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PROTEIN PURIFICATION, SEQUENCING, AND cDNA CLONING OF P68
A SPERM SURFACE PROTEIN

by

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in partial fulfillment of the requirements for the degree of
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University of Ottawa
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ABSTRACT

P68/62, an ~ 68 kDa doublet, is a sperm surface protein whose ability to bind to the egg zona pellucida has been documented *in vitro* and *in vivo* (Tanphaichitr *et al.*, 1992, 1993). This study aimed to purify P68/62 from boar sperm for P68 protein microsequencing and to isolate the cDNA for P68 from boar testis. Early attempts were made to isolate the P68 cDNA from boar testis using reverse-transcription PCR and primers based on previously obtained P68 peptide sequences. One PCR product, clone A, showed 82% and 78% overall nucleotide and amino acid homology to human cleavage signal 1 (HCS1) protein, respectively. Clone A is most likely the pig homolog of HCS1. No other clones sequenced showed any potential as the P68 cDNA clone. Therefore, more P68/62 was purified to obtain more protein sequences for future P68 cDNA cloning work. P68/62 was isolated from a boar sperm protein extract using chromatofocusing in conjunction with fast pressure liquid chromatography. Pure P68/62 fractions were defined by silver-stained SDS-PAGE and immunoblotting results using antiSLIP1, antiRSA, and anti-keratin. Approximately 0.5 μ g of pure P68 was obtained for microsequencing. Trypsin digestion of P68 and sequencing of four peptides resulted in sequences highly homologous to a casein peptide and three different regions of the human testis arylsulfatase-A protein. Preliminary analyses and studies suggest that AS-A and the casein peptide are components of the P68 polypeptide.

This thesis is dedicated to my loving family and Jonathan Soboloff

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CHAPTER 1

INTRODUCTION

1.1 Events of fertilization

In the mammalian system, binding of capacitated, acrosome-intact sperm (i.e., sperm that have not yet undergone the acrosome reaction, Appendix A) to the glycoprotein matrix surrounding the egg, the zona pellucida (ZP), is considered the initial step of the sperm-egg interaction. For fertilization to be successful, many processes occur before this binding as well as after. Before sperm-egg binding, the sperm undergo a series of changes that occur both in the male and female reproductive systems. In the seminiferous tubules of the testis, differentiation of spermatogenic cells is essential for the formation of the organelles necessary for motility (i.e., a tail) (Bleil, 1991). The assembly of the molecular machinery required for recognition and penetration of the ZP, as well as for egg plasma membrane fusion (e.g., an acrosome) also occurs at this time (Bleil, 1991). However, spermatozoa are still immature and require transport to the epididymis where they are stored and undergo maturation. As they pass through the caput epididymis and then mature in the environment of the cauda epididymis, sperm become proficient at forward motion (Bleil, 1991). Sperm surface changes and completion of chromatin condensation concludes the maturation process (Bleil, 1991; Krzanowska, 1982; Pellicciari *et al.*, 1983).

In order to gain ZP binding ability and hyperactivated motility, sperm undergo capacitation once they reach the female reproductive tract. Capacitation can also be induced *in vitro* by incubating sperm in media supplemented with albumin (Yanagimachi,

1994). Among the changes that occur during capacitation, plasma membrane proteins and sugar residues are altered to expose egg receptors (Berger, 1990; Fraser, 1984; Koehler, 1981; Miller and Ax, 1990; Singer *et al.*, 1985); proacrosin is processed to acrosin (Parrish and Polakoski, 1981); a cholesterol efflux causes a decrease in the membrane cholesterol:phospholipid ratio (Davis, 1981; Davis *et al.*, 1980; Langlais and Roberts, 1985, Suzuki, 1988; Suzuki and Yanagimachi, 1989), resulting in increased plasma membrane fluidity; and the increased membrane fluidity facilitates a gradual Ca^{2+} influx (Fraser, 1982; Suarez, 1993) and allows sperm motility to become hyperactivated (Katz *et al.*, 1989; Burkman, 1990). It is at this point that the sperm are ready to interact with the oocyte.

In the female reproductive system, the oocyte also undergoes changes before it encounters sperm. Before release into the oviduct, oocytes continue to mature despite being developmentally arrested at meiosis I. During this time, cumulus cells are proliferating, forming layers surrounding the oocytes (Wassarman and Albertini, 1994). Other oocyte changes that occur during this growth phase include the appearance of cortical granules and the formation of the zona pellucida (Wassarman and Albertini, 1994). Both of these structures are important for the prevention of polyspermy. The oocyte completes meiosis I but is arrested again at metaphase II until fertilization by the sperm. Leading up to metaphase II, cell cycle control proteins undergo biochemical changes that are necessary for egg activation at fertilization (Wassarman and Albertini, 1994).

Fertilization involves a series of steps that allow the binding of multiple numbers

of sperm to the egg, but permits only one to penetrate and fertilize the egg proper. First, the sperm must penetrate the cumulus cell layers surrounding the egg. In order to do so, the sperm must be capacitated and acrosome intact (Austin, 1960; Cummins and Yanagimachi, 1986). If the sperm are not capacitated or if they have already undergone an exocytotic event called the acrosome reaction (Appendix A), they are able to attach to the cumulus surface, but are unable to penetrate it (Yanagimachi, 1994). The cumulus mass is composed of cells and a matrix whose major component is hyaluronic acid (Yanagimachi, 1994). PH-20, a guinea pig sperm surface protein, has been shown to contain a hyaluronidase domain and its activity is essential for sperm penetration of the cumulus mass (Gmachl and Kreil, 1993; Lin *et al.*, 1993, 1994). Therefore, this protein, and homologs in other species, may be responsible for penetration of the cumulus mass by digesting hyaluronic acid.

Once through the cumulus mass, the oocyte's ZP is the next barrier that the sperm must penetrate. In most cases, the ZP acts as a barrier to inter-species gamete interactions, induces the acrosome reaction, and is involved in the prevention of polyspermy. In all mammals studied, the ZP is a matrix that consists of a few cross-linked glycoproteins (Wassarman, 1988; Liang and Dean, 1993; Harris *et al.*, 1994; Dunbar *et al.*, 1991). Studies suggest that sperm first bind to the carbohydrate groups of the ZP (Florman *et al.*, 1984; Florman and Wassarman, 1985; Bleil and Wassarman, 1988; Macek and Shur, 1988; Rosiere and Wassarman, 1992). There are many small variations in the carbohydrate composition of the ZP (Hedrick and Wardrip, 1981). Presumably, differences in the oligosaccharide chains between species dictates which

species will be able to bind. In this way, the difference in the carbohydrate moieties of these glycoproteins is believed to be the main determinant of species binding specificity (Hedrick and Wardrip, 1981; Wassarman, 1993). Furthermore, the homology of ZP peptide sequences among various species is high (Wassarman, 1993; Harris *et al.*, 1994), reducing the likelihood that inter-species interactions could be regulated by protein interactions. However, since the peptide sequence dictates how the sugar residues are presented on the ZP, it may have an indirect regulatory role.

The initial binding of the ZP to sperm occurs via specific proteins. Since studies of the mouse and the pig ZP proteins are the most extensive, the following description will be limited to these models. Among the mouse ZP proteins (ZP1, ZP2, and ZP3), ZP3 and ZP2 are the primary and secondary sperm receptors, respectively (Bleil and Wassarman, 1980; Wassarman *et al.*, 1985; Bleil *et al.*, 1988; Mortillo and Wassarman, 1991). In the pig system, the proteins are named ZP1, ZP2, ZP3, and ZP4 (Dunbar *et al.*, 1981; Dunbar *et al.*, 1985; Wardrip and Hedrick, 1985). Pig ZP3, upon endo- β -galactosidase digestion (EBGD), produces two proteins, ZP3 α and ZP3 β , and a polylactosamine (Yurewicz *et al.*, 1987). ZP3 α is the primary sperm receptor, although its sperm binding ability may be improved by its complexing to ZP3 β (Sacco *et al.*, 1989; Yurewicz *et al.*, 1993; Yurewicz and Sacco, 1996).

In the case of the primary zona receptor(s) on the sperm, none have been confirmed, but many candidates, including ones which could interact with ZP sugar residues (e.g., glycosyltransferases and glycohydrolases), have been proposed (see section 1.3 below). Due to the large number of sperm proteins that have shown an ability to bind

to the ZP, the current model for sperm-ZP interaction (Tanphaichitr *et al.*, 1998, Snell and White, 1996; Thaler and Cardullo, 1996; Bérubé and Sullivan, 1994; Wassarman, 1995) suggests that the interaction may involve either simultaneous or sequential protein action and that no sperm protein is single-handedly responsible for anchoring the sperm to the zona pellucida. This belief is supported by the fact that of transgenic male mice with null mutations for a candidate zona binding protein (Galt), 10% are still fertile and impregnate females (Lu and Shur, 1997; Lu *et al.*, 1997).

There are conflicting views on the region of the sperm head that binds first to the zona pellucida. The convex ridge has been identified as the region of initial sperm-ZP binding (Yanagimachi, 1994; Vazquez *et al.*, 1989; Mortillo and Wassarman, 1991), although other studies provide evidence that initial binding occurs at the postacrosomal region of the sperm head with final ZP binding at the convex ridge (Chen and Cardullo, 1994). The latter view is supported by the presence of ZP-binding sites in the postacrosomal or equatorial region (Baltz *et al.*, 1988; Aarons *et al.*, 1992;). Regardless, once primary binding has been established on the ZP surface, the sperm must penetrate the ZP. This is accomplished following the acrosome reaction, an exocytotic event that is triggered by sperm binding to ZP3 (Bleil and Wassarman, 1983; Wassarman, 1990; Kopf and Gerton, 1991). The acrosome reaction is also required before sperm can fuse with the egg plasma membrane (Yanagimachi, 1994).

Aggregation of sperm surface proteins, which may be mediated by the ZP, has been implicated in the activation of signal transduction, concluding with the acrosome reaction (Leyton and Saling, 1989; Bleil and Wassarman, 1983). The signal transduction

is mediated by the sperm G_i mechanism (Gong *et al.*, 1995; Ward and Kopf, 1993). A Ca^{2+} influx (Arnoult *et al.*, 1996; Florman *et al.*, 1989); pH increase (Florman *et al.*, 1989); and increases in cAMP (Ward and Kopf, 1993), IP_3 and diacylglycerol (Roldan *et al.*, 1994) levels occur. The end result of the signal transduction pathway is the fusion of the outer acrosomal and plasma membranes at various points along the acrosomal cap, and the exposure of the inner acrosomal membrane. Exposure of the inner acrosomal membrane reveals proteins (e.g., proacrosin/acrosin) that assist with maintaining secondary binding to the ZP2 (Wassarman, 1993; Yanagimachi, 1994). The acrosome contents are also released. The contents include hydrolytic enzymes that may help with zona penetration such as: proacrosin/acrosin, hyaluronidase, N-acetylglucosaminidase, and arylsulfatase (Yanagimachi, 1994). The exact mechanism of ZP penetration is still unknown.

Once the sperm have successfully penetrated through the ZP, they quickly cross the perivitelline space to bind to and fuse with the egg plasma membrane. The surface of the egg plasma membrane is covered with microvilli except for the area immediately overlying the meiotic spindle. Fusion occurs first between the equatorial region of the sperm head and the area of the egg that is covered by microvilli (Yanagimachi, 1994). An interesting discovery suggests that one of the mechanisms for this interaction may involve fertilin, which exists on the mammalian sperm surface (Primakoff *et al.*, 1987; Blobel *et al.*, 1990), and integrins, which are found on the oocyte plasma membrane (Almeida *et al.*, 1995; Myles and Primakoff, 1997). Recent studies of egg plasma membrane sulfolipid immobilizing protein 1 (SLIP1) (Ahnonkitpanit *et al.*, submitted)

suggest that another mechanism may involve the binding of sperm sulfogalactosylglycerolipid (SGG) to egg SLIP1. The sperm is subsequently incorporated into the egg.

Upon fusion with the sperm, the oocyte becomes activated. In sea urchins, during the first few seconds following sperm fusion, the oocyte plasma membrane depolarizes preventing further sperm fusion until a more permanent block, the cortical granule reaction, is initiated (Yanagimachi, 1994). This fast block to polyspermy is presumed to occur in mammals, although it has never been examined (Yanagimachi, personal communication). Following the presumed fast block, a signal transduction cascade initiates the cortical granule reaction. During this reaction, cortical granules present just below the egg membrane surface are exocytosed. Like the sperm acrosome, the cortical granules contain hydrolytic enzymes. These enzymes are released and cause the hardening of the ZP due to modifications made to ZP2 and ZP3 (the zona reaction) (Gwatkin, 1977). The effect on the ZP was called "hardening" due to the original observation that there was a decrease in zona pellucida solubility by chymotrypsin *in vitro* (Gulyas and Yuan, 1985). More specifically, ZP2 is partially digested by a protease and it is thought that glycosidases digest the O-linked oligosaccharides of ZP3 (Bleil *et al.*, 1981; Moller and Wassarman, 1989; Wassarman, 1993). This hardening traps any sperm in the ZP and prevents any more from binding to it (Wolf, 1981; Sato, 1979). The ZP also loses its ability to induce the acrosome reaction in sperm (Bleil and Wassarman, 1980; Bleil and Wassarman, 1983). Therefore, it is a mechanism that permanently prevents additional sperm from fertilizing the oocyte (the slow block to polyspermy).

To complete the fertilization event, the nucleus of the sperm decondenses and develops into a pronucleus (Yanagimachi, 1994). The egg continues its progression through meiosis II and then develops another pronucleus (Yanagimachi, 1994). Both pronuclei migrate to the same area of the egg and the DNA in both pronuclei is replicated such that each chromosome is composed of two sister chromatids (Yanagimachi, 1994). The nuclear envelope breaks down, a single spindle fibre is formed, and mitosis is completed by the separation of each chromatid, followed by cytokinesis (Yanagimachi, 1994). The stable nature of the ZP proteins surrounding the fertilized egg protects the embryo until it implants in the uterine wall. Studies have shown that embryos that lack a zona pellucida or that have a damaged one cannot survive the passage through the oviduct (Nichols and Gardner, 1989).

1.2 Zona pellucida proteins

The zona pellucida (ZP) is the very porous, extracellular matrix that surrounds all mammalian oocytes. As mentioned in the preceding section, there are only a few glycoproteins that make up this extracellular matrix. Collectively, these proteins are involved in sperm binding to the oocyte, induction of the acrosome reaction in bound sperm, prevention of post-fertilization polyspermy, and protection of the embryo until it implants in the uterine wall (Wassarman, 1988; Liang and Dean, 1993). These proteins are synthesized and secreted by the developing oocyte and form an ordered pattern of long, interconnected filaments (Greve and Wassarman, 1985). The mammalian zona proteins that have been studied to date are from the mouse, hamster, pig, human, rabbit,

monkey, dog, cat, sheep, and cow systems (Bleil and Wassarman, 1980; Dunbar *et al.*, 1980, 1981; Brown, 1986; Oikawa *et al.*, 1987; Shabanowitz and O'Rand, 1988; Harris *et al.*, 1994). The mouse model is often used as the point of reference because it is the most fully characterized in terms of both ZP protein function and cDNA/genomic sequences, and the information on the peptide sequences and other biochemical properties can be correlated to protein function.

In the mouse, the zona pellucida is comprised of three acidic sulfated glycoproteins: ZP1, average molecular weight (m.w.) = 200 kDa; ZP2, average m.w. = 120 kDa; and, ZP3, average m.w. = 83kDa. ZP2 and ZP3 are monomers, whereas ZP1 is a dimer of polypeptide chains that are held together by disulfide bonds (Bleil and Wassarman, 1980). The three glycoproteins are organized such that ZP2 and ZP3 form repeating heterodimers, the long filaments of the ZP, and neighbouring filaments are connected via ZP1, which is responsible for maintaining the ZP structure (Wassarman, 1988). It has been shown that all the ZP proteins are glycosylated (Bleil and Wassarman, 1980) and some evidence suggests that they are also sulfated (Shimizu *et al.*, 1983) and terminally sialylated (Wassarman, 1988). It has also been shown that the apparent molecular weights of the polypeptide moieties of the ZP1 dimer, ZP2, and ZP3 are only 75 kDa, 81 kDa, and 44 kDa, respectively, based on SDS-PAGE of ZP precursors or by deglycosylation of the proteins (Wassarman, 1988). Therefore, the difference in size indicates that there is heavy glycosylation of the proteins as well as other post-translational modifications to the peptides.

All three proteins contain N-linked (i.e., via asparagine residues) complex

oligosaccharides (Wassarman, 1988). It has been determined that ZP2 has six, and ZP3 has three or four, N-linked oligosaccharides per molecule (Wassarman *et al.*, 1986; Greve *et al.*, 1982; Salzmann *et al.*, 1983; Wassarman *et al.*, 1985). ZP2 and ZP3 also contain O-linked (i.e., via serine and threonine residues) oligosaccharides (Wassarman, 1988). However, there is no consensus on the number. One research group has estimated that there are three O-linked oligosaccharides per ZP3 molecule (Nagdas *et al.*, 1994). It is unknown whether ZP1 contains any O-linked oligosaccharides.

The organization of the pig zona pellucida is similar. Recent studies have shown that it is composed of three major proteins, pZP1 (90kDa), pZP3 α , and pZP3 β , each approximately 50 kDa (Yurewicz *et al.*, 1987). Based on their respective gene sequences, these three glycoproteins correspond to the mouse proteins ZP2, ZP1 and ZP3, respectively (McLeskey *et al.*, 1998). However, partial cleavage of pZP1 by reduction of its intermolecular disulfide bonds can produce two products, pZP2 and pZP4 (Hasegawa *et al.*, 1994). All of the ZP proteins are glycosylated, although it has only been ascertained that ZP2 and ZP3 contain both O- and N-linked oligosaccharides (Wassarman, 1988; Nagdas *et al.*, 1994). Approximately two-thirds of the N-linked ZP3 oligosaccharides have high molecular weights and a large content of sialic acid, galactose, N-acetylglucosamine, and sulfate groups to make them acidic (Yurewicz *et al.*, 1991). O-linked oligosaccharides of ZP3 are mainly tetrasaccharides with peripheral sulfated polyglucosamines (Yurewicz *et al.*, 1992). Both ZP1 and ZP3 are sialylated (Wassarman, 1988). Unlike the mouse system, less is known about the interaction between the glycoproteins to form the ZP structure. ZP3 α and ZP3 β have been shown to interact

(Yurewicz and Sacco, 1996) but nothing is known about ZP1's structural role.

In the mouse system, ZP3 is the primary sperm receptor (Wassarman, 1995). Its carbohydrate moiety has been implicated as the active binding component, although there is some debate as to what specific structure(s) is (are) involved. Conflicting results have implicated both the O-linked (Florman and Wassarman, 1985) and N-linked oligosaccharides (Yamagata, 1985). Furthermore, it may involve terminal α -galactose residues (Bleil and Wassarman, 1988) or N-acetyl β -glucosamine residues (Nagdas *et al.*, 1994; Shur and Hall, 1982a,b) of O-linked oligosaccharides.

Pig functional studies have shown that ZP3 α plays a significant role in sperm binding, but that ZP3 β is also important (Sacco *et al.*, 1989; Yurewicz *et al.*, 1993; Bagavant *et al.*, 1993). ZP3 β by itself does not bind to the sperm plasma membrane, although it may complex with ZP3 α to act synergistically in sperm binding (Yurewicz *et al.*, 1993). Investigations into the functional domain(s) of the primary ZP receptor proteins have also revealed that the sugar residues may play an important role in sperm binding. More specifically, it has been shown that it is the neutral N-linked oligosaccharide chains of ZP3 α (Noguchi *et al.*, 1992, Yonezawa *et al.*, 1995), and possibly only the N-terminal halves (Gupta *et al.*, 1996), that are important. However, other studies have suggested that the O-linked oligosaccharide chains of ZP3 α are also important (Yurewicz *et al.*, 1991). Therefore, in the pig system as with in the mouse, no conclusions have yet been drawn regarding the active sperm-binding sugar residues.

1.3 Putative zona pellucida-binding proteins

Since the characteristics of the mouse and pig ZP have been well-explored, a large body of research is now focused on determining the sperm protein(s) involved in ZP binding. Numerous putative ZP-binding proteins have been described in the mouse, pig, human, guinea pig, hamster, and rabbit systems. Many of these sperm surface proteins are enzymes. No single protein has been shown conclusively to be the major ZP receptor despite the fact that functional studies have suggested that several of these proteins are important for ZP binding.

