, it is unclear whether the dense bodies that we have identified are in fact the same or separate aggregations to those associated with rosette formation. Nevertheless, this seems likely, as they do possess a similar amorphous morphology and pattern of distribution within the epididymis.

In previous studies, we have speculated that the formation of dense bodies may be an effective way of simultaneously delivering a number of proteins to spermatozoa through the mediation of the chaperones that they contain (HSPD1, HSPE1, HSP90B1, and possibly others). This notion is consistent with emerging evidence that extracellular chaper- ones are not only able to bind and transport a myriad of "cargo" proteins, but that the interaction of these complexes at the surface of cells leads to the subsequent uptake of the chaperone-cargo protein complex (reviewed in [59]). This conclusion is also commensurate with the observation that the electron-dense material responsible for rosette formation contains CRISP1, a glycoprotein secreted by the corpus epididymis that is involved in sperm maturation [57]. Thus, the proposed ability of this material to embed the sperm head and bind several cells together may increase the efficiency of such transfer processes. However, the mechanism by which proteins are translocated from the dense bodies to either the surface or intracellular compartments of the sperm remains to be determined.

If HSPE1 participates in gamete interaction, as we have hypothesized for the chaperones HSPD1 and HSP90B1, then these proteins would be expected to colocalize on the apical surface of the head of live, capacitated spermatozoa. Using immunofluorescent staining of capacitated caudal spermatozoa, we have demonstrated that this was the case. Additional studies employing a flow cytometry assay not only confirmed the surface localization of the chaperones, but also revealed a dramatic increase in the level of surface exposure of HSPE1 during capacitation. In this context, HSPE1 was only observed on the surface of approximately 25% of uncapacitated cells, but was detected on virtually all capacitated spermatozoa. Although the superficial expression of both HSPD1 and HSP90B1 is also correlated with sperm capacitation [14], the level of exposure of these chaperones is much less than that

observed for HSPE1. Such findings not only implicate HSPE1 in the capacitation-dependent acquisition of zona binding potential [11, 60], but also indicate the possible use of this protein as a marker to monitor the capacitation status of spermatozoa. Further support for the functional significance of HSPE1 in relation to fertilization was advanced by the demonstration that anti-HSPE1 antibodies inhibited sperm- zona pellucida interaction in a concentration-dependent manner. Nevertheless, the possibility that HSPE1 serves as a receptor to directly mediate sperm-zona pellucida interaction has been discounted on the basis that recombinant HSPE1 protein failed to adhere to the zona pellucida and competitively inhibit sperm interaction. Rather, these data support the hypothesis that HSPE1 is localized within close proximity of the zona receptor, and may be involved in chaperone-mediated assembly of a zona-receptor complex, as we have previously proposed for its cochaperone, HSPD1 [11].

Although chaperones were originally thought to be restricted to organelles within the cell, recent reports are highlighting their role on the cell surface. These include cancer cells, blood cells, fibroblasts, hematopoietic stem cells, and neural progenitors [12, 41, 61-66]. Our evidence that the surface expression of HSPD1, HSPE1, and HSP90B1 is involved in acquisition of zona binding potential in mouse spermatozoa [11, 14] has highlighted another novel extracel- lular role for these important chaperone proteins. Further studies into the identity of the chaperone client proteins may yield important insights into the molecular basis of sperm maturation and fertilizing potential.

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