1.3.1 Mouse zona pellucida-binding proteins

The major mouse ZP-binding proteins that have been identified are: N-acetylglucosamine:β1,4-galactosyltransferase (Galt), sp56, tyrosine kinase substrate (otherwise known as p95 or zona receptor kinase (ZRK)), trypsin inhibitor sensitive site (TIS), and mannosidase. Galt is one of the more promising sperm proteins because: it is found on the surface of live sperm (Gong *et al.*, 1995); it interacts selectively with ZP3 (Miller *et al.*, 1992); purified Galt, inhibitors of Galt, and antibodies to Galt each block sperm-egg binding *in vitro* (Shur and Neely, 1988); immunoaggregation of Galt leads to the acrosome reaction (Macek *et al.*, 1991); when it is overexpressed, sperm bind more purified ZP3 and are hypersensitive to the ZP-induced acrosome reaction (Youakim *et al.*, 1994); and, modification of N-acetylglucosamine on ZP oligosaccharides, Galt's binding site, by an enzyme found in cortical granules prevents zona binding (Miller *et al.*, 1993). However, the precise location, the extent of acrosome reaction by cross-linking of the

enzyme, and the species-specificity of zona binding still need to be determined.

sp56 was identified as a ZP receptor by its preferential binding to ¹²⁵I-radiolabelled ZP3 via a photoactivatable crosslinker (Bleil and Wassarman, 1990). sp56 also binds tightly to ZP3 affinity columns and to the ZP3 oligosaccharides that have been identified as binding to sperm (Bleil and Wassarman, 1990; Cheng et al., 1994). However, as to be expected, it does not bind to the zona pellucida of fertilized eggs (Bookbinder et al., 1995). sp56 is unique to the testis and its mRNA is detectable only in spermatids and testicular spermatozoa (Bookbinder et al., 1995). Purified exogenous sp56 blocks sperm-egg binding (Bookbinder *et al.*, 1995) and has been shown to be a peripheral plasma membrane protein that is localized to the heads of fixed, acrosome intact sperm (Bleil and Wassarman, 1990; Cheng *et al.*, 1994). However, recent electron microscopic results have challenged the notion that sp56 is on the sperm surface; rather, it has been shown to be an acrosomal protein (Foster *et al.*, 1997). Furthermore, it still remains to be shown whether or not sperm-egg binding can be disrupted by specific sp56 antibodies, and whether it is linked to a transmembrane protein involved in the induction of the acrosome reaction.

Studies of tyrosine kinase substrate, or p95 or zona receptor kinase (ZRK), have suggested that it is another enzyme that also plays a role in zona binding. And unlike sp56, it may be involved directly in the initiation of the signal transduction pathway (non-G_i mechanism) leading to the acrosome reaction. p95, so-named for its size based on SDS-PAGE migration, is found on the sperm head and a monoclonal antibody against it inhibits sperm-egg binding (Leyton *et al.*, 1995). Partially purified p95 binds only to ZP3

(Leyton and Saling, 1989). Interestingly, regarding its possible role in initiating the acrosome reaction, it is phosphorylated on tyrosine and the levels of phosphorylation increase upon exposure to solubilized ZP (Leyton and Saling, 1989a). It was suggested that p95 is a tyrosine kinase and, therefore, autophosphorylates itself (Burks *et al.*, 1995). In the presence of specific protein tyrosine kinase inhibitors, p95 is not phosphorylated and the acrosome reaction does not occur (Leyton *et al.*, 1992). However, it still remains to be shown that the inhibitors were acting directly on p95. In addition, many mouse sperm tyrosine-phosphorylated proteins exist that are ~95 kDa by SDS-PAGE and that seem to have different properties. One example is a p95 hexokinase (Kalab *et al.*, 1994; Visconti *et al.*, 1996). Finally, if mouse p95 is to be considered a ZP receptor, ZP3 binding to p95 on live mouse sperm, and species-specific ZP binding still needs to be shown.

Little information has been gathered on another sperm surface enzyme believed to be involved in ZP binding, trypsin inhibitor-sensitive site (TIS). It was originally characterized as a protease on the mouse sperm head whereby exposure to trypsin inhibitors prevented the sperm from fertilizing the egg (Saling, 1981). However, it is not trypsin since TIS is unable to hydrolyze known trypsin substrates (Benau and Storey, 1987). When capacitated mouse sperm were exposed to either natural or synthetic trypsin inhibitors and then added to eggs, the sperm showed a decreased ZP binding ability in a dose-dependent manner (Saling, 1981). Furthermore, it was shown that TIS is involved in ZP binding and not ZP penetration, since inhibitor treatment prevented ZP binding only (Saling, 1981). How exactly this protease may function in ZP binding is unknown.

Mannosidase is another sperm surface enzyme that has been implicated in sperm-ZP binding. Functional studies were performed in the mouse (Cornwall *et al.*, 1991), but activity has been detected in other species including the rat, human, and hamster (Tulsiani *et al.*, 1989; Tulsiani *et al.*, 1990). Most of the research has been performed using the rat model. In rats, it was shown to be active primarily towards mannose-containing oligosaccharides (Tulsiani *et al.*, 1989, 1990) and several reports have suggested that mannose residues on the zona pellucida may be involved in sperm-zona binding (Shalgi *et al.*, 1986; Lambert, 1984). In the rat model system, mannosidase is a 115 kDa integral membrane protein in its mature form (Tulsiani *et al.*, 1993) that is localized to the periacrosomal region of the live and fixed sperm head (Tulsiani *et al.*, 1995). The enzyme activity is controlled such that it increases as the rat sperm mature in the epididymis (i.e., it exists originally in an inactive form that, by the completion of maturation, is activated as a result of proteolytic processing) (Tulsiani *et al.*, 1995). Evidence that suggests mannosidase may play a role in sperm-ZP binding include: in the rat, it is active at the physiological pH range where fertilization takes place (Tulsiani *et al.*, 1989); the catalytic domain is oriented towards the outer rat sperm surface (Tulsiani *et al.*, 1989); inclusion of mannosidase inhibitors or a competitive mannose-like substrate in sperm-egg coincubates inhibited sperm mannosidase activity and decreased the number of sperm bound per egg in a dose-dependent manner (Cornwall *et al.*, 1991); furthermore, the inhibition did not result from a negative effect on motility or acrosome integrity; treatment of hamster oocytes with concavalin A, a lectin that binds to high mannose and/or hybrid oligosaccharides, prevents sperm-egg binding (Oikawa *et al.*, 1974); and

pretreatment of rat oocytes with jack-bean mannosidase showed nearly complete inhibition of sperm-egg binding (Shalgi *et al.*, 1986). What still remains to be shown is any species specificity of mannosidase binding, direct mannosidase binding to ZP3, and whether it is capable of initiating the acrosome reaction once bound to the zona pellucida.

1.3.2 Pig zona pellucida-binding proteins

AP, proacrosin/acrosin, Sp38, and zonadhesin have been identified as ZP-binding proteins in the pig system. AP is a ~52 kDa integral membrane protein that is located over most of the sperm head surface on live and fixed cells (Peterson *et al.*, 1991). It is considered to be a porcine ZP-binding protein because of its ability to bind to, and block, antibodies that normally inhibit sperm-egg binding (Peterson and Hunt, 1989). In addition, pretreatment of solubilized zonae with an AP dispersion was able to block the zonae's ability to subsequently inhibit the binding of a sperm-egg coincubate (Peterson *et al.*, 1991). Experimentation suggests that AP binds to ZP3 α -EBGD, the primary sperm receptor of pig ZP, and specific anti-AP antibodies inhibit sperm-egg binding *in vitro* (Peterson *et al.*, 1991). However, it has not been shown directly that AP binds to ZP3. Studies to determine whether AP binds only to unfertilized eggs or can trigger the acrosome reaction must still be performed.

Proacrosin, the zymogen form of the sperm-specific serine protease acrosin, has also been identified as a ZP-binding protein (Jones and Brown, 1987; Brown and Jones, 1987). Its presence has been detected not only in pig, but in various species that include bull, guinea pig, goat, ram, rabbit, and humans (Polakoski and Parrish, 1977; Brown and

Harrison, 1978; Green, 1978; Hardy *et al.*, 1989; Meizel and Mukerji, 1976; Tobbias and Schumacher, 1977). Some active acrosin has been found on the acrosome of intact sperm (Tesarik *et al.*, 1988, 1990), but mainly it is localized in the acrosome and is exposed following the acrosome reaction (Richardson *et al.*, 1991). During the acrosome reaction, some acrosin is released but a large portion remains bound to the sperm membrane (Richardson *et al.*, 1991). Therefore, it is most likely involved in the secondary, instead of the primary, ZP binding event. In any case, inhibitory studies using anti-acrosin monoclonal antibodies demonstrate acrosin's involvement in *in vitro* fertilization (De Ioannes *et al.*, 1990). Acrosin has been shown to bind to the ZP with a specificity for fucose (Topfer-Petersen and Henschen, 1987, 1988). Proacrosin/acrosin binds to the ZP of a variety of different species (Hardy and Garbers, 1994). Therefore, it must function in conjunction with other molecules that confer species-specificity as well as primary zona binding. Although it remains controversial, studies have shown that acrosin may also participate in the penetration of the zona (Urch *et al.*, 1985; Elce *et al.*, 1986).

Sp38, named after its apparent molecular size by SDS-PAGE analysis, has similar properties to proacrosin: it is found only in the testis (Mori *et al.*, 1995), ZP binding is prevented by the same inhibitors (Mori *et al.*, 1993), and it is exposed on the sperm head surface following the acrosome reaction. More specifically, it was shown that Sp38 was localized to the anterior, intraacrosomal region of the fixed sperm head prior to its release following the acrosome reaction (Mori *et al.*, 1995). It binds in a Ca^{2+} -dependent manner to the 90 kDa glycoprotein of pig zona pellucida (Mori *et al.*, 1993), pZP1, which has been suggested to be involved in the secondary sperm binding event (McLeskey *et al.*,

1998). It is still uncertain whether or not Sp38 binds to the sulfated sugar residues of the ZP protein. Interestingly, in ZP binding studies, it was shown that the binding of Sp38 to the zona pellucida was inhibited by the addition of proacrosin or a synthetic Sp38 peptide that is similar to a region of proacrosin (Mori *et al.*, 1995). Consequently, the precise role of Sp38 *in vivo*, and as it relates to proacrosin, is not yet clear. Since the amount of Sp38 increases over the course of sperm maturation in the testes (Mori *et al.*, 1995), its case as an important sperm protein is strengthened. Proacrosin has a high affinity for the ZP, but it is lost upon its processing to acrosin; whereas, Sp38 constantly binds to the ZP but at a lower affinity. Therefore, the current hypothesis (Mori *et al.*, 1995) is that a tight ZP-binding of acrosome-reacted sperm occurs first via proacrosin. Following proacrosin activation, a loose binding mediated by Sp38 replaces it. Sp38 is also an attractive molecule for assisting in the penetration of sperm through the zona pellucida. However, many more studies are required to confirm either theory.

Sperm zonadhesin is a promising primary binding protein. It is a 150 kDa protein under nonreducing conditions that dissociates to 105 and 45 kDa subunits after disulfide bond reduction (Hardy and Garbers, 1994). It was isolated as a ZP-binding protein from porcine membranes based on its ability to bind to an affinity matrix of porcine ZP. Not only does it bind to porcine ZP, it binds in a species-specific manner (Hardy and Garbers, 1994). The protein sequence contains a potential ZP-binding site: a stretch of repetitive sequences rich in proline and threonine that are characteristic of mucins, which regulate cell adhesion (Hardy and Garbers, 1995). The cDNA for the protein has been cloned and revealed that the protein is an integral membrane protein. Also, it contains a domain

similar to pre-pro-von Willebrand factor (Hardy and Garbers, 1995), which binds to sulfogalactosylceramide (SGC) (Roberts *et al.*, 1986) and heparin, the latter being a promoter of the acrosome reaction *in vitro* (Parrish *et al.*, 1989). These findings suggest a possible role in signal transduction, but this has not been demonstrated (Hardy and Garbers, 1995). Northern blot screening of various pig tissues detected zonadhesin in the testis only. More specifically, protein expression was localized to haploid spermatids (Hardy and Garbers, 1995). Although zonadhesin is presently the only protein known to bind ZP in a species-specific manner (Hardy and Garbers, 1995; Snell and White; 1996), competitive inhibition studies are required to support the necessity for zonadhesin in the binding process. Lastly, studies are still required to demonstrate that zonadhesin binds only to unfertilized eggs.

1.3.3 Human zona pellucida-binding proteins

Hu9 and SP-10 are promising ZP-binding proteins in humans. Hu9, a 95 kDa protein, may be the human homolog of mouse p95/ZRK. Hu9 is also a transmembrane protein tyrosine kinase that is recognized by both an antibody to phosphotyrosine and a monoclonal antibody to an extracellular domain of mouse p95/ZRK (Burks *et al.*, 1995). Since it had been suggested that p95 may be hexokinase (Kalab *et al.*, 1994), it was shown that Hu9 is not a hexokinase. The cDNA sequence for the human 95 kDa protein was cloned and the predicted amino acid sequence contained features found in all protein tyrosine kinases (Burks *et al.*, 1995). Like mouse p95/ZRK, Hu9 kinase activity was stimulated by exposure to human ZP3 (Burks *et al.*, 1995; McLeskey *et al.*, 1998).

Expression of Hu9 was detected only in the testis. By *in situ* hybridization of a cross-section of the testis, it was localized in the seminiferous tubules, suggesting that it is specific to the germ cells (Burks *et al.*, 1995). Functional studies showed that sperm-egg binding could be blocked by the preincubation of oocytes with synthetic Hu9 peptides (Burks *et al.*, 1995). Direct localization of Hu9 on the sperm as well as a demonstration of direct species-specific binding between Hu9 and ZP3 would strengthen the evidence that Hu9 is an important sperm protein for ZP binding. Furthermore, some doubt has been raised regarding Hu9's true identity. Hu9 cDNA sequence has a very high homology to a putative human proto-oncogene, *c-mer* (Bork, 1996; Tsai and Silver, 1996), and resequencing of the Hu9 clone by another research group found differences from the reported sequence (Jay Baltz, University of Ottawa, personal communication). Therefore, the Hu9 cDNA sequence must be reconfirmed.

SP-10 is a testis-specific acrosomal protein that was first detected in humans (Herr *et al.*, 1990), but since then it has been detected in many other mammalian species. It has been suggested that SP-10 is involved in fertilization because: it was detected in over 200 mature sperm samples and, thus, may be a conserved protein (Herr *et al.*, 1990); SP-10 is expressed in the testis, and not in somatic tissues (Anderson *et al.*, 1987; Freerman *et al.*, 1994); a monoclonal antibody specific to the protein is also non-reactive to somatic tissue antigens and inhibits fertilization based on the hamster egg penetration assay (Anderson *et al.*, 1987); preliminary unpublished data showed reduced fertility rates in female baboons receiving an SP-10 vaccine; and SP-10 was detected on the human sperm head before and after the acrosome reaction (Herr *et al.*, 1990; Foster *et*

al., 1994). The presence of SP-10 on the sperm before and after the acrosome reaction may indicate that it is involved in a later stage of ZP binding (Coonrod *et al.*, 1996).

The evidence for SP-10's role in ZP interactions is still weak and its function is vague. To substantiate its role as an important ZP-binding protein, further experimentation is required to determine what SP-10 is interacting with on the oocyte. Such studies could demonstrate direct binding to ZP2. SP-10's interaction with the ZP is not known to be species-specific or specific to unfertilized oocytes.

1.3.4 Guinea pig zona pellucida-binding proteins

One of the first proteins characterized was PH-20, a 64 kDa protein. The gene is conserved among mammals (mouse, rat, hamster, rabbit, bovine, monkey, and human genomic DNA) (Lathrop *et al.*, 1990), as is expected for a functionally important protein. Furthermore, female guinea pigs immunized with PH-20 showed a complete block of *in vivo* fertility (Primakoff *et al.*, 1988). PH-20 is expressed only in the testis (Primakoff *et al.*, 1988; Lin *et al.*, 1994) and is found on the surface of both acrosome-intact and acrosome-reacted sperm (Cowan *et al.*, 1986). Interestingly, this protein has a hyaluronidase domain that has been shown to be involved in sperm penetration of the cumulus cells surrounding the oocyte (Lin *et al.*, 1994), and a soluble hyaluronidase is found in the acrosome that is structurally related to PH-20 (Hunnicuttt *et al.*, 1996). Therefore, on acrosome-intact sperm, PH-20 may function to help sperm reach the ZP.

On acrosome-reacted sperm, PH-20 is involved in maintaining ZP binding. The amount of exposed PH-20 on the sperm head following the acrosome reaction is

increased and it has been implicated in the maintenance of secondary ZP-binding (Cowan *et al.*, 1986; Primakoff *et al.*, 1985; Thaler and Cardullo, 1995). A monoclonal antibody against PH-20 strongly inhibits ZP binding (Primakoff *et al.*, 1985). After the acrosome reaction, PH-20 is present on the sperm head, but it has relocated to the inner acrosomal membrane via a Ca^{2+} -dependent mechanism (Myles and Primakoff, 1984; Cowan *et al.*, 1991). Its new location is consistent with its role in secondary binding. Studies have shown that this ZP binding activity and the hyaluronidase activity are performed by different regions of the protein (Hunnicuttt *et al.*, 1993, 1996).

Although species-specific binding has not been demonstrated, analysis of the carboxy terminal of the PH-20 gene sequence showed that it is highly divergent between species (Ramaraao *et al.*, 1994). Therefore, specificity may result from differences in this region. However, it is unknown whether this region affects the binding domains. In addition, studies showing direct PH-20 binding to the ZP and studies demonstrating selective binding to unfertilized eggs are still required.

1.3.5 Hamster zona pellucida-binding proteins

A promising glycoprotein in hamsters is P26h. This protein is so-named based on its apparent size by SDS-PAGE: ~26 kDa. It is a promising zona binding protein because: it is localized over the acrosome on the live and fixed sperm head (Bérubé and Sullivan, 1994); it shows high affinity for the ZP glycoproteins (Sullivan and Bleau, 1985); it is not expressed in other hamster tissues such as brain, heart, lung, spleen, liver and kidney (Bérubé and Sullivan, 1994); it is synthesized by the caput epididymis and

added to the sperm surface during epididymal maturation (Sullivan and Robitaille, 1989; Robitaille *et al.*, 1991), the developmental stage when sperm acquire the ability to bind to the zona pellucida (Cooper, 1986); anti-P26h antibodies inhibit sperm-ZP binding *in vitro* (Bérubé and Sullivan, 1994); and, male hamsters immunized against P26h are infertile due to the production of specific anti-P26h antibodies (Bérubé and Sullivan, 1994). A P26h homolog has been found in human sperm, P34H, that has demonstrated similar results in the localization and antibody inhibition studies (Boué *et al.*, 1994).

It was found that hamster ZP proteins bound to P26h, but could not bind to sperm proteins from ram, bull, and stallion sperm (Sullivan and Bleau, 1985). From this, the authors concluded that P26h binding is species-specific. However, further proof of species specificity (i.e., demonstrating that P26h does not bind to ram, bull, and stallion ZP proteins) would make that argument stronger. To strengthen the evidence that P26h is a true ZP receptor, demonstration that P26h does not bind to the ZP of fertilized eggs should also be shown. It is not known whether the protein is involved in signal transduction leading to the acrosome reaction.

1.3.6 Rabbit zona-binding proteins

Finally, a number of rabbit sperm proteins have been characterized. Rabbit sperm autoantigen (RSA) and rabbit testis galactosyl receptor (RbTGr) have been the most studied. RSA denotes a family of 3-5 low molecular weight (14-18 kDa) glycoproteins found on rabbit sperm. RSA was identified as a ZP-binding protein by their location on the sperm surface and by the inhibition of ZP binding caused by specific polyclonal and

monoclonal antibodies against it. Before the acrosome reaction, RSA is located in various regions of the sperm head, but afterwards it is specifically located in the anterior postacrosomal region (O'Rand and Fisher, 1987). The fact that the protein relocates to the region of secondary binding suggests that RSA, like PH-20, may assist in maintaining sperm-ZP binding following the acrosome reaction. Treatment of sperm with specific antibodies to RSA resulted in decreased sperm binding to the zona and a complete block of sperm penetration *in vitro* and *in vivo* (O'Rand, 1981; O'Rand *et al.*, 1984). Conversely, *in vivo* capacitated sperm treated with solubilized zonae were unable to bind anti-RSA (O'Rand, 1981). In the presence of RSA, the binding of capacitated sperm to intact ZP was inhibited (O'Rand *et al.*, 1988). RSA binding to the ZP was shown directly to validate the inhibition results. Finally, it was revealed that RSA binds to various sulfated carbohydrates (O'Rand *et al.*, 1988) and, therefore, may interact with the sugar residues of the zona proteins. The species-specificity of the interaction and a RSA specificity for non-fertilized eggs has not yet been demonstrated.

The mRNA sequence and expression of Sp17, the 17 kDa member of the RSA family, has been determined (Richardson *et al.*, 1994). Sp17 mRNA is found in rabbit, mouse and human testes, but not in any of the somatic tissues tested. Its deduced amino acid sequence featured relevant similarities to other protein domains: the N-terminal of human testis cAMP-dependent protein kinase and the key amino acids for a C-type lectin galactose binding domain.

Rabbit testis galactosyl receptor (RbTGr), a galactose-binding protein, consists of two major proteins that are 54 and 49 kDa in size (Abdullah *et al.*, 1991). It is

immunologically related to the asialoglycoprotein receptor of the rat liver, but performs a different function since RbTGr binds to solubilized rabbit zonae whereas the liver receptor does not (Abdullah *et al.*, 1991). Studies in the rat system has shown that it is expressed on Sertoli, spermatogenic and mature sperm cells (Abdullah and Kierszenbaum, 1989). In mature rat and human sperm, it is located on the membrane surface overlying the acrosome region and it contains a Ca^{2+} -dependent carbohydrate-binding site (Abdullah and Kierszenbaum, 1989; Goluboff *et al.*, 1995).

The evidence to support RbTGr's role in ZP binding remains incomplete. With the availability of RbTGr-specific antibodies, sperm-ZP binding inhibition experiments should be performed to support the current evidence. In addition, similarly to RSA, the species-specificity of the interaction and a specificity for unfertilized eggs must still be demonstrated.

1.4 Sulfogalactosylglycerolipid (SGG)

SGG, also known as seminolipid, is a sulfated glycolipid that is found in mammalian germ cells (6-8% of total testis lipids) and, to a lesser extent, in the brain (0.2% of total lipids) (Murray *et al.*, 1980; Farooqui, 1981; Ishizuka and Yamakawa, 1985). The specific functions of SGG are unknown. Like its structural analog, sulfogalactosylceramide (SGC), SGG may be involved in cell-cell interactions. It has been shown to bind to selectin (Suzuki *et al.*, 1993), a molecule involved in the adhesion of white blood cells, and preliminary results have also implicated SGG in the sperm-ZP binding event (Kamolvarin *et al.*, 1996; White *et al.*, in preparation). Pretreatment of

sperm with anti-SGG antibodies significantly reduced ZP binding in a dose-dependent manner (Kamolvarin *et al.*, 1996).

Sulfated molecules, especially sugar residues, have been shown to be important in sperm-ZP binding (Jones, 1991). Therefore, the sulfate group of SGG may be the active region of the lipid. This is supported by the observation that pretreatment of sperm with partially purified seminal plasma arylsulfatase-A (AS-A), which desulfates SGG, significantly reduces sperm-ZP binding. The sperm's ZP binding ability approached normal levels if heat-inactivated AS-A was used in place of AS-A (White *et al.*, in preparation).

Given its negatively charged sulfate group, SGG is able to bind cations such as Ca^{2+} (Tupper *et al.*, 1994). In a model system, when Ca^{2+} binds to membrane-bound SGG, the membrane fluidity is increased, which may be required for the acrosome reaction (Tupper *et al.*, 1994). In addition, the increase in membrane fluidity may play a role in ZP receptor movement on the sperm head following ZP binding.

During spermatogenesis, SGG is first observed in zygotene spermatocytes (Lingwood, 1985) and remains metabolically stable for the remainder of spermatogenesis (Kornblatt, 1979), sperm maturation and during *in vitro* capacitation (Lingwood, 1986; Tanphaichitr *et al.*, 1990). Desulfation of sperm SGG (~15%) occurs only when sperm become immotile or senescent (Tanphaichitr *et al.*, 1990). Interestingly, abnormalities in SGG synthesis can result in sterility (Lingwood *et al.*, 1985). On mature rat and mouse sperm, it has been immunolocalized to the sperm head. In the mouse, SGG is found all over the sperm head and on the midpiece of caput epididymal sperm, whereas caudal

epididymal sperm display SGG on the postacrosomal region, concave and convex ridges of the sperm head, and on the midpiece (Lingwood, 1986; Tanphaichitr *et al.*, 1993; White *et al.*, in preparation).

SGG has a specific binding protein that is also present in similar areas of the sperm head, named sulfoglycolipid immobilizing protein 1 (SLIP1). The binding has been illustrated *in vitro* (Lingwood, 1985; Tanphaichitr *et al.*, 1998), but the kinetics of the SLIP1-SGG interaction has not yet been determined. Sperm SGG is also involved in sperm-egg plasma membrane binding. Studies have shown sperm-egg plasma membrane binding decreased in a dose dependent manner when sperm were pretreated with anti-SGG antibodies (Ahnonkitpanit *et al.*, submitted). The relative amounts of sperm SGG and egg SLIP1 have not been ascertained, but quantitation of sperm SGG and sperm SLIP1 indicated that the molar ratio of SGG to SLIP1 is approximately 70,000:1 (Gadella *et al.*, 1992; Tanphaichitr *et al.*, 1993). The ability of exogenous SLIP1 to bind to the cell surface at the same location as SGG supports that SGG may be present in excess of SLIP1 (Lingwood and Nutikka, 1991; Moase *et al.*, 1997). In addition to binding to ZP proteins, a current hypothesis is that the excess SGG may assist the movement of SLIP1 in the sperm plasma membrane by providing free binding sites across the sperm surface (Moase *et al.*, 1997). This hypothesis must still be proven experimentally.

1.5 SLIP1 (P68/62)

Immunoblotting studies revealed that, like SGG, SLIP1 is found only in mammalian germ cells and, to a lesser extent, in the brain (Law *et al.*, 1988; Murray *et al.*,

1980; Ishizuka and Yamakawa, 1985). The 68-kDa sperm surface glycoprotein is most likely a peripheral membrane protein because it can be extracted from the sperm head with a low salt solution (Law *et al.*, 1988). However, this extract amounts to an estimated 50% of the total testicular germ cell SLIP1. Since detergent is required for complete solubilization, a proportion of SLIP1 may be anchored in the plasma membrane. No molecular weight difference was detected between the differentially-extracted forms (Law *et al.*, 1988). When the testes of various species were screened with antiSLIP1, SLIP1 was detected in the rat, mouse, hamster, guinea pig, rabbit, dog, pig, bull, rooster, frog, and fish (Law *et al.*, 1988). The presence of SLIP1 across such a wide range of species suggests that SLIP1 may be evolutionally conserved and, therefore, functionally important.

Immunofluorescence studies using cross sections of the rat testis tissue revealed the presence of SLIP1 next to the basal membrane. Therefore, it is likely that SLIP1 is present in spermatogenic cells of an early stage (Lingwood, 1986). In fixed caudal epididymal rat sperm, indirect immunofluorescence studies show that SLIP1 is concentrated at the convex ridge (Lingwood, 1986). In mature live mouse sperm, immunofluorescence studies as well as protein A-immunogold electron microscopic studies on live mouse sperm have confirmed SLIP1's presence on the sperm plasma membrane (Tanphaichitr *et al.*, 1993; Moase *et al.*, 1997). On live acrosome intact mouse sperm, direct immunofluorescence indicated that SLIP1 is present in the postacrosomal region of the plasma membrane of the sperm head. However, in indirect immunofluorescence studies, it can be translocated to the convex ridge by

immunoaggregation caused by the bivalent antibodies used (Tanphaichitr, 1993; Moase *et al.*, 1997). As mentioned previously, the initial sperm head site for ZP-binding may be the convex ridge or the postacrosomal region with final movement to the convex ridge (see Section 1.1). Based on the latter hypothesis, the results of the SLIP1 immunofluorescence localization studies are consistent with those postulated for a ZP-binding molecule.

SLIP1 has been shown to bind to the ZP (Tanphaichitr *et al.*, 1993) and, therefore, may be involved in the initial event of sperm-egg binding, but not as a “true receptor” (Snell and White, 1996). A true receptor would bind only to the ZP before the egg is fertilized. Analyses of the SLIP1-ZP interaction in fertilized mouse eggs indicate that ZP modifications that occur after fertilization do not inhibit the binding of SLIP1. SLIP1 binding to the ZP can still occur at various stages of early mouse embryogenesis (i.e., 2-cell to morulae stage) and minimally at the blastocyst stage (Tanphaichitr *et al.*, 1998). Furthermore, it has been suggested that a true ZP receptor induces the acrosome reaction upon immunoaggregation (Leyton and Saling, 1989). The aggregation of SLIP1 on the sperm head by exposure of sperm to antiSLIP1 IgG did not induce the acrosome reaction (Moase *et al.*, 1997). In addition, it is unlikely that SLIP1 is involved directly in cell signalling because it is a peripheral membrane protein. SLIP1 may act simply as an adhesive molecule prior to, or in cooperation with, other receptors on the sperm surface that bind to the zona pellucida.

Also consistent for a candidate ZP-binding molecule is the fact that sperm-egg binding is inhibited by the presence of exogenous SLIP1 or pre-exposure of sperm to

antiSLIP1 antibodies. When sperm and eggs were co-incubated *in vitro* in the presence of low concentrations (0.4 nM) of exogenous rat testis SLIP1, binding was significantly decreased (Tanphaichitr *et al.*, 1993). A significant decrease was also observed when the eggs were pre-incubated with SLIP1 before exposure to the sperm. When sperm were pretreated with affinity purified antiSLIP1 IgG, binding of sperm to the ZP was inhibited. Direct binding of SLIP1 to the ZP of whole intact eggs as well as to isolated ZP was confirmed (Tanphaichitr *et al.*, 1993).

The interaction of SLIP1 with the ZP was also shown *in vivo*. Sperm retrieved from the cauda epididymis or vas deferens were treated with antiSLIP1 IgG or preimmune serum and then used to artificially inseminate superovulated female mice. AntiSLIP1 IgG-treated sperm showed a 25% fertilization rate as compared to 80% in mice inseminated with preimmune serum IgG (Tanphaichitr *et al.*, 1992).

Interestingly, SLIP1 is also present on the periphery of zona-free eggs (Ahnonkitpanit *et al.*, submitted). In all cases, SLIP1 was localized over all areas of the egg except for the region above the chromosomes. In addition, It has been shown to play a role in sperm-egg plasma membrane binding, but not fusion (Ahnonkitpanit *et al.*, submitted). Since the region above the chromosomes is where the second polar body is extruded at fertilization, and sperm do not bind in this region, the lack of SLIP1 there may be to prevent incorrect sperm binding.

In light of recent findings, SLIP1 may actually be a combination of proteins and various isolation methods may yield different components. SLIP1 was originally isolated from a rat testis homogenate by its ability to bind specifically to an SGG affinity column

(Lingwood, 1985). A simpler and more efficient method was developed whereby the protein was initially extracted from rat testicular cells using only a low salt buffered solution of ATP, EDTA, and sucrose (AES solution) (Law *et al.*, 1988). A second step to the AES extraction was added to isolate purer SLIP1 (Lingwood and Nutikka, 1991). This step involved separating SLIP1 from the AES protein extract on a DEAE cellulose column with a salt gradient. The major SLIP1 fractions were pooled and applied to a Cibachron blue column where pure SLIP1 was eluted using a buffered ATP solution. This SLIP1, or SLIP1 isolated by SGG affinity column chromatography, still maintained its ability to bind to SGG (Boulanger *et al.*, 1995).

Albumin sequence was obtained on two separate occasions when SLIP1 was purified by AES extraction followed by DEAE cellulose chromatography (Lingwood, personal communication; Moase and Tanphaichitr, personal communication).

Lingwood's recent results suggest that a component of SLIP1 may be testis heat shock protein 70 (tHsp70) (Boulanger *et al.*, 1995). Studies from our lab indicate that there is a unique 68 kDa SLIP1 protein and a 62 kDa antigenically-related SLIP1 protein, P68/62, that has SGG and ZP-binding activities. P68/62 was isolated by AES extraction followed by chromatofocusing (Tanphaichitr *et al.*, 1998). Notably, antiSLIP1 still cross-reacted with P68/62, but P68/62 has been shown to be different from albumin and tHsp70 (see below). Therefore, "SLIP1" may be composed of at least these three proteins.

It has been shown that all three antiSLIP1 cross-reactive proteins (albumin, tHsp70, and P68/62) are distinguishable from each other based on various characteristics. These characteristics are: 1) only albumin cross reacts with anti- albumin antibodies; 2)

only albumin and tHsp70 cross-react with anti- Hsp74.5 (an antibody raised against recombinant *M. hyopneumoniae* Hsp); 3) P68/62 and tHsp70 are located on the sperm head and bind ATP, unlike albumin; 4) when added exogenously to gamete coincubates, tHsp70 inhibited sperm-ZP binding by up to 50% vs. 75% by P68, and the concentration needed for this maximum inhibition was 10 μ M vs. 10 nM, respectively; 5) albumin does not inhibit sperm-egg binding; and, 6) tryptic peptide sequences of P68 are not homologous to the other proteins. The component of interest to the current study was P68/62, as defined by the method of isolation, because it is this component of SLIP1 that contains a ZP- and SGG-binding activity. Therefore, I will refer to "P68/62" based on this definition and distinguish it from "SLIP1", which contains additional non-ZP binding components .

1.6 Hypothetical role of sperm P68/62

It has been shown that sperm P68/62 is involved in sperm-ZP binding both *in vitro* and *in vivo* (Tanphaichitr *et al.*, 1993, 1992). But, it would be expected that P68/62 co-operates with other sperm proteins that confer species-specificity and binding specificity to unfertilized eggs, as well as with an initiator of the acrosome reaction following ZP binding. The role of P68/62 may be similar to that of the rabbit sp17 and RbTGr proteins (Richardson *et al.*, 1994; Abdullah *et al.*, 1991) in that they are lectin-like molecules that assist in maintaining sperm-ZP binding. Similarly, P68/62 may function like selectin, an adhesive molecule involved in the lymphocyte homing event (Laskey, 1992; Butcher and Picker, 1996). Briefly, in the initial stages of this process, adhesive

contact between white blood cells and the endothelial surface is mediated by selectins. Once this primary adhesion has occurred, tight binding and the initiation of a signal transduction cascade follows. Interestingly, selectin, like P68/62, binds SGG *in vitro* (Suzuki *et al.*, 1993).

It has been suggested that SGG's role may be to provide a lawn on which P68/62 can migrate from its initial to final location (Moase *et al.*, 1997). Consistent with this hypothesis is the fact that the fluidity of SGG is increased when it interacts with Ca^{2+} (Tupper *et al.*, 1994). As Ca^{2+} is present on the sperm surface (Ruknudin, 1989) and in the female reproductive tract for various pre-fertilization sperm activities (Fraser, 1987; Yanagimachi, 1994; Cummins and Yanagimachi, 1986; Saling *et al.*, 1978), it may also assist in increasing sperm membrane fluidity for P68/62 relocalization. P68/62 may require relocalization as a result of ZP binding (see Discussion). The mechanism of P68/62's interaction with SGG still must be elucidated.

1.7 Research objectives

Since P68/62 has been implicated in sperm-ZP binding, uncovering its ZP binding domains and the specificity of its ZP binding is of interest before investigating P68/62's interactions with other sperm surface molecules. To this end, the immediate goal of this research project was to isolate the P68/62 cDNA for functional studies. Since no P68/62, or homolog, DNA sequences are known, they must be deduced from amino acid sequencing results of the purified protein. However, P68/62 is present in very low amounts on the sperm head (0.0003 pg/sperm; Tanphaichitr *et al.*, 1993) and although the

current method used in the lab to purify P68/62 is the most effective, the yield of purified protein is low. Therefore, a major obstacle is purifying large quantities of P68/62 for amino acid sequencing.

Our P68/62 purification method involves the chromatofocusing of a boar sperm protein extract. Boar ejaculate was used because boars provide a very large amount of sperm per ejaculate (a ~ 200 ml sperm-rich fraction from 1-2 boars). Ejaculate was used instead of testis tissue because sperm are the only cells present in the ejaculate and the fluid component is composed of seminal plasma, which can be separated easily from sperm by centrifugation. This method also reduces the levels of albumin, otherwise found in blood serum in the testis. Finally, since we are interested in determining the functional role of P68/62, P68/62 that is extracted from sperm may be in its most functionally mature form.

Protein sequencing had been attempted for SLIP1 purified from an SGG affinity column, but it was unsuccessful. It is believed that the reason for the failure was that SLIP1 is most likely N-terminal blocked (Lingwood, personal communication). Therefore, it was questionable that N-terminal P68/62 sequence could be obtained. Since internal cDNA sequences are preferable for cloning work anyways, the P68/62 preparation would be digested with trypsin and selected peptides would be sequenced. Sequences of low degeneracy (if possible) would be used to synthesize oligonucleotides for cDNA cloning of P68/62.

Protein sequencing and cDNA cloning of P68/62 had been attempted previously in our lab (Moase and Tanphaichitr, unpublished results). P68/62 was purified by

chromatofocusing of a protein extract (Moase *et al.*, 1997) and tryptic peptide sequences were obtained (in collaboration with the NRC, Ottawa), one with a significant homology to albumin. Based on these sequences, only highly degenerate nucleotide probes could be synthesized and early P68/62 cloning efforts by cDNA library screening were not successful.

Originally, the goal of this research project was to assist in this cDNA cloning of P68/62 using a PCR technique instead of library screening. Early results were also unsuccessful. Because the amino acid sequencing of purified P68/62 (on which all the cloning work was based) had been performed only once, it was decided that the focus of this thesis work should be redirected towards purifying more P68/62 and to repeat the protein sequencing. It was hoped that the new sequences may be less degenerate, more specific to P68/62, and provide more cloning tools to facilitate the cloning work.

The long-term research goals of the lab are to use the P68/62 cDNA to determine the functional binding domain(s) of P68/62, whether it's specific sugar residues or region(s) of the protein. More precisely, the cDNA will be used to produce recombinant P68/62 proteins whereby different regions of the protein have been deleted. The effect of such deletions on ZP binding would be assessed in order to locate the key binding domains for future study and the development of an immunocontraceptive.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Biological samples

Boar semen was kindly collected for us by Mr. Roger Barden, Centre for Food and Animal Research, Agriculture and Agri-food Canada, Ottawa, Ontario, as described previously (Fiser *et al.*, 1991). Pig testis tissue was collected from freshly slaughtered animals (Nepean, Ontario). Mouse spleen total RNA was generously donated by Dr. Peter Walker, Loeb Research Institute, University of Ottawa, Ottawa, Ontario. Human lung genomic DNA was obtained from Dr. Douglas Gray, University of Ottawa, Ottawa, Ontario. Human skin keratins were extracted from human toe skin cells as follows: a 0.5 cm² sample of skin from the side of a human toe was pressed in Laemmli's sample buffer for SDS-PAGE (Laemmli, 1970), heated at 95°C for 10 minutes, and then stored at -20°C between use.

Protein extraction, SDS-PAGE and Immunoblotting Reagents

All reagents required for P68/62 extraction from boar sperm were purchased from Sigma Chemical Company. SDS-PAGE electrophoresis-grade reagents were purchased from Bio-Rad Laboratories (Bio-Rad Laboratories, Inc., Richmond, CA). SDS-PAGE prestained and unstained low molecular weight protein standards were also purchased from BioRad Laboratories. The RSA protein standard was purchased from Cappel (Organon Teknika Inc., Scarborough, Ontario).

For the Chaudhuri and Green silver stain method (1987), acetic acid, glutaraldehyde, sodium carbonate, silver nitrate, formalin, ammonium hydroxide and glycerol were purchased from Sigma Chemical Company; urea was purchased from Bio-Rad; and sodium hydroxide and methanol were purchased from Fisher (Ottawa, Ontario). Kodak rapid fix, solution A and B, (Eastman Kodak Company, Rochester, New York) was used to destain the silver stained gels.

Protein electroblotting buffer was prepared using electrophoresis-grade Tris-HCl and Glycine (Bio-Rad Laboratories) or CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid) buffer from Sigma Chemical Company. Methanol was purchased from Fisher.

The antibodies used for the immunoblotting studies were obtained from different sources. The antiSLIP1-CL antibody, a rabbit polyclonal antibody directed against rat testis SLIP1 purified by SGG affinity column chromatography (Lingwood, 1985), was generously provided by Dr. Cliff Lingwood, Department of Microbiology, Hospital for Sick Children, University of Toronto, Toronto, Ontario. The antiserum was formylated, to reduce nonspecific ionic binding, as described previously (Lingwood, 1985). Briefly, the serum is diluted 1:1 in phosphate-buffered saline (PBS, 137 mM NaCl, 8 mM NaH_2PO_4 , 2.7 mM KCl, 1.5 mM KH_2PO_4 , pH 7.4) and 37% formaldehyde is added to a final concentration of 1%. The mixture is incubated at 4°C for 30 minutes and then dialyzed against PBS overnight.

The antiRSA antibody is a Cappel rabbit polyclonal IgG directed against rat serum albumin (RSA) (Organon Teknika Inc.). The anti-keratin antibody (Zymed Laboratories, Inc., San Francisco, CA) is a whole rabbit antiserum directed against bovine muscle

epidermal keratin with a wide-spectrum cross-reactivity to human keratins. A rabbit polyclonal antibody directed against recombinant human AS-A (produced as previously described, Stein *et al.*, 1988) was generously provided by Dr. V. Gieselmann, Christian Albrechts University, Kiel, Germany.

All the primary antibodies were diluted in 5% skim milk (Carnation Instant Skim Milk Powder, Nestlé, Don Mills, Ontario) in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6). The working dilutions for each antibody were 1:500 for antiSLIP1-CL and anti-keratin, and 1:1000 for antiRSA and antiAS-A.

The secondary antibody used for all immunoblotting was a goat anti-rabbit antibody conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL). The antibody was diluted 1:4000 in Tris-buffered saline before use.

Molecular Biology Reagents

All RNA extraction reagents were purchased from Sigma Chemical Company and dedicated for this work. Agarose and alkaline agarose gel electrophoresis reagents were of analytical grade and also purchased from Sigma Chemical Company. DNA sequencing gels were prepared using electrophoresis grade reagents from Bio-Rad Laboratories, except for boric acid and EDTA, which were purchased from Sigma Chemical Company.

The degenerate PCR primers (A1, A2, B1, B2) were based on previously obtained P68 peptide sequences (Appendix B). These primers, specialized oligonucleotide probes, the clone-specific custom-made PCR primers, as well as the custom-made DNA sequencing primers were synthesized using the Oligo 1000 DNA Synthesizer (Beckman,

Fullerton, CA) with phosphoramidites (Beckman) and inosine (Glen Research, Sterling, VA). The AE2 positive control PCR primers, which amplify a conserved coding region of the mouse anion exchanger 2 (AE2) protein, were generously donated by Dr. Jay Baltz, Loeb Research Institute, University of Ottawa, Ottawa, Ontario. The β -actin positive control PCR primers were obtained from Stratagene (La Jolla, CA). T3 and T7 promoter-based DNA sequencing primers and lambda gt11 forward and reverse DNA sequencing primers were obtained from Sigma Chemical Company.

A UNIZAP unamplified mouse testis cDNA library (Stratagene) was created by Dr. Nuch Tanphaichitr, Loeb Research Institute, University of Ottawa, Ottawa, Ontario. All reagents used for the replica plating and screening of the PCR products transformants were purchased from Sigma Chemical Company, except for the preparation of Denhardt's reagent. Denhardt's reagent was prepared using Ficoll (Pharmacia, Uppsala, Sweden) and polyvinylpyrrolidone and bovine serum albumin, fraction V (Sigma Chemical Company). Radioactive nucleotides were purchased from Amersham (Amersham Canada Ltd., Oakville, Ontario).

2.2 Total RNA extraction from boar testis

To avoid RNase activity, all solutions for RNA work were prepared using DEPC-treated Milli-Q water (Millipore Canada, Nepean, Ontario) and all glassware, spatulas, and stir bars were treated with DEPC-water and autoclaved, or baked for 4 hours at 250°C. Total RNA was extracted from boar testis tissue using a protocol based on that of Chomczynski and Sacchi (1987). This protocol was used instead of an RNA isolation kit

in order to have better control over the quality of RNA that was obtained.

Boar testes were removed from the animal immediately after slaughtering, divested of their outer capsule, and 3-5 gram pieces of tissue were individually wrapped in aluminum foil and immediately snap-frozen in liquid nitrogen. Approximately 0.3 g pieces of frozen tissue were homogenized in a cold denaturing solution (4 M guanidine isothiocyanate, 0.5% N-lauryl sarcosine, 0.025 M sodium citrate, pH 7.0, 0.78% β -mercaptoethanol) using a Brinkman Polytron (Westbury, NY). To the homogenate was added a 1:10:2 volume ratio of 2 M sodium acetate, pH 4, H₂O-saturated phenol, and chloroform/isoamyl alcohol (49:1). The homogenate was centrifuged at 500 g at 4°C for 20 minutes (Beckman TJ-21 rotor). The aqueous phase was transferred to a new tube and the RNA was precipitated overnight at -20°C using ice cold 100% isopropanol. The RNA was pelleted by centrifugation as described above and dried under vacuum. The pellet was resuspended in denaturing solution and re-precipitated in 1 volume of ice cold 100% isopropanol overnight at -20°C. The RNA solution was centrifuged as before, the pellet was washed with cold 75% ethanol, and re-centrifuged. The pellet was partially dried under vacuum and resuspended in 1 mM EDTA/10 mM Tris-HCl, pH 7.4.

The RNA was quantitated from the A₂₆₀ (Spectronic 1201, Milton Roy, Rochester, NY). The quality of the preparation was determined by the 260/280 nm absorbance reading ratio and by ethidium bromide staining of an aliquot electrophoresed in a denaturing agarose gel (Sambrook *et al.*, 1989).

2.2.1 mRNA isolation from boar testis total RNA

mRNA was isolated from the total RNA preparation using the PolyAtract mRNA Isolation System II kit (Promega, Madison, WI). Briefly, a biotinylated-oligo(dT) probe is incubated with the RNA for 30 minutes at 65°C. Then, the RNA solution is added to streptavidin-linked paramagnetic particles and incubated at room temperature for 10 minutes. The mRNA-bound paramagnetic particles are captured along one side of the tube using a magnetic tube stand and then the supernatant is removed. The particles are washed four times with 0.1x SSC (150 mM NaCl, 17 mM sodium citrate) using the magnetic tube stand to capture the particles. After the final wash, the mRNA is eluted from the particles by resuspending, and gently mixing, the particles in water. The final mRNA solution is removed following magnetic capture of the particles and quantitated using the A_{260} .

2.3 cDNA synthesis from boar testis mRNA and mouse spleen total RNA

cDNA was synthesized by reverse transcription of 4 μ g of boar testis mRNA or 20 μ g mouse spleen total RNA. All components of the reverse transcription cocktail were chilled on ice for 15 minutes then combined in the following order: 5x enzyme buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 100 mM DTT (Sigma Chemical Company), 10 mM dNTP mix (0.8 mM of each dNTP, Perkin-Elmer, Foster City, CA), 20 units RNasin (Promega), 200 units/ μ g RNA of M-MLV reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), 200 ng/ μ g RNA of random primer dN₆ (Boehringer Mannheim, Mannheim, Germany), 4 μ g mRNA or 20 μ g total RNA, and

DEPC-treated water to a final volume of 25 μ l. The cocktails were mixed and microcentrifuged briefly to collect all liquid at the bottom of the tube.

To check the efficiency of cDNA synthesis, 2.5 μ l of the above cocktails were transferred to another tube containing 10 mCi/ml 35 S dATP (Amersham). All cDNA synthesis reaction tubes were incubated at room temperature for 10 minutes and then at 37°C for 2 hours. Following this incubation, protein was removed using a modified phenol/chloroform method and the cDNA was collected (Ausubel *et al.*, 1992). Briefly, an equal volume of phenol buffered with 10 mM EDTA/10 mM Tris-HCl, pH 7.5 was added, mixed and microcentrifuged at 16 000 g for 5 minutes. The upper aqueous phase was transferred to a new tube and an equal volume of 24:1 chloroform/isoamyl alcohol was added, mixed and microcentrifuged again. cDNA was precipitated from the upper aqueous phase by the addition of a 1/10th volume of 3M sodium acetate, pH 5.5, and two volumes of 100% ethanol. The solution was mixed and incubated at -20°C overnight. The pelleted cDNA was air dried briefly before being resuspended in 20 μ l Milli-Q water.

The radiolabelled cDNA products were electrophoresed at 20 V for 5.5 hours on a 1.2% alkaline agarose gel (Sambrook *et al.*, 1989) following heating at 65°C for 10 minutes. The gel was then soaked in 7% TCA at room temperature for 30 minutes with shaking. The gel was dried under vacuum (BioRad Laboratories) and exposed to film (X-OMAT, Eastman Kodak Company) for 2 hours. On the autoradiograph, the efficiency of the reverse transcription reaction was assessed by the identification of strong signals for a variety of different sized cDNA species.

2.4 Genomic DNA isolation from boar testis

Genomic DNA was isolated from boar testis using the Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. As for the extraction of total RNA, the boar testes were removed from the animal immediately after slaughtering, divested of their outer capsule, and 3-5 gram pieces of tissue were individually wrapped in aluminum foil and immediately snap-frozen in liquid nitrogen. From these pieces of tissue, 10-20 mg pieces of testis tissue were used as starting material for DNA extraction. As suggested by the manufacturer, Proteinase K (60 μ g, Boehringer Mannheim) was used during the initial cell lysis to degrade any protein and maximize the DNA yield.

2.5a Polymerase Chain Reaction (PCR) of cDNA prepared from boar testis mRNA

All materials were dedicated for PCR use to avoid human DNA contamination. The pipets, pipet tips, Eppendorf tubes, autoclaved Milli-Q water, 10x Taq DNA polymerase buffer (Perkin-Elmer), 25 mM $MgCl_2$, 1.25 mM each dNTP mix (Perkin-Elmer), and overlay wax or mineral oil (Sigma Chemical Company) were UV-treated for 35 min before use to induce thymine dimers in any contaminating DNA that may have been present. The generation of thymine dimers would make the amplification of contaminating DNA more difficult during the subsequent polymerase chain reaction.

A 100 μ l reaction cocktail containing a final concentration of 1x polymerase buffer, 2.5 mM $MgCl_2$, 1.25 mM each dNTP mix, 45 pmol of each primer, 10 μ l of the 20 μ l total of cDNA synthesis products, and Milli-Q water was pre-mixed on ice and

overlaid with wax or mineral oil. The cocktail was "hot started" for 15 minutes at 95°C (PTC-100 Thermal Cycler, MJ Research, Inc., Watertown, MA). Then, 2.5 U of Taq polymerase (Perkin-Elmer) was added and the following PCR program was initiated: 40 cycles of 1.5 minutes at 94°C (denaturation step), 2.0 minutes at 37°C (primer annealing step), and 3.0 minutes at 72°C (DNA extension step). The program concluded with a final DNA extension step for 10 minutes at 72°C, followed by an indefinite temperature hold at 4°C.

2.5b Touchdown-Polymerase Chain Reaction (TD-PCR) of cDNA prepared from boar testis mRNA or from a UNIZAP unamplified mouse testis cDNA library

The reaction mix was prepared as for the standard PCR described in 2.4a except the total reaction volume was decreased to 50 μ l and 3 μ l of the 20 μ l cDNA synthesis reaction or 3 μ l of the cDNA library (1.4×10^6 pfu/ml) was used. The PCR program was modified such that there was a total of 37 cycles in which the denaturation and extension steps were the same as above, but the annealing temperature began at 49°C and was decreased by 3°C every other cycle until it reached 37°C, at which time this annealing temperature was maintained for another 30 cycles. The program also concluded with a 10 minute final extension step at 72°C followed by an indefinite temperature hold at 4°C.

2.5c PCR amplification of a testis TD-PCR clone, clone A, in human lung and boar testis genomic DNA

In a 100 μ l reaction, 0.75 μ g of either human lung genomic DNA or boar testis genomic DNA were pre-mixed on ice with a final concentration of 1x polymerase buffer, 2.5 mM MgCl₂, 1.25 mM each dNTP mix, 50 pmol of each clone A custom-made primer (see Appendix C), and Milli-Q water. The cocktail was overlaid with mineral oil and “hot started” for 10 minutes at 95°C. Then, 2.5 U of Taq polymerase (Perkin-Elmer) was added and the following PCR program was initiated: 30 cycles of 94°C for 1.5 minutes, 51°C for 1 minute, and 72°C for 2 minutes. After 30 cycles, a final DNA extension step was carried out at 72°C for 10 minutes followed by an indefinite temperature hold at 4°C.

2.6 PCR product analysis

The PCR products were electrophoresed at 100 V for 3-3.5 hours on a 1% or 2% agarose gel containing 0.004% ethidium bromide to visualize the bands. Only products from the TD-PCRs were subcloned for further analysis. Major separable bands were cut from the gel, eluted from the gel slice by microcentrifugation through sterile glass wool, and subcloned into PCR II vector according to the manufacturer’s protocol (TA Cloning Kit, Invitrogen, San Diego, CA). Competent cells (OneShot cells, Invitrogen) were transformed with the ligation reaction mix according to the manufacturer’s instructions and plated on LB agar medium containing X-Gal (40 mg/ml) and ampicillin (50 μ g/ml) for overnight incubation at 37°C. White colonies were selected for culture in LB medium

containing ampicillin (50 $\mu\text{g/ml}$) and then plasmid DNA was isolated using a Wizard miniprep kit (Promega).

Many remaining TD-PCR products were not easily separable from each other by gel electrophoresis. Therefore, another technique was used to subclone these TD-PCR products. They were randomly ligated into the PCRII vector by using a 1 μl aliquot of the PCR reaction mix in the ligation reaction. Competent cells were transformed with the ligation mix and then grown on LB/ampicillin agar plates as described above.

The clones needed to be screened quickly for those containing PCR products with one of each primer (A1 or A2) at each end. Therefore, the colonies were replica plated onto Nytran filters (Schleicher & Schuell, Keene, NH) (Sambrook, *et al.*, 1989). The cells on the filters were lysed using a solution of 5x SSC, 0.5% SDS, and 1 mM EDTA (pH 8.0) at 50°C for 45 minutes. Then, the filters were prehybridized with BLOTTO (Johnson *et al.*, 1984) + 6x SSC at 40°C for 2.5 hours. The A1 and A2 PCR primers (40 pmol each) were end-labelled with ^{32}P -dATP using T4 polynucleotide kinase (Pharmacia). For each pair of duplicate filters, one filter was incubated overnight (~ 19 hours) with the A1 primer probe and the other filter was incubated overnight with the A2 primer probe. The filters were washed four times in 2x SSC/ 1% SDS for 5 minutes at room temperature, then in 1x SSC/ 1% SDS for 40 minutes at 50°C and exposed to film. The autoradiographs of duplicate filters were compared and only the colonies that showed positive hybridization with both probes were selected for plasmid insert analysis. Such colonies were cultured in LB/ampicillin and plasmid DNA was isolated as described above.

The approximate sizes of the plasmid inserts of all clones were determined by restriction enzyme digestion with EcoRI (Pharmacia) followed by electrophoresis on a 1.2% agarose gel containing ethidium bromide at 70V for 1.5 hours. DNA sequencing of plasmid inserts was performed by PCR using the Cyclist DNA Sequencing Kit (Stratagene, La Jolla, CA). Sequencing grade plasmids were prepared using the Qiagen midiprep kit (Qiagen, Santa Clarita, CA). The inserts were sequenced from the ends towards the middle using M13 universal primers or T3 and T7 primers. Primers for sequencing internal regions of the inserts were designed using the Oligo Primer Analysis Software, version 4.0 (National Biosciences Inc., Plymouth, MN). Some sequencing was also performed by the Ontario Regional Cancer Centre, University of Ottawa, Ottawa, Ontario. Database homology searches for all sequencing results were accomplished using the NCBI BLAST service.

2.7 Extraction of P68/62 from boar sperm

P68/62 was extracted from boar sperm based on a previous report (Tanphaichitr *et al.*, 1998). The sperm rich fraction of fresh boar ejaculate (~200 ml from 1-2 boars) was centrifuged at 500 g for 25 minutes at 37°C to remove the seminal plasma. The pelleted sperm were washed twice in PBS, pH 7.4, at 37°C by gentle resuspension and centrifugation. Great care was taken to resuspend the sperm pellets by slow pipetting up and down in order to avoid plasma membrane damage, which could result in a release of acrosomal proteases. Then, a crude extract of P68/62 was prepared by resuspending the sperm pellet in 9 volumes of AES extraction solution (1 mM ATP, 1 mM EDTA, 0.34 M

sucrose, 20 mM Tris-HCl, pH 7.6, and 0.2 mM N- ϵ -p-tosyl-L-lysine chloromethylketone hydrochloride (TLCK)) at 4°C for 1 hour with constant rocking. The extract was centrifuged (GPR Centrifuge) at 500 g for 25 minutes at 4°C to pellet the sperm. The supernatant was removed and ultracentrifuged using the L8-70M Beckman ultracentrifuge and Ti60 rotor (100 000 g, 1 hour, 4°C). Finally, aliquots (25 ml) of the supernatant were dialysed extensively against 4 litres of Milli-Q water for ~ 26 hours with 2-3 water changes, and then lyophilized to dryness (Labconco, Kansas City, MO).

2.8 AES extract protein quantitation

The amount of protein in the boar sperm AES extract solution was quantitated for chromatofocusing using the Bio-Rad Protein Microassay (Bio-Rad Laboratories). Several dilutions (0, 2, 5, 10, 15 mg/ml) of bovine serum albumin (BSA) (Sigma Chemical Company) in Milli-Q water were prepared in duplicate, based on an A_{280} of 0.67 for a 1 mg/ml solution. Duplicate AES extract samples were diluted 1:9 in Milli-Q water for protein quantitation. All protein concentrations were quantitated according to the assay protocol and the AES extract concentration was determined based on the BSA standards results.

2.9 P68/62 purification by chromatofocusing

At first, P68/62 was purified from the boar sperm AES extract using a chromatofocusing protocol as reported previously (Tanphaichitr *et al.*, 1998). Previous attempts to purify SLIP1 had resulted in the co-purification of albumin (Lingwood,

personal communication; Tanphaichitr, unpublished results). Therefore, the chromatofocusing conditions below were optimized to maximize the recovery of P68/62 as well as to separate P68/62 from albumin. Briefly, approximately 225 μg of lyophilized AES extract, quantitated using the Bio-Rad Protein Microassay, was reconstituted in 1.5 ml of Milli-Q water and chromatofocused in a Bio-Rad column (20 x 1.1 cm) (Bio-Rad Laboratories) containing Polybuffer Exchanger 94 resin (Sigma Chemical Company) pre-equilibrated with 0.025 M imidazole-HCl, pH 7.4 (Sigma Chemical Company). Proteins were eluted from the column with 225 ml of Polybuffer 74 (Sigma Chemical Company), pH 4.0, followed by 50 ml of 1 M NaCl, and finally 50 ml of 1 N HCl. Fractions (4 ml) were collected at a rate of 0.2 ml/min.

Since the majority of P68/62 was not eluted by the Polybuffer wash (a decrease in pH from 7.4 to approximately 4.5), its pI was estimated to be less than 4.5. Therefore, to expedite P68/62 elution, the chromatofocusing procedure was modified such that the column resin was initially equilibrated to pH 5 using 0.025 M piperazine (PIPES) buffer. However, albumin's pI is approximately 4.7-5.0 (Lehninger, 1975; Bundschuh et al., 1992) and so it still may be eluted with P68/62. To safeguard against this, a shallow chromatofocusing pH gradient from 5 to 4 was used. This increased the separation resolution of P68/62 from albumin (and other proteins) by allowing more time for all the albumin to be eluted in the PIPES buffer. To increase the loading capacity, the Bio-Rad column size was increased to 1.5 x 30 cm. This change made it possible to load twice as much AES extract per run. Finally, to reduce the time required from 2 days to 4 hours per run, fast pressure liquid chromatography (FPLC) was adopted.

For FPLC, the lyophilized AES extract was reconstituted in 2 ml of Polybuffer 74 for chromatofocusing (LKB-Pump P-500, LKB Controller LCC-500 Plus, Pharmacia) in a Pharmacia column (1.9 x 26 cm) containing the Polybuffer Exchanger 94 resin pre-equilibrated with 0.025 M piperazine, pH 5. To ensure uniform column packing, a 3 mg solution of cytochrome c (Sigma Chemical Co.) in Milli-Q water was loaded and followed as it passed through the resin. The AES extract was chromatofocused only if the cytochrome c band (yellow) had migrated through the column as a tight band. The fraction size during the chromatofocusing run was reduced from 4 to 2 ml to improve P68/62 resolution even more. Fractions were collected at a rate of 1 ml/min.

Fraction profiles of the pH and A₂₈₀ readings were compiled for each chromatofocusing run. Fractions were assessed for P68/62 purity by SDS-PAGE and immunoblotting with antiSLIP1-CL, antiRSA, and, in some cases, anti-keratin antibodies. An aliquot (200 μ l) of selected protein fractions was desalted in MeOH, CHCl₃, and Milli-Q water as described previously (Wessel and Flügge, 1984). The protein samples were suspended in Laemmli's Sample Buffer for SDS-PAGE (Laemmli, 1970) followed by silver staining or immunoblotting (see below). The quantity of P68/62 on the silver-stained SDS-PAGE gels was estimated by densitometric comparison to co-electrophoresed RSA of various known amounts. Fractions of different degrees of P68/62 purity were pooled, dialysed extensively against Milli-Q water (2 days with five to six 4-litre water changes per day), and lyophilized to dryness.

2.10 SDS-PAGE, protein gel silver staining, and immunoblotting

Proteins were electrophoresed on duplicate 0.75 mm-thick 10% or 12% polyacrylamide gels (Laemmli, 1970) using the MiniProtean II minigel apparatus (Bio-Rad Laboratories). One gel was silver stained (using the Bio-Rad Silver Stain kit and following the manufacturer's instructions, or following the protocol of Chaudhuri and Green, 1987) and the other was electroblotted to nitrocellulose (Towbin and Gordon, 1984). Briefly, the proteins were electroblotted to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.) in transfer buffer composed of 192 mM glycine, 20% methanol, and 25 mM Tris-HCl, using Bio-Rad's MiniProtean II apparatus (Bio-Rad Laboratories, Inc.). The transfer was carried out at 250 mA for 30 minutes at approximately 4°C.

Before antibody detection, nonspecific binding sites on the nitrocellulose were blocked with 5% skim milk powder (Carnation) in TBS for 1 hour at room temperature or overnight at 4°C. The blots were incubated for 1 hour at room temperature with the primary antibody, diluted in the same blocking medium. The blot was washed of excess antibody by three quick and vigorous washes in TBS followed by 2 x 10 minute washes under less vigorous agitation. Next, the blots were exposed to the horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature, followed by washes as described above. Antigen-antibody binding was detected by chemiluminescence using the ECL (Enhanced Chemiluminescence) detection kit (Amersham Canada Ltd., Oakville, Ontario).

Special modifications for the final attempt at P68 microsequencing

To improve the solubilization of the final lyophilized P68/62 pools in a small

volume of Laemmli's SDS-PAGE sample buffer, a special procedure was developed. To slowly rehydrate the lyophilized protein sample, 7 μ l of Milli-Q water was added and it was stored at 4°C overnight. Then, aliquots (10 μ l) of 10 M urea, to a final concentration of 4.7 M, were added to ensure complete solubilization. Next, 4x Laemmli's SDS-PAGE sample buffer, pH 6.8 was added such that the final concentration would be 1x. The protein solution was acidic, so to correct the pH, 1 μ l of 1.5 M Tris-HCl, pH 8.8 was added. Finally, this solution was sonicated for 3 minutes in a sonicating water bath (Branson Ultrasonic Cleaner, Branson, Connecticut), heated at 95°C for 3 minutes, microfuged briefly to collect the liquid at the bottom of the tube, and electrophoresed immediately.

P68/62 chromatofocusing fractions and the final P68/62 pools for sequencing were electrophoresed in 12% polyacrylamide SDS gels. The polyacrylamide gels were made with piperazine di-acrylamide (PDA) (Bio-Rad Laboratories) instead of methylene-bis-acrylamide (bis) to increase protein resolution and to reduce background silver staining. Electrophoresis was carried out at 35 mA per gel for 1.5 hours. SDS-PAGE was performed such that the migration of the P68 band was consistent. This was achieved by pouring all gels with a consistent proportion of stacking and resolving regions (~ 1.5 cm and 4.5 cm high, respectively) and carrying out the electrophoresis until the prestained albumin protein standard reached a premarked location on the apparatus (i.e., 2.2 cm through the resolving gel).

Following SDS-PAGE, P68/62 for sequencing was electroblotted to Problott PVDF (polyvinylidene difluoride) membrane (ABI, Inc., Foster City, CA). The protein

was blotted in CAPS buffer (10 mM CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid), 10% methanol) at 125 mA for 1.5 hours at room temperature. The blot was rinsed briefly in Milli-Q water followed by 100% methanol. Protein bands were visualized by Amido Black staining (Bio-Rad Laboratories) for 2 minutes (instead of the manufacturer's suggestion of 1 minute) and destained with Milli-Q water. The extra staining time made it possible to clearly visualize the protein band without a comparable increase in background staining. The P68 band was excised from the membrane with a new scalpel blade.

To determine the correct position of the P68 band, a small portion of one side of the lane was excised and immunoblotted with antiSLIP1, antiRSA, and anti-keratin antibodies. The banding pattern for each immunoblotting experiment was compared to the banding pattern on the remaining Amido Black-stained blot. In this way, the location of only the antiSLIP1 cross-reactive P68 band was singled out.

The gel, after transfer, was silver stained (Chaudhuri and Green, 1987) to determine the amount of protein remaining in the gel. The use of PDA in the preparation of the gels, reduced background gel staining and the silver stain development period could be extended to detect very low protein levels.

2.11 Peptide microsequencing of P68

The protein digestion and microsequencing work was performed by John Leszyk at the Worcester Foundation Microsequencing Facility (Worcester Foundation for Biomedical Research, Shrewsbury, MA). The blotted P68 was digested *in situ* with

trypsin. Peptides were selected for sequencing based on the amount of peptide present (according to the peak areas of an HPLC chromatogram) and the length of the peptides (based on the mass spectrometry data).

2.12 Special keratin contamination precautions

To decrease contamination from skin and hair keratins during the P68/62 preparation, special precautions were taken during protein purification, fraction analysis, and preparations for sequencing. A cap, gloves, and a smock were worn at all times. Glassware, stir bars, spatulas, and all electrophoresis equipment (where possible) were soaked overnight in RBS-35 (Pierce), followed by a rinse with Milli-Q water and then methanol. Where this regimen could not be followed, the equipment was cleaned with methanol. Sterile, individually packaged pipettes (Fisher, Nepean, ON) and unopened packages of single-use items were used. New, high grade reagents were used to prepare all solutions. Solutions were filtered through a 0.45 μm filter (Nalgene, Rochester, NY) where possible.

Before using the FPLC equipment for chromatofocusing, the loading sample loop was replaced with a new one and the system was flushed with 8 M urea to solubilize any keratin, followed by a flushing with 1 M HCl and Milli-Q water. As handling of the dialysis tubing was shown to increase the amount of keratin present, the tubing was cured and used with the least amount of handling. To prevent contamination by airborne particles, the curing and storage of the tubing was carried out in covered containers. New gloves were worn when it had to be handled and the openings were not touched. Before

use, all solutions, FPLC fractions, final P68/62 preparations, dialysis tubing, and urea rinses of all the equipment were tested for the presence of keratin by silver stained SDS-PAGE and parallel immunoblotting using an anti-keratin antibody.

CHAPTER 3

RESULTS

3.1 RNA Extraction from Boar Testis

Total RNA was extracted from frozen boar testis tissue (approximately 0.9 g each time) according to a modified protocol based on that of Chomczynski and Sacchi (1987). From the A_{260} reading, the RNA yield was estimated to be 1.85 mg, based on an A_{260} of 1 for a 40 $\mu\text{g/ml}$ RNA solution. This preparation had an $A_{260/280}$ of 1.8, indicating that the preparation was of acceptable purity. Ratios of 1.9 to 2.0 indicate highly purified preparations of RNA (Ausubel *et al.* 1992). The presence of contaminating protein would lower the ratio, since proteins have a peak absorption at 280 nm. To confirm the quality of the preparation and to ensure that the RNA was not degraded, 2 lanes of 10 μg of the preparation was electrophoresed in a 1.2% denaturing agarose gel (Fig. 1). The visualization of the 28S and 18S rRNA bands, 4.4 kb and 2 kb, respectively, are used to estimate the sizes and amounts of the remaining RNA species. The ethidium bromide-stained RNA appeared in the gel lanes as a long smear extending above and below clear rRNA bands. The 28S band was approximately twice as intense as the 18S band, indicating the RNA was intact. No low molecular weight spots of RNA were visible, indicating an absence of degraded RNA.

3.1.1 mRNA Isolation from Boar Testis Total RNA

From 2.4 mg of boar testis total RNA, 82 μg of mRNA was isolated (i.e., 3.4 % of

Fig. 1- RNA Extraction from Boar Testis

RNA was extracted from approximately 0.9 g of frozen tissue using guanidine isothiocyanate. Shown is a 1.2% denaturing agarose gel of the extracted RNA stained with ethidium bromide. Each lane contains 10 μ g of RNA. The 28 S and 18S rRNA bands are indicated by arrowheads.

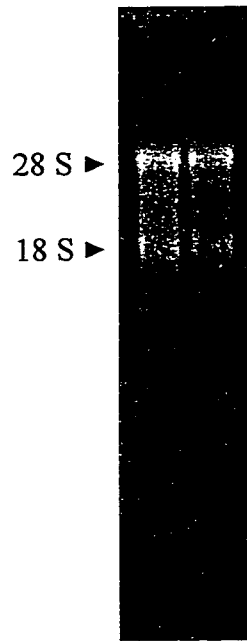


Figure 1

the total RNA). Since mRNA is known to represent approximately 3-5% of the total RNA (Alberts et al., 1989; Stryer, 1988), it was likely that most mRNA species were present in this preparation. Therefore, it was used as the template RNA for cDNA synthesis.

3.2 cDNA Synthesis by Reverse Transcription of PolyA(+) RNA

cDNA was synthesized from boar testis mRNA using random primers. A control preparation of total RNA from mouse spleen (containing 28S and 18S rRNA species for size comparisons) was also used in a separate reaction. Mouse spleen RNA was selected as the negative control RNA because SLIP1 had not been detected previously in this tissue (Lingwood, personal communication). Therefore, presumably, P68/62 should not be amplified in the subsequent PCR using the cDNA. In order to visualize the products by autoradiography, ³⁵S dATP was added to a separate aliquot (2.5 μ l) of each reaction mixture to radioactively label the cDNA. Shown in Figure 2 is the autoradiograph of a 1.2 % alkaline agarose gel of the radiolabelled cDNA products from boar testis mRNA and mouse spleen total RNA (control). Clearly visible in the mouse spleen cDNA synthesis products were the bands resulting from the 28S and 18S rRNA species. For both sample lanes, the long smear of cDNA products extended above and below the rRNA marker bands, indicating that various cDNA species of different sizes were obtained.

Fig. 2- Reverse Transcription of PolyA(+) RNA

³⁵S-labelled cDNA from reverse transcription of boar testis mRNA (lane 1) and from mouse spleen total RNA (lane 2) was electrophoresed in a 1.2% alkaline agarose gel and subjected to autoradiography. The cDNA bands resulting from the 28S and 18S rRNA species in the mouse spleen total RNA are indicated by the arrowheads.

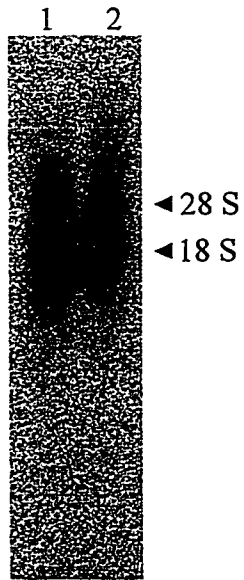


Figure 2

3.3 P68/62 cDNA Cloning by the Polymerase Chain Reaction (PCR)

Various techniques and materials were used in an attempt to isolate the P68/62 coding sequence. After several trials using different PCR conditions, the first working strategy (see section 2.5a) employed a standard 40 cycle PCR program with cDNA from boar testis mRNA as the template. cDNA synthesized from mouse spleen total RNA was used as a negative PCR control since P68/62 is not expressed in the rat spleen (Law *et al.*, 1988). It should be noted, however, that this strategy was presumptive because these expression studies were performed at the protein level and nothing is known about the presence or absence of RNA transcripts in the spleen. Four different primer pair combinations were used, based on previously obtained SLIP1 peptide sequences (see Appendix B). Three of these primer combinations yielded PCR products that were visualized on an ethidium bromide-stained 2% agarose gel (Fig. 3). However, a comparison of the electrophoretic migration of the boar testis products and the mouse spleen products showed that none of the former products' sizes were different from the mouse negative control products. Therefore, it was ventured that the boar testis PCR products were, in fact, due to non-specific primer amplification.

To improve the likelihood of specific primer amplification, "touch-down PCR" (Don *et al.*, 1991) was initiated. Using this method, primer annealing is allowed under the most stringent annealing temperatures first, followed by a gradual decrease in stringency over subsequent cycles. In this manner, the correctly primed sequence gets an amplification "head start" (and is, therefore, more abundant) over mis-primed amplification products. The best results were obtained when cDNA was synthesized from

Fig. 3- RT-PCR Products from Boar Testis mRNA

A 100 μ l reaction cocktail was prepared containing either cDNA synthesis products from boar testis mRNA or mouse spleen total RNA (negative control). Shown is an ethidium bromide-stained 2% agarose gel of the PCR products. Lanes: 1-4, 6- PCR with testis cDNA template; 7-11- PCR with mouse spleen cDNA template; 1,8- A1+A2 primers; 2,9- A1+B2 primers; 3,10- B1+A2 primers; 4,11-B1+B2 primers; 5- PCR kit positive control; 6,7-AE2 primers.

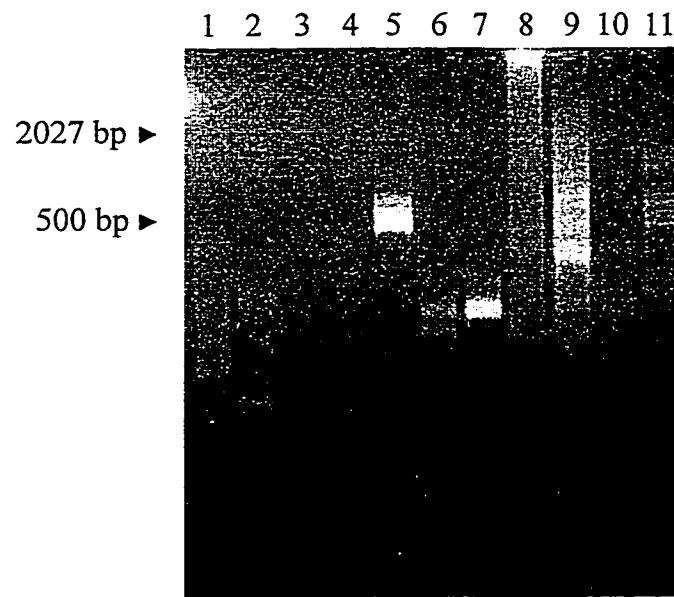


Figure 3

4 μg of poly A(+) RNA and used for the TD-PCR with 45 pmol of each primer, 2.5 mM MgCl_2 , 2.5 U Taq polymerase, and 200 μM of each dNTP. However, this PCR resulted in many amplification products, as visualized by ethidium bromide-stained 2% agarose gel electrophoresis of the products (Fig. 4). The TD-PCR products ranged in size from 75 to 2000 bp. It was still possible that some of the PCR products resulted from false priming, since the primer annealing temperature was decreased significantly. None of the bands could be discounted since the expected product size was unknown. Therefore, as many of the products as possible had to be sequenced.

Some PCR products were clearly separable from the rest. One such band (Fig. 5) was excised directly from the gel, eluted from the gel slice, then subcloned in a plasmid vector for sequencing. Digestion of the plasmid with EcoRI (to release the insert) confirmed that PCR product A had been successfully ligated into the vector. Sequencing of the PCR product revealed that nearly the full length of the antisense strand (i.e., the opposite strand to the strand containing the PCR primer peptide sequences) had 82% and 88% nucleotide identity to two adjacent regions of human cleavage signal 1 (HCS1) protein, and 73% and 83% amino acid identity (78% and 92% similar), respectively. The sense strand didn't have any strong homologies and contained many stop codons (see Appendices C, D, and E). Therefore, TD-PCR clone A is presumably the boar homolog of HCS1 protein.

To confirm that clone A was not a result of human DNA contamination in the TD-PCR tube, a PCR was performed using genomic DNA from boar testis and human lung in separate reaction tubes and primers were designed to hybridize to two regions of the boar

Fig. 4- Touchdown-PCR of Boar Testis cDNA

The cDNA synthesized from boar testis mRNA and mouse spleen total RNA (negative control) was used as the template DNA in touchdown PCR. Shown are ethidium bromide stained 2% agarose gels of the PCR products. The size of some bands of the DNA molecular weight markers are indicated in panel A. In panel B, the 500 bp PCR kit positive control product is indicated by the arrowhead. **Panel A:** The template DNA used is indicated above the lanes. Lanes: 1- lambda DNA, HindIII fragments; 2- primers A1+A2; 3- primers A1+B2; 4- primers B1+A2; 5- primers B1+B2; 6- PCR kit positive control (lambda DNA with primers that amplify a 500 bp product); 7- primers A1+A2; 8- primers A1+B2; 9- primers B1+A2; 10- primers B1+B2. **Panel B:** Positive control PCR reactions. Lanes: 11- PCR kit positive control; 12, 13- boar testis cDNA and primers that amplify a ~200 bp region of a membrane anion exchanger cDNA (AE2 primers).

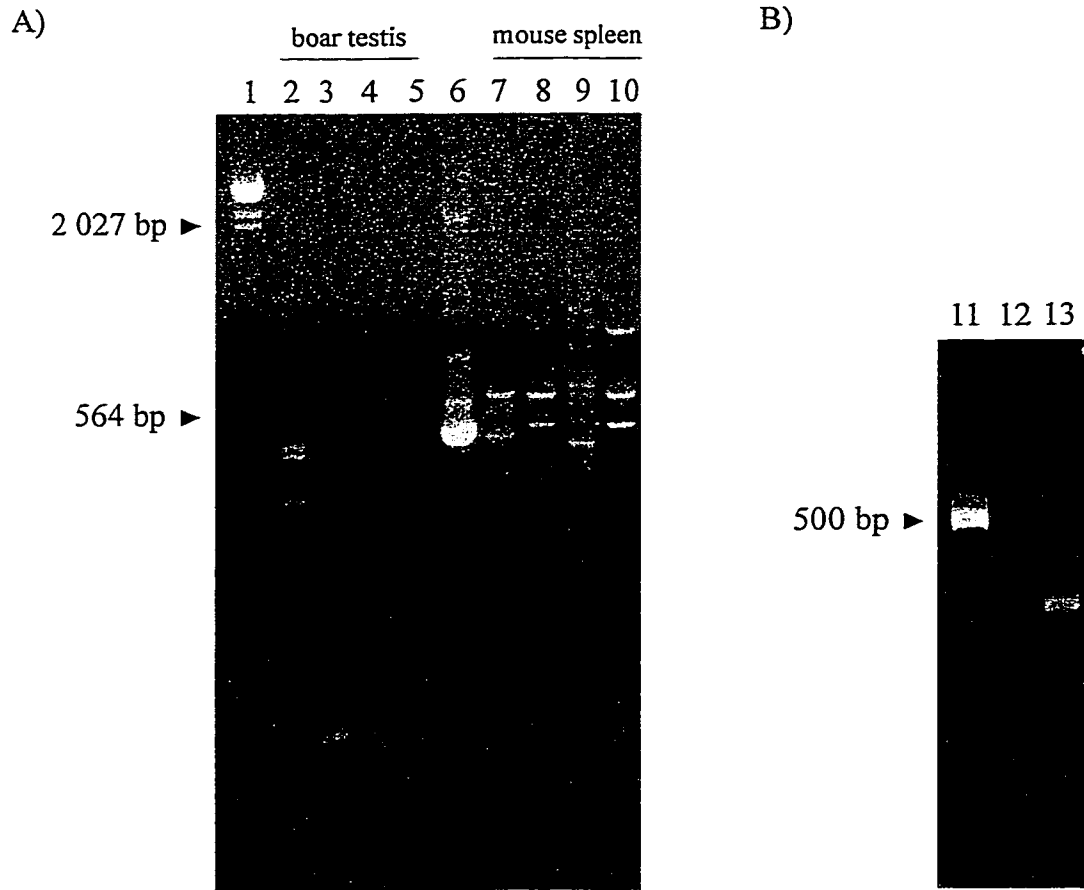


Figure 4

Fig. 5- Isolation of Touchdown-PCR Products from Boar Testis cDNA

The PCR products from touchdown-PCR of boar testis cDNA (see fig.4) were originally electrophoresed in a 2% agarose gel (Panel A) and stained with ethidium bromide for analysis. To isolate a unique product, band A, the products were re-electrophoresed in a 1% low melting agarose gel and stained with ethidium bromide (Panel B). The position and size of some DNA molecular weight markers, as well as the position of band A and the 500 bp PCR positive control product, are indicated. Lanes: Panel A: 1- TD-PCR products using both primers (A1+A2), 2-TD-PCR products using only primer A1, 3- TD-PCR products using only primer A2, 4- 500 bp PCR kit positive control Panel B: 1- 1 kb DNA ladder size standards, 2- TD-PCR products using both primers.

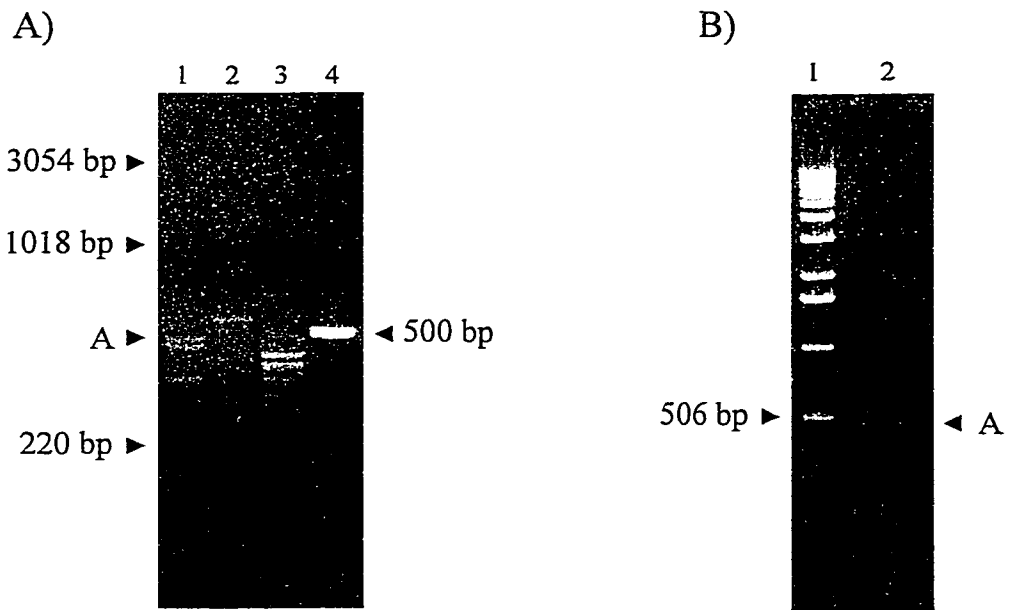


Figure 5

clone A DNA sequence that were the most unlike the HCS1 sequence (Genbank accession number M61199). In this way, it was hoped that the only amplicon would be from the boar testis DNA sample. However, the most unique regions between the two sequences still had 65 and 76% homology, so some human DNA amplification could occur. These regions were from bases 69 to 94 of clone A (bases 480 to 505 of HCS1) and 175 to 199 (bases 586 to 610 of HCS1), respectively. If the boar clone A was a real amplicon and not a result of human DNA contamination, the boar testis PCR product should match the boar clone A sequence and not the HCS1 sequence. However, it should be noted that the difference between the boar and human DNA sequences over the 130 bp amplified region amounted to 7 base changes, excluding the primer sequences.

A 1% ethidium bromide-stained agarose gel of the PCR products (Fig. 6) showed that a strong band of the expected size (130 bp), product Y, was produced using the boar testis DNA. A similar sized human DNA band was also visible, product Z, but in very low amounts. Also, there was a small amount of a slightly larger boar testis DNA product (~250 bp), product X.

From the stained gel, all the PCR products were excised, eluted, and subcloned. DNA sequencing results of both the human lung and boar testis PCR products revealed that the full DNA sequences of products Y and Z were identical to clone A (Fig. 7). The internal DNA sequence for product Z should have matched the HCS1 sequence, so sequencing of the human DNA product was repeated and, again, the sequence was identical to that of clone A. Therefore, since no difference was detected between the boar and human PCR products, no definitive conclusion can be drawn regarding the possibility

Fig. 6- PCR of Boar Testis and Human Lung Genomic DNA

Genomic DNA isolated from boar testis and human lung was used as template DNA for a PCR using boar testis clone A-based primer sequences. The ethidium bromide-stained 1% low melting agarose gel of the PCR products is shown. Lanes: 1- 1 kb DNA ladder, 2- human lung genomic DNA PCR products, 3- boar testis genomic DNA PCR products, 4- Hind III digested lambda DNA size marker. Arrow heads indicate PCR products Z and Y of the expected size, and an extra boar testis product, X, of slightly larger larger size. All products were excised directly from the gel for DNA sequencing.

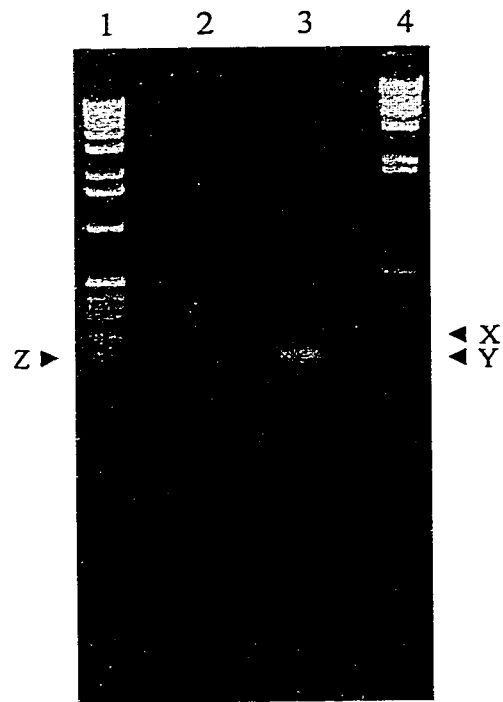


Figure 6

Figure 7- Comparison of the Nucleotide Sequences of Products Obtained from Clone A-Directed PCR

The double underlined sequences are the sequences for the PCR primers used, A-1 and A-2. The sequences of PCR products Y and Z (Y,Z) matched the clone A sequence (A) identically between the region of the two primers. PCR product X (X) was larger in size due to the binding of the 3' end primer to the underlined sequence instead of to the 3' double underlined sequence. The entire sequence of product X matched clone A exactly.

A:			5' <u>CC</u>	<u>CATCTGATAC</u>
X,Y,Z:			CC	CATCTGATAC
A:	<u>CAAGCAAGAA</u>	<u>AGTATTCCGA</u>	GCATCGGTGG	CCCTAACACC
X,Y,Z:	CAAGCAAGAA	AGTATTCCGA	GCATCGGTGG	CCCTAACACC
A:	AACTGCTCCA	TCTAGAACAG	GCTCTGTGCA	GACACCTCCA
X,Y,Z:	AACTGCTCCA	TCTAGAACAG	GCTCTGTGCA	GACACCTCCA
A:	GAAGTAGAAA	<u>GTTCTGAGGA</u>	<u>AGTTGGAGCA</u>	<u>GTTGAGGAGG</u>
X,Y,Z:	GAAGTAGAAA	GTTCTGAGGA	AGTTGGAGCA	GTTGAGGAGG
A:	CCTTAGAGGC	TGTAGGACCT	AAATCTGAAG	TGGAGAAAGA
X:	CCTTAGAGGC	TGTAGGACCT	AAATCTGAAG	TGGAGAAAGA
A:	GCATGGAAAA	ATCTCATTAT	TGCCAGCTGC	<u>TGAGGAAGTA</u>
X:	GCATGGAAAA	ATCTCATTAT	TGCCAGCTGC	TGAGGAAGTT
A:	<u>CATAAAAATG</u>	<u>TGGGGCAGGA</u>	3'	
X:	GGAGCAGTTG	AGGAG		

Figure 7

that clone A resulted from human DNA contamination. Sequencing results of product X revealed that its larger size was a result of incorrect annealing of one of the PCR primers outside of its intended binding site. Interestingly, the complete sequence still matched that of boar clone A, strengthening the case that the clone A sequence is from boar DNA.

As for the remaining TD-PCR products, an aliquot of the reaction mixture was used for random ligation of the products into a plasmid cloning vector. The clones were screened for those containing PCR products with one of each PCR primer sequence at each end, indicating correct primer annealing. Thirty-four positive clones were identified (Fig. 8). EcoRI digestion of the plasmid vectors to release the inserts revealed that actually fifteen of these “positive” clones did not contain an insert. They were discarded and the remaining clones were grouped based on similar insert size and a representative clone was sequenced. The sizes of the subcloned products ranged from approximately 200 bp to 1200 bp. These sizes coincided with the size range seen on the ethidium bromide-stained gel of the original TD-PCR products. Four clones were sequenced to completion. Another eight clones were partially sequenced (~ 150-200 bases from each end) and seven clones have not been sequenced. It is possible that some of these unsequenced clones are the same as those that have been sequenced, since they are of similar sizes.

Most of the sequenced products did not contain an open reading frame in the PCR primers' reading frame. The number of stop codons varied from 1 to 10. Resequencing of the clones with only a few stop codons confirmed the presence of the stop codons and, therefore, did not result in the generation of an open reading frame (Table 1).

Fig. 8- Identification of Clones Containing PCR Products with Both Primer Sequences

Transformant colonies were grown and replica plated onto nitrocellulose filters. The filters were probed successively with each of the PCR primers used and exposed to film. Positive clones were identified by matching primer hybridization signals on the duplicate filters. Furthermore, these clones also had to hybridize to the other primer probe on the duplicate filters. Top panels: Film was exposed to the filters for 45 hours. Lower panels: Film was exposed to the filters for 5 days. A1: PCR primer A1 probe, A2: PCR primer A2 probe. Note: the filter signals are mirror images of each other due to the replica plating technique. Arrowheads demonstrate a positive clone for both screens.

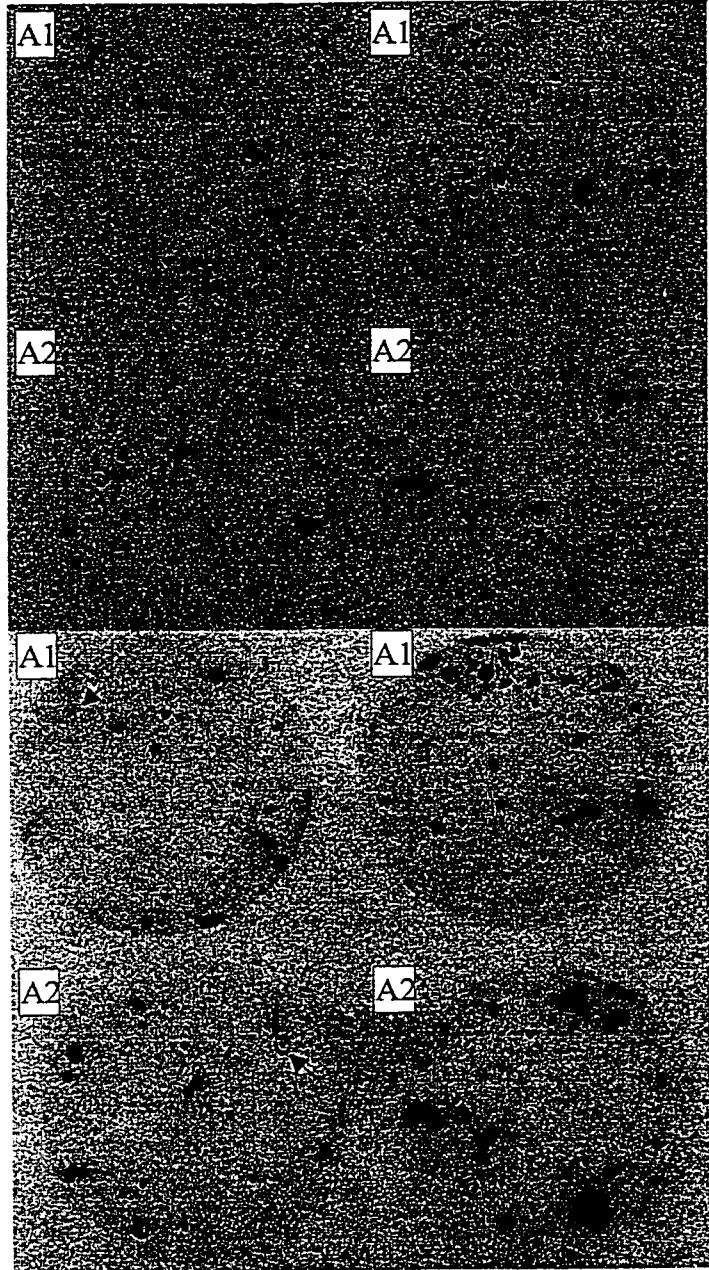


Figure 8

TD-PCR amplification of the SLIP1 cDNA sequence was also attempted using an aliquot of an unamplified mouse testis cDNA library instead of the boar testis cDNA. Five PCR products were obtained, as revealed by agarose gel electrophoresis (Fig. 9). They ranged in size from 130 to 305 bp (Table 1). The product bands were clearly separable from each other, so they were excized directly from the gel for subcloning. Results from DNA sequencing of these clones revealed that all the paired PCR primer sequences were in different reading frames. Except for clone H, all clones contained stop codons in at least one of the two primer reading frames. Nonetheless, clone H was not a valid PCR product, since the two primer sequences were in different reading frames. BLAST homology searches were performed for all clones, but of the matches found (Table 1), none were high percentage matches.

3.4 Purification of P68/62 from boar sperm

3.4.1 Extraction of P68/62 from boar sperm

Previously, SLIP1 had been extracted from various species of sperm using a low concentration of an ATP/EDTA/sucrose (AES) solution (Lingwood, 1985; Law *et al.*, 1988; Tanphaichitr *et al.*, 1993). The same solution has been effective in extracting P68/62 from boar sperm (Tanphaichitr *et al.*, 1998). The current work also used the AES solution to extract P68/62 from the sperm-rich fraction of fresh boar ejaculate. This sperm-rich fraction (200 ml) contained motile, acrosome-intact sperm at a concentration of approximately 552 million sperm per ml. From this fraction, approximately 320 μ g of

Fig 9- Touchdown-PCR Products from a UNIZAP Unamplified Mouse Testis cDNA Library

A 3 μ l aliquot of the library was used in a 50 μ l reaction volume. Shown is A) an ethidium bromide-stained 1% agarose gel of the PCR products. The primers used are indicated for each lane. B) a 1.5% stained agarose gel of the PCR products generated from the A1+A2 primer pair only. The amount loaded was 1.5 times that of lane 2. Five products (E to I) were cut out of this gel and ligated into a plasmid vector for sequencing. The sizes of some molecular weight marker bands are indicated. Lanes: 1- A1+A2 primers, 2- B1+B2 primers, 3- A1 primer only, 4- A2 primer only, 5- B1 primer only, 6- B2 primer only, 7- AE2 primers, 8- 500 bp PCR kit positive control, 9- no template DNA, A1+A2, 10- no template DNA, B1+B2, 11- 1 kb DNA ladder marker, 12- A1+A2 (same as lane 2).

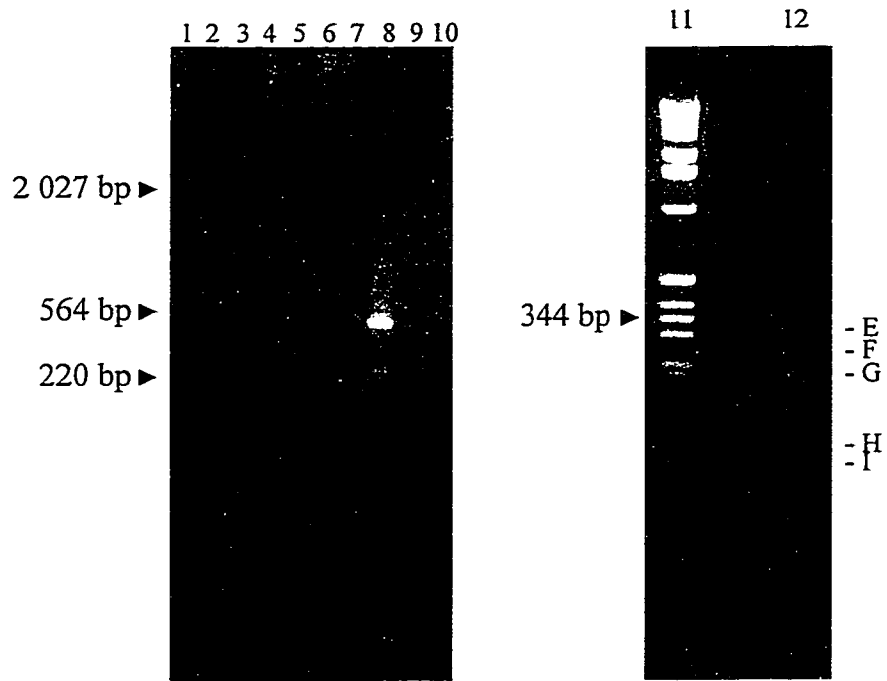


Figure 9

total protein was extracted when quantified using the Bio-Rad Protein Microassay (according to the manufacturer's instructions). Using the A_{280} reading to measure the amount of protein, the quantity would be consistently 1000-fold higher. This discrepancy was resolved later when the amount of purified P68/62 was calculated using the A_{280} reading or a densitometric comparison to rat serum albumin protein standards following silver-stained SDS-PAGE (Fig. 10). The results of the densitometric analysis were in agreement with the Bio-Rad Protein Microassay results. It is not known what was distorting the A_{280} readings.

Silver-stained SDS-PAGE of ~ 0.3 % of the protein extract showed that many other proteins had also been extracted from the sperm, making it impossible to identify the P68/62 doublet (Fig. 11). Immunoblotting with antiSLIP1 confirmed that P68/62 was present in the protein extract. However, the cross-reactive band is weak because the amount of P68/62 in the extract is low and only a small amount of the AES extract was blotted. Immunoblotting with the antiRSA antibody revealed that a great amount of albumin (also 68 kDa in size) was also present. Therefore, an additional purification step is required in order to obtain pure P68/62.

3.4.2 Chromatofocusing of the boar sperm AES protein extract

A single-step chromatofocusing technique was used to isolate P68/62 from the other proteins of the AES extract. Originally, this 2-day procedure involved collecting 4 ml fractions at a rate of 0.2 ml/minute from an anion exchange column. Protein elution occurred as a pH gradient from 7 to 4 was created on the column,

Fig. 10- Densitometric Analysis and P68/62 Quantitation from Rat Serum Albumin (RSA) Protein Standards

An aliquot of purified P68/62 and various known amounts of an RSA protein standard were electrophoresed in a 12% polyacrylamide gel and the gel was silver stained. Lanes 1-4: 2, 5, 10, and 15 ng of RSA, respectively; lane 5: 15 μ l of purified P68/62.

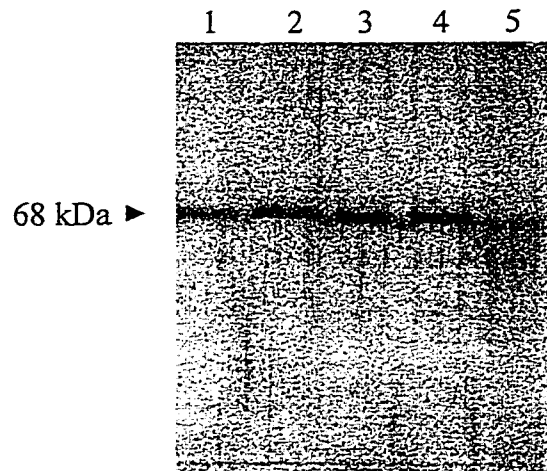


Figure 10

Fig. 11- Detection of P68/62 in the Boar Sperm AES Extract

An approximately 0.3% aliquot of the boar sperm AES extract was electrophoresed in two parallel 10% polyacrylamide gels. One gel was silver stained and the other was electroblotted to nitrocellulose membrane for immunoblotting studies. Shown is the AES extract aliquot that had been subjected to SDS-PAGE followed by silver staining (lane "extract"), as well as the results of immunoblotting with antiSLIP1 and antiRSA antibodies in the parallel sample (lane "antiSLIP1" and "antiRSA", respectively). Arrowheads indicate the relative positions of the P68/62 cross-reactive bands for the antiSLIP1 immunoblot and the RSA band of the antiRSA immunoblot.

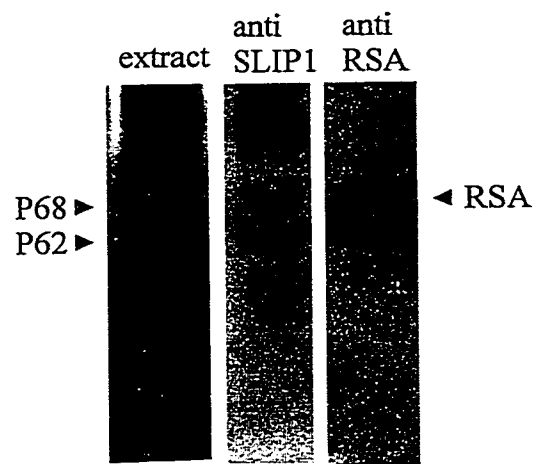


Figure 11

followed by a wash with 1 M NaCl, and finally a wash with 1 M HCl (Tanphaichitr *et al.*, 1998; see Methods).

The pH and A_{280} profiles of fractions selected from various stages throughout the run remained relatively constant from one run to the next. P68/62 was consistently eluted in the 1 M NaCl and 1 M HCl washes as a 68 and 62 kDa doublet (Tanphaichitr *et al.*, 1998). The 68 and 62 kDa proteins were usually present in equal amounts, as determined by silver-stained SDS-PAGE, but antiSLIP1 immunoblotting resulted in a stronger P68 signal. Immunoblotting with antiSLIP1 and antiRSA of selected fractions from various elution points showed that the salt and acid fractions did indeed contain pure P68/62 and no, or very little, albumin.

As mentioned for the AES extract protein measurements, the A_{280} readings were consistent from one run to the next, but they still overestimated the amount of protein present in the fractions. Therefore, the amount of pure P68/62 was estimated roughly by densitometric analysis of silver stained gels containing P68/62 fractions and known amounts of a rat serum albumin protein standard. A linear density gradient was observed for 2 to 20 ng of the stained albumin standard. Therefore, the amount of a pure P68/62 fraction could be estimated from small amounts of silver stained P68/62. The yield of P68/62 from one chromatofocusing run was ~100 ng from ~64 μ g of AES extract. In order to increase the amount of protein that could be separated per run, a larger column (1.5 x 30 cm) was used. Twice as much AES protein extract could be loaded per run. Consequently, the yield of purified P68/62 increased to ~200 ng.

FPLC was adopted to drive the chromatofocusing column. Previously, the

solutions were passed through the column using a peristaltic pump. Using FPLC, the time required was reduced from 2 days to 4.5 hours. In combination with FPLC use, protein resolution per fraction was improved by reducing the size of the fractions from 4 to 2 ml. The smaller fraction sizes increased the separation of the fractions containing P68/62 and other proteins from those containing only P68/62.

At first, the pH gradient was eliminated from the FPLC program because P68/62 was eluting consistently in the 1 M salt wash. The program began with a salt gradient from 0-2 M followed by a column wash with 1 M HCl. However, using this protocol, an increased amount of albumin was detected in each fraction throughout the run. A pH gradient from 5-4 was included before the salt gradient to elute any albumin. As a result, the amount of albumin in the salt fractions was reduced and some fractions were completely free of albumin. Therefore, the pH gradient did seem to play a role in albumin removal before P68/62 fractions were eluted with the salt gradient. In all subsequent FPLC-chromatofocusing runs, a pH gradient was included before the salt gradient.

The pH and A_{280} profiles of the fractions were similar to the profiles before the use of FPLC (Fig. 12). Based on the silver-stained SDS-PAGE results and the immunoblotting results with antiSLIP1 and antiRSA, P68/62 was still eluting in the salt and acid fractions. More specifically, P68/62 was eluted once the salt concentration reached around 1.4-1.7 M NaCl and in the acid fractions (Fig. 13).

Part way through the P68/62 purification process, it came to our attention that human keratins, some of which are also 68 and 62 kDa in size, are a common

Fig. 12- Comparison of the Elution Profiles for Peristaltic Pump Chromatofocusing vs. FPLC Chromatofocusing

Shown in Panel A) is a standard P68/62 elution profile for the method used previously in the lab to isolate P68/62. The elution profile of the current method is presented in Panel B). In panel A), the addition of 1 M NaCl and 1 M HCl to the column is indicated. Most of the pure P68/62 is eluted at this time. Similarly, in panel B), the salt concentration on the column increases gradually to a maximum level at approximately the same fraction as the salt addition in A). 1 M HCl is added at around the same fraction as in A). Although the size of the A_{280} protein peaks are not an accurate representation of the amount of protein present, the position of the peaks remained relatively constant between runs and could be used as a guide to the fractions containing pure P68/62.

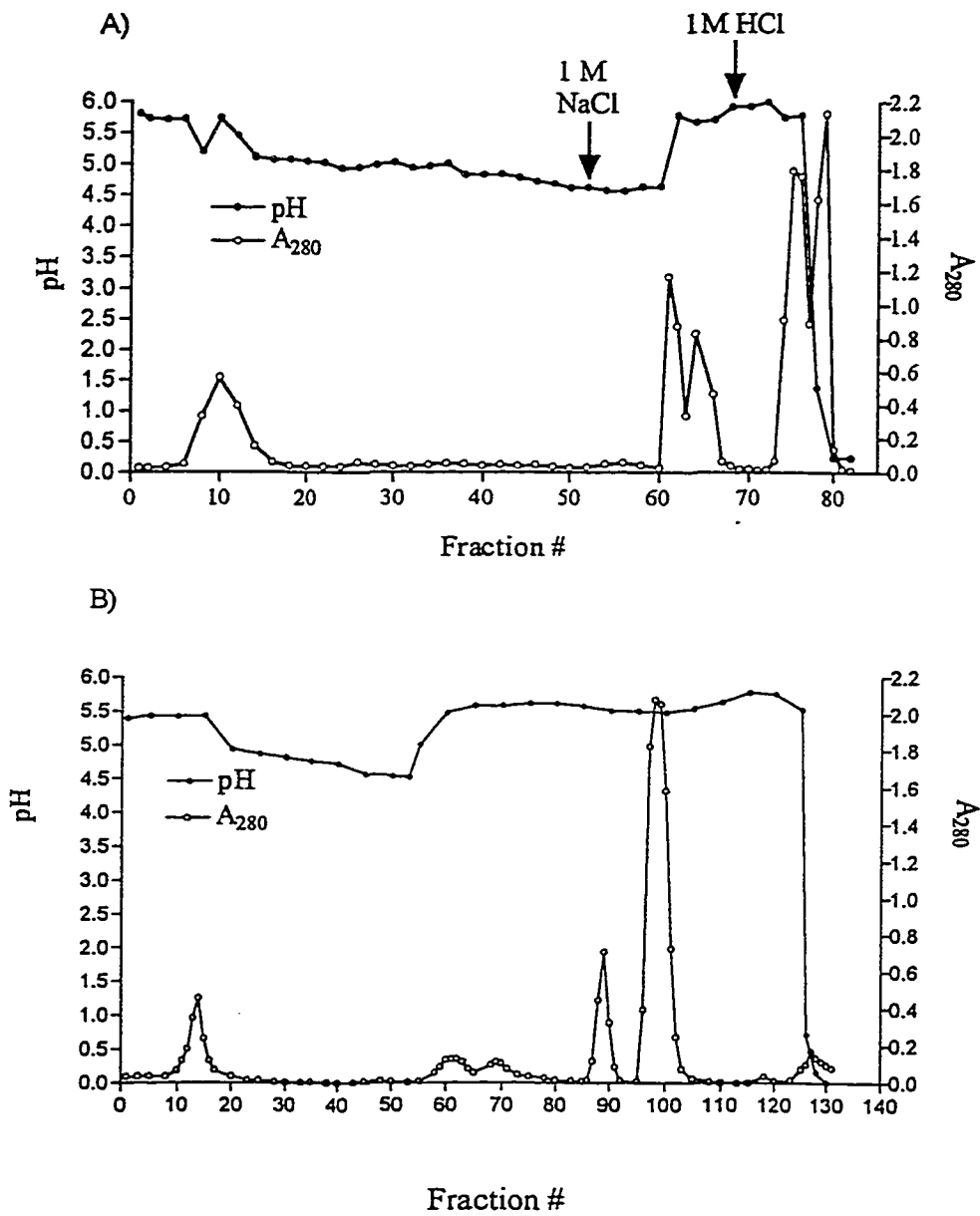


Figure 12

Fig. 13- Analysis of Boar Sperm P68/62 Fractions Eluted by Chromatofocusing

The AES boar sperm protein extract was chromatofocused using fast pressure liquid chromatography. Selected fractions were analyzed from various points during the chromatofocusing run. The upper panel shows the results of silver stained SDS-PAGE of these fractions. The middle panel are the immunoblotting results using antiSLIP1 and the lower panel are the antiRSA immunoblotting results. Comparison of the results indicates that the most pure P68/62 fractions elute in the high salt (1.4-1.7 M NaCl) and acid fractions. Lanes: R- 50 ng RSA, AES- ~40 ng boar sperm AES extract, 1 to 127- chromatofocusing fractions. Above the lane designations, "pI" refers to the elution of fractions during the pH gradient, "NaCl" refers to fractions eluted once the salt gradient had begun, and finally, the fractions eluted in "HCl". P68/62 fractions eluted in high salt have also marked by the extra bar above the salt fractions.

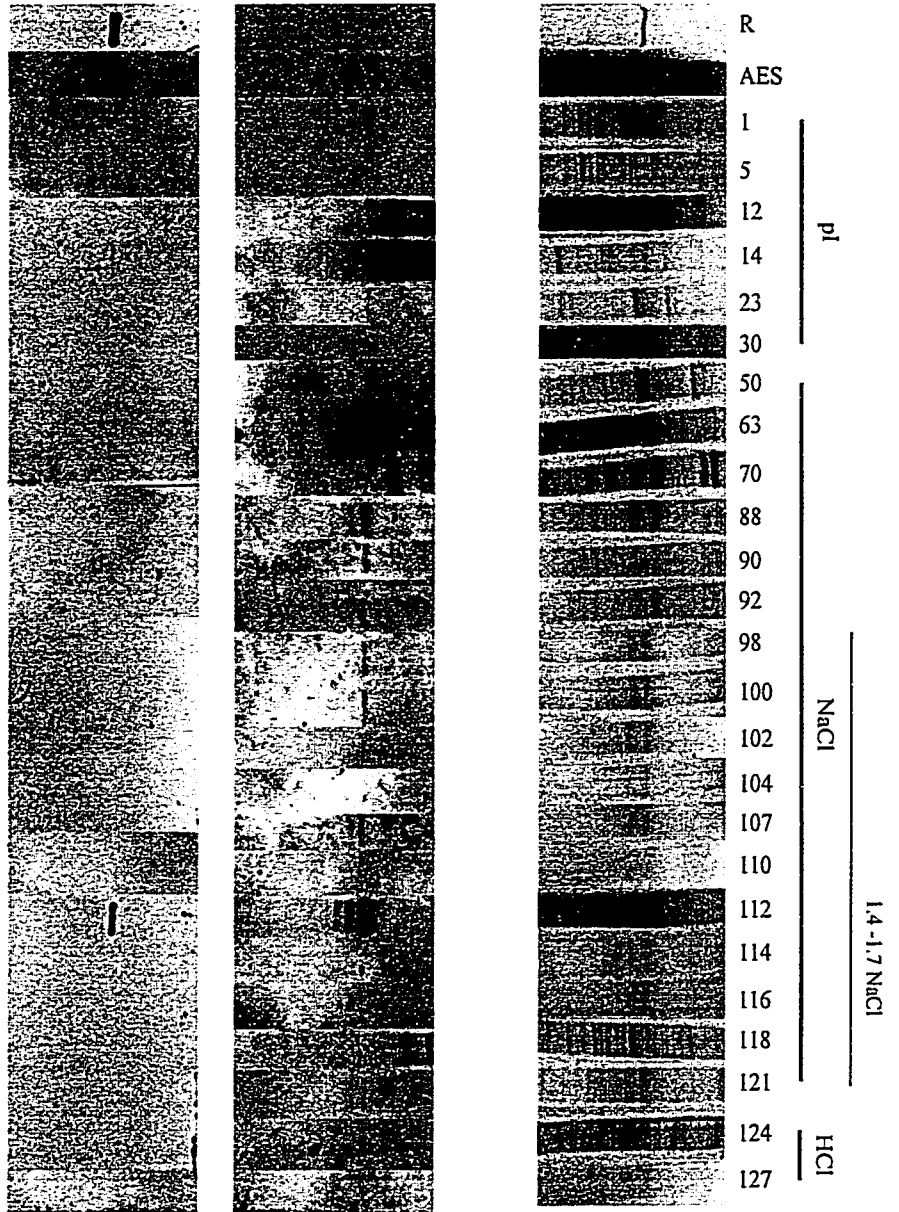


Figure 13

contaminant of protein work. To ensure that there were no keratins in the P68/62 preparation, immunoblots of previous FPLC fractions were stripped and re-probed using an anti-keratin polyclonal antibody. Surprisingly, many fractions did cross-react with the antibody. However, the major cross-reactive bands were present at, or just below, the P68 and P62 bands, depending on the percentage of acrylamide in the gel and the length of time of electrophoresis (Fig. 14). Therefore, the anti-keratin antibody recognizes a slightly different sized doublet that can be separated from the P68/62 doublet recognized by antiSLIP1. These anti-keratin cross-reactive bands were detected with antiSLIP. Therefore, these results indicated that the anti-keratin antibody does not cross-react with P68/62, but that the antiSLIP1 antibody does cross-react with keratin. Therefore, from that point on, all previous fractions that might be used for protein sequencing were first tested with the anti-keratin antibody and all subsequent chromatofocusing fractions were tested with antiSLIP1 first, then anti-keratin, and finally antiRSA.

3.5 P68 tryptic peptide microsequencing

3.5.1 P68 preparation and blotting to PVDF membrane

Since sequencing was going to be carried out from P68 blotted to and then excised from a PVDF membrane, it was vital that P68 could be separated from all other proteins by SDS-PAGE. P68/62 salt fractions from 11 rounds of boar sperm extractions were pooled based on different grades of P68/62 purity (Fig. 15). None of the acid fractions were used for sequencing because there was the possibility that acid hydrolysis had modified the amino acids. The criteria for each salt fraction pool was

Fig. 14- Immunoblotting of Purified Boar Sperm P68/62 with AntiSLIP1 and Anti-keratin Antibodies

Shown are boar sperm AES extract FPLC chromatofocusing fractions that were subjected to SDS-PAGE in a 10% polyacrylamide gel and immunoblotted with antiSLIP1 and anti-keratin antibodies. Strong antiSLIP1 cross-reactivity of a ~ 68 kDa band is observed. A slightly faster migrating band cross-reacted with the anti-keratin antibody. Both blots reveal a lower doublet band in most fractions.

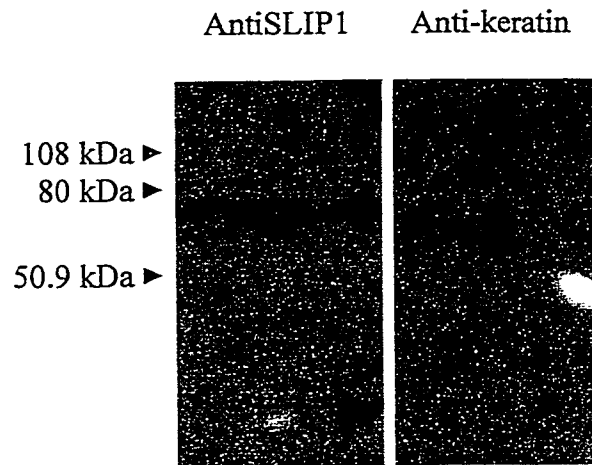


Figure 14

Fig. 15- Comparison of the Various Grades of P68/62 Purity for the First Attempt at P68 Microsequencing

P68/62 fractions collected from chromatofocusing of AES boar sperm protein extracts were subjected to SDS-PAGE followed by silver staining or electroblotting to nitrocellulose membrane. The membranes were immunoblotted with antiSLIP1, anti-RSA, and anti-keratin. The purity of the fractions was assessed based on the silver-stained gels and the combination of immunoblotting results. Shown above are the combined results for two fractions from each of the four pools used (Grades I, II, III, and IV). The position of the P68 band in each case is indicated by the arrowhead.

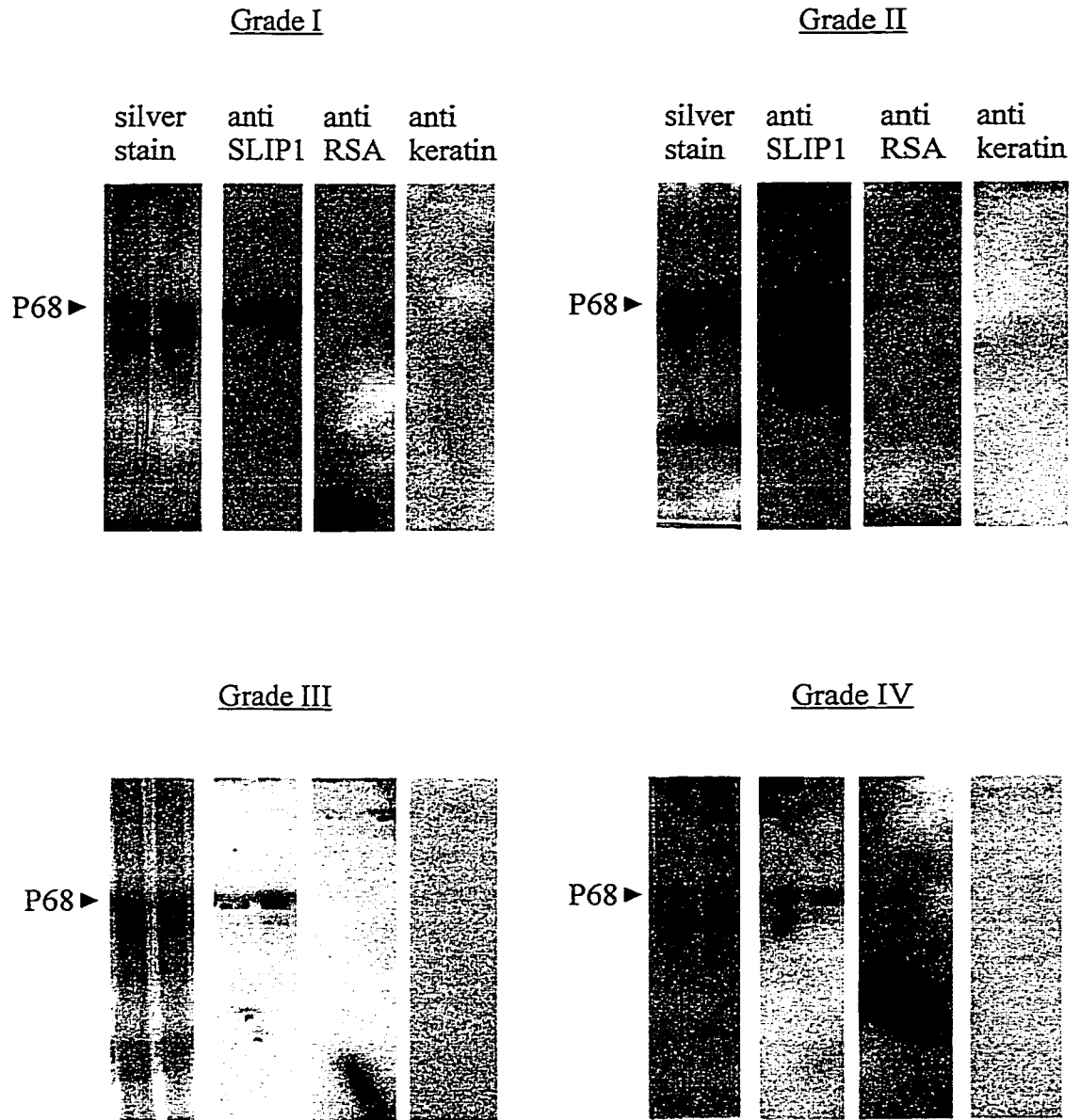


Figure 15

as follows: Grade I- pure P68/62 (i.e., no other protein bands on the silver stained gels and only antiSLIP1 cross-reactivity on the immunoblots); Grade II- only antiSLIP1 cross-reactivity on the immunoblots, but on the silver stained gels, P68/62 is present with a few other protein bands that are clearly separable from the P68 band; and, Grade III- still only antiSLIP1 cross-reactivity on the immunoblots, but there are a few more protein bands present with P68/62, as compared to the Grade II pool, and these extra bands are close to the P68 protein band.

Since the pooled P68/62 fractions could be in a salt solution as high as 2 M NaCl (from the end of the chromatofocusing run), the eluted protein fraction pools required desalting before they could be electrophoresed. To determine the extent of dialysis required to reduce the ionic strength of the protein solution, sham dialyses of 25 ml of a 2 M NaCl solution containing 500 ng RSA against Milli-Q water were performed. Osmolality readings were used to measure the change in ionic strength. It was found that after 1.5 days with six water changes, the osmolality had been reduced from an extrapolated value of 3620 mOsM to 497 mOsM, the equivalent to 0.28 M (Fig. 16). The protein loss was monitored using the A_{280} and was found to be minimal: the initial reading was 0.676 and the final reading was 0.672. All three P68/62 pools were dialysed separately according to the optimized protocol and lyophilized.

A grade IV pool of partially purified P68/62 fractions (Fig. 15), similar to the grade III pool, was also dialysed and lyophilized like the other pools. This pool would be used to test all the optimized protocols to be used for the preparation of P68 for sequencing: from dialysis and lyophilization to resolubilization, electrophoresis,

Fig. 16- Determination of the Osmolality of NaCl Solutions

NaCl solutions of known concentrations were measured in an osmometer to determine the osmolality. The concentrations of the salt solutions assayed were 0.1, 0.2, 0.3, 0.4, and 0.5 M. The standard curve was used to determine the osmolality of two separate 2M salt solutions following dialysis according to our method for preparing P68/62 fractions (see Methods). The osmolality of the solutions was measured (504 and 497 mOsM), which, according to the standard curve results, was equivalent to salt concentrations of 0.281 and 0.277M, respectively. Therefore, the dialysis had reduced the ionic strength of the solution significantly.

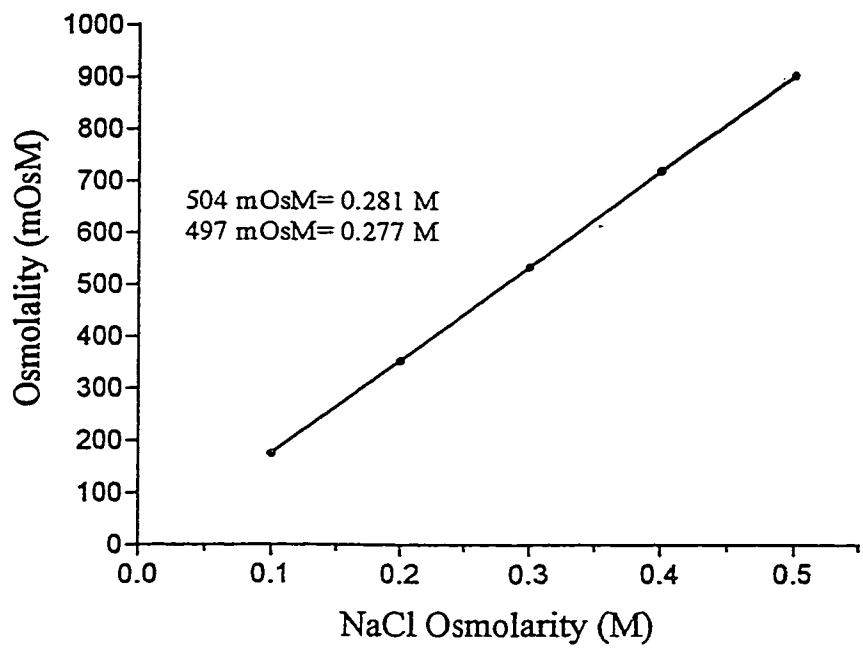


Figure 16

electrotransfer, and PVDF membrane staining. Approximately 1 μg of protein was collected for this pool in order to accurately represent the amount of P68/62 that would be used for sequencing.

Dialysis of the grade IV pool followed by lyophilization to dryness was as successful as the RSA trial. However, the partially purified P68/62 was very difficult to resolubilize in a small volume (max. 40 μl) of Laemmli's sample buffer for SDS-PAGE. A white precipitate was clearly visible when the sample buffer was mixed with the protein powder. In addition, the colour of the pH indicator in the sample buffer changed from a deep blue to yellow, indicative of an acidic solution. Despite efforts to completely solubilize the precipitate by step-wise heating, increasing the pH, the addition of 10 M urea, using a more concentrated sample buffer, or the step-wise addition of the sample buffer components, the insoluble precipitate remained. Even when resorting to the use of larger volumes of sample buffer, there was always a clearly visible unknown precipitate. Therefore, since the grade IV pool could not be salvaged, the grade III pool was used to determine how best to resuspend the lyophilized protein in a small volume before resolubilizing the grade I and II pools.

The best solution, and the one used for pools I and II, was to resolubilize the powder in whatever volume of sample buffer was required, adjust the pH of the solution (which had become acidic) with 1.5 M Tris-HCl, pH 8.8, then acetone precipitate the protein, and resuspend the final protein pellet in a small volume of SDS-PAGE sample buffer. The pH of the final sample was corrected using 1-2 μl of 1.5 M Tris-HCl, pH 8.8 when necessary. Before loading, the protein solution was heated step-wise to avoid

protein precipitation and quick-spun to pellet any remaining precipitate. Only the supernatant was electrophoresed.

At the start of SDS-PAGE, the sample moved very slowly through the stacking gel relative to the protein standards and the protein sample widened to almost double its normal width over the course of electrophoresis (Fig. 17). The migration of the protein sample relative to the prestained protein standards did not change.

The final transfer conditions to PVDF membrane were also optimized using RSA trials (Fig. 18). It was important to determine the best conditions for maximum transfer of protein to the membrane in the shortest period of time. Using RSA as the transferred protein, the transfer was most efficient at 125 mA for 1.5 hours at room temperature in the CAPS buffer system. But, since different proteins may behave differently, the RSA-optimized transfer conditions were then tested with the grade III P68/62 pool. The transfer conditions worked just as well with the partially purified P68/62 as for albumin (Fig. 19).

The amount of protein suggested for microsequencing following *in situ* digestion of the blotted protein was 50-100 picomoles (or 3.4-6.8 μg of a 68 kDa protein), although successful sequencing had been accomplished with as little as 20 pmol (1.36 μg of a 68 kDa protein)(John Leszyk, personal communication). The total amount of P68 from the first grade pool was approximately 927 ng. If the second grade pool was included (424 ng), the amount of sequenceable P68 was raised to approximately 1.35 μg . Therefore, to ensure that the minimum requirement was met, these two pools were combined and blotted to PVDF for sequencing. The bands were easily identified following Amido

Fig. 17- SDS-PAGE of the Grade III P68/62 Pool

The grade III P68/62 pool was electrophoresed in a 10% SDS-polyacrylamide gel and silver stained. RSA (50 ng) was co-electrophoresed as a size protein standard (lane 1). The grade III pool of P68/62 (~ 105 ng, lane 2) was used to test the resolubilization method for the lyophilized protein pool for P68 microsequencing.

1 2



Figure 17

Fig. 18- Trial Electrotransfers of Rat Serum Albumin (RSA) for the Optimization of P68 Transfer Conditions

RSA preparations (1 and 4 μg) were subjected to SDS-PAGE in one 10.5% polyacrylamide gel and electroblotted to PVDF membrane under different transfer conditions. The transfer gels were silver-stained to detect any remaining protein and the protein on the membrane was visualized by Amido Black staining. **Top row:** Silver stained gels- Lanes: 1- 1 μg RSA, 2- 4 μg RSA, **Bottom row:** Amido Black stained membranes- Lanes: 3- 1 μg RSA, 4- 4 μg RSA. Column A: Silver stained gel of RSA samples without electroblotting. Transfer conditions: Column B- 250 mA, room temperature, 15 minutes; Column C- 125 mA, room temperature, 1.5 hours; Column D- 375mA (i.e., maximum), 4°C, 30 minutes. The position of the RSA band is indicated.

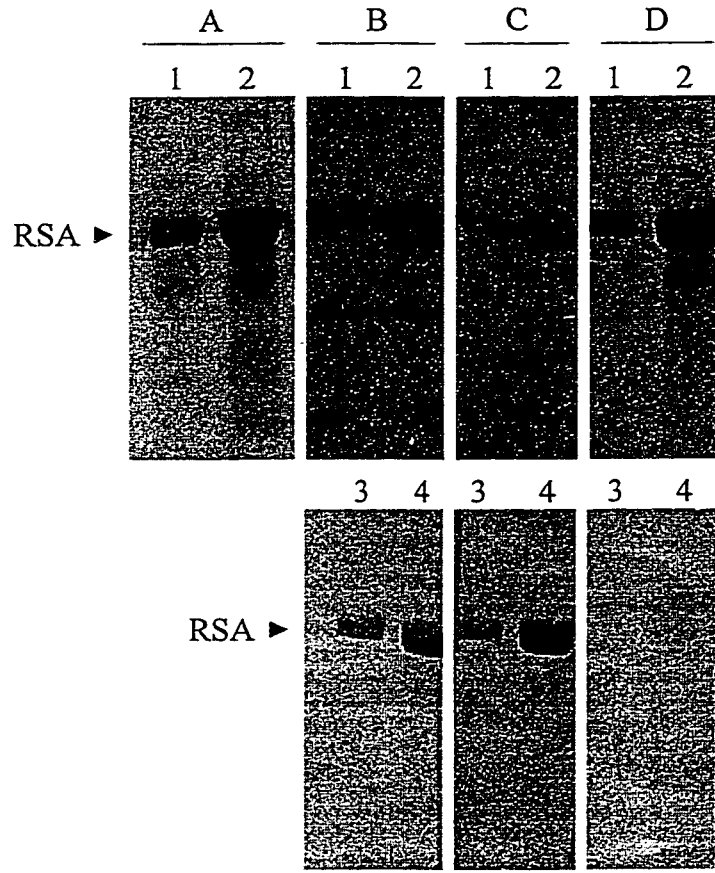


Figure 18

Fig.19- Trial P68/62 electrotransfer to PVDF for microsequencing

The grade III P68/62 pool was electrophoresed in a 10% SDS gel and electroblotted to PVDF membrane using the RSA-optimized transfer conditions (125 mA, 4°C, 1.5 hours). Following electroblotting, the gel was silver stained to detect any remaining protein and the membrane was immunoblotted with antiSLIP1 to detect P68/62. Lanes: 1- 100 ng RSA, 2- 140 ng P68/62. Protein standard size markers are indicated by the arrowheads in kilodaltons.

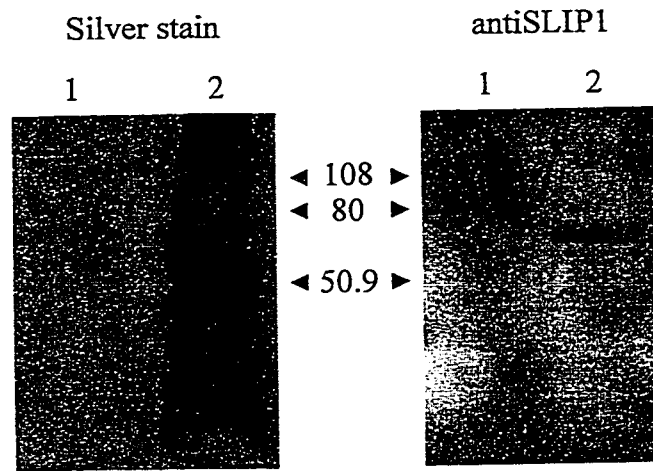


Figure 19

Black staining of the blot and once the excess liquid was wicked from the surface. The positions of all bands were determined mainly by this method. As expected, the P68/62 bands were wide and thin and P68 was slightly lower than the RSA protein standard (Fig. 20).

The SDS-PAGE and electroblotting of P68/62 grades I+II were performed using special precautions to avoid any protein contamination of the P68/62 sample (see Methods). However, the sequencing results of the P68 band indicated that it was keratin. Despite the fact that the P68/62 pool had been checked for the presence of keratin using the anti-keratin antibody and despite the use of a special protocol when working with the final protein pool, the first tryptic peptide sequenced was a 100% match, over the 18 amino acids sequenced, to the tail region of human keratin 9 (Fig. 21). Due to the length of the match and since the tail region of all keratins are very unique, there was not much chance that the purified protein was something other than keratin 9. Also, the chromatogram of the P68 tryptic digest matched almost identically to that of a keratin tryptic digest (Fig. 22). Therefore, no other peptides from the P68 digest were sequenced.

Keratin 9 is found on the palms of the hands and the soles of the feet. Therefore, the implications of the sequencing result needed to be settled- i.e., had a novel location and function for sperm keratins been discovered, or was the result due to human keratin contamination during P68 purification? Conclusive results from different studies determined that it was the latter. IIF studies showed no sperm staining of pig and mouse sperm using the anti-keratin antibody, although hair follicle cells stained positively (White and Tanphaichitr, unpublished results). Furthermore, a sample of the AES extract

Fig. 20- Amido Black-Stained PVDF Membrane of Transferred RSA and P68/62

Samples of RSA and P68/62 were electrophoresed in a 10% polyacrylamide SDS-gel and electroblotted to PVDF membrane. Shown are the results of Amido Black staining of the membrane following protein transfer. The lanes labelled "std" contain low molecular weight prestained protein standards. Arrowheads indicate the positions of the stained RSA, P68, and P62 bands.

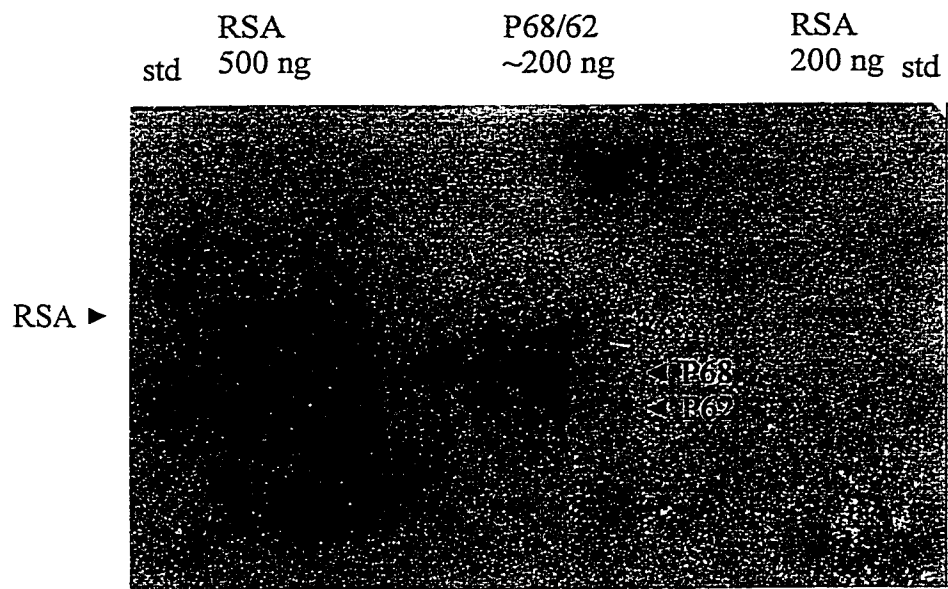


Figure 20

Fig. 21- Amino Acid Sequence Comparison of a P68 Tryptic Peptide and Human Keratin

The amino acid sequence of a P68 tryptic peptide obtained by microsequencing is a 100% match to a peptide found in the human keratin sequence. The homologous region is bolded in the keratin sequence. This region is located in the unique tail domain (underlined) of the protein.

P68 Peptide Sequence (18 amino acids): GGSGG SYGGG GSGGG YGG

Human Keratin Amino Acid Sequence:

(SWISS-PROT Protein Sequence Database, Accession number P35527)

MSCRQFSSSY	LTSGGGGGGG	LGSGGSIRSS	YSRFSSSGGR
GGGGRFSSSS	GYGGGSSRVC	GRGGGGSFGY	SYGGGSGGGF
SASSLGGGFG	GGRGFGGAS	GGGYSSSGGF	GGGFGGGSGG
GFGGGYGS GF	GGLGGFGGGA	GGGDGGILTA	NEKSTMQELN
SRLASYLDKV	QALEEANNDL	ENKIQDWYDK	KGPAAIQKNY
SPYYNTIDDL	KDQIVDLTVG	NNKTLLDIDN	TRMTLDDFRI
KFEMEQLNRQ	GVDADINGLR	QVLDNLTMEK	SDLEMQYETL
QEELMALKKN	HKEEMSQLTG	QNSGDVNVEI	NVAPGKDLTK
TLNDMRQEYE	QLIAKNRKDI	ENQYETQITQ	IEHEVSSSQ
EVQSSAKEVT	QLRHGVQELE	IELQSQLSKK	AALEKSLEDT
KNRYCGQLQM	IQEQISNLEA	QITDVRQEIE	CQNQEYSLLL
SIKMRLEKEI	ETYHNLLEGG	<u>QEDFESSGAG</u>	<u>KIGLGGRGGS</u>
<u>GGSYGRGSRG</u>	<u>GSGGSYGGGG</u>	<u>SGGGYGGGSG</u>	<u>SRGGSGGSYG</u>
<u>GGSGSGGGSG</u>	<u>GGYGGGSGGG</u>	<u>HSGGSGGGHS</u>	<u>GGSGGNYGGG</u>
<u>SGSGGGSGGG</u>	<u>YGGGSGSRGG</u>	<u>SGGSHGGGSG</u>	<u>FGGESGGSYG</u>
<u>GGEEASGSGG</u>	<u>GYGGGSGKSS</u>	HS	

Figure 21

Fig. 22- Tryptic Peptide Chromatograms of P68 and Keratin

In situ digestion of blotted P68 with trypsin was performed. The peptides were eluted from the blot and separated by capillary HPLC (Worcester Foundation for Biomedical Research, Shrewsbury, MA). The upper panel shows the chromatogram resulting from the P68 digest. The lower panel is an independent tryptic digest of a keratin protein for chromatogram peak comparison.

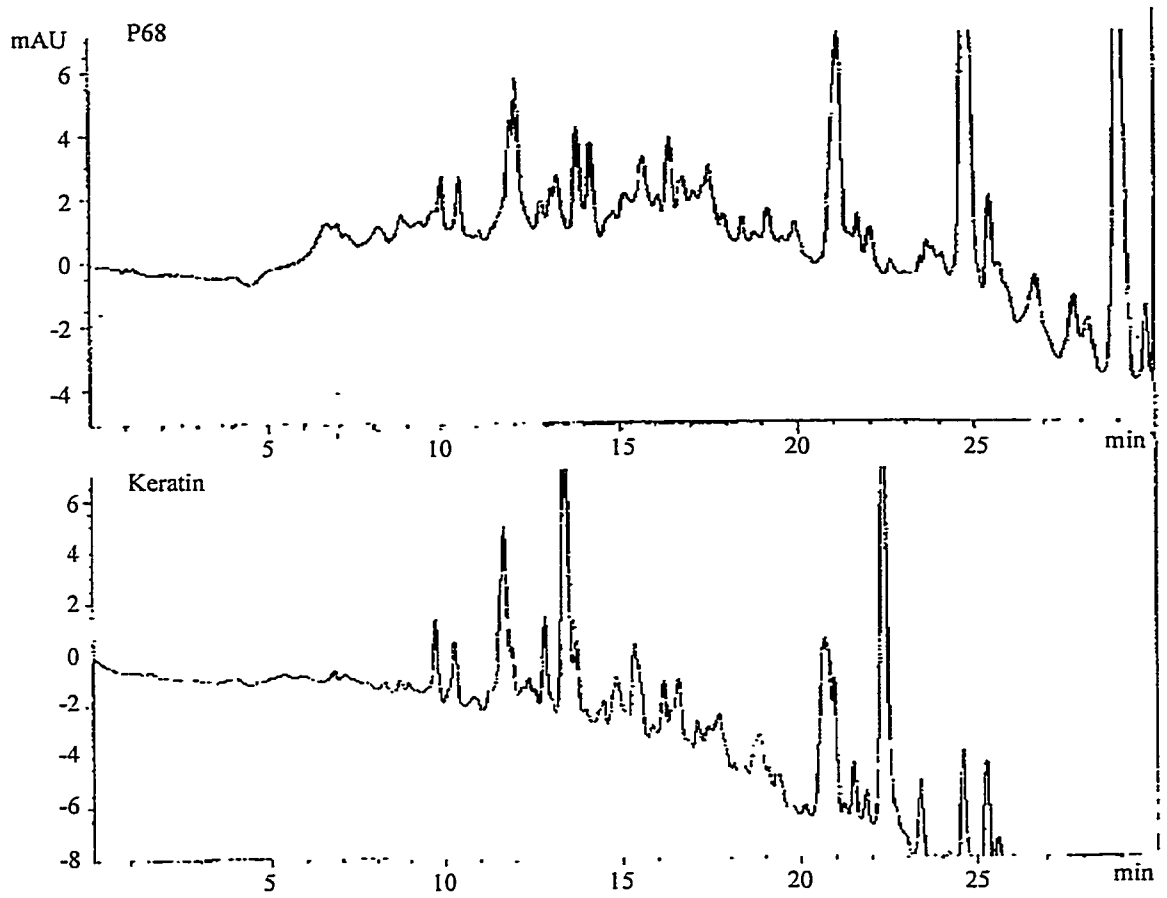


Figure 22

used to isolate P68/62 did not exhibit any anti-keratin cross-reactive 68 kDa proteins. Older blots of P68/62 fractions from the lab's first protein sequencing attempt also failed to show any cross-reactive 68 kDa proteins when probed with anti-keratin antibodies, although the 62 kDa band did cross-react (Fig. 23). Conversely, the recent P68/62 fractions that had shown only antiSLIP1 cross-reactive bands were re-probed with new anti-keratin antibody. It was revealed that the lower part of the 68 kDa antiSLIP1 cross-reactive bands contained keratin, as well as that the majority of keratin was present around the 62 kDa band (Fig. 14).

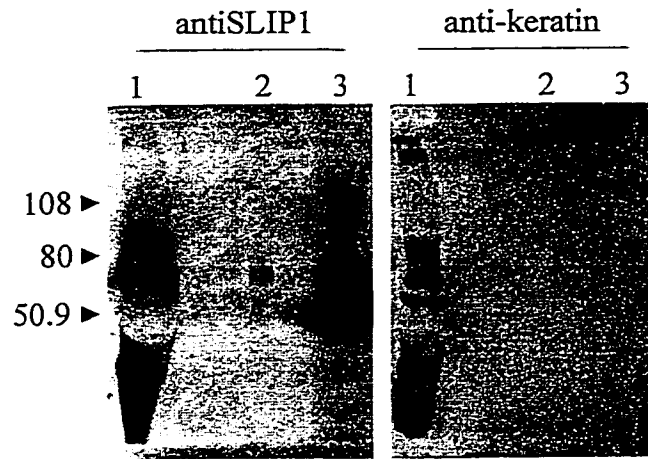
Starting with boar sperm collection, all the materials, equipment, and procedures used in the P68/62 purification process were extensively tested. The testing ascertained that some anti-keratin cross-reactive proteins were found in a wash of the FPLC injection port and sample loop (Fig. 24). However, keratin contamination had most likely occurred by the re-use of gloves and during handling of the dialysis tubing. Gloves were worn at all times during the procedures, but they were usually re-used. Therefore, keratin could have been deposited on the outside of the gloves when removing and then refitting the same pair. As this action was done repeatedly with the same pair of gloves, the amount of keratin on the gloves could increase to significant levels. The transfer of keratins to the protein solutions would be facilitated during dialysis procedures, where the opening of the dialysis tubing is manipulated with the hands.

Therefore, the P68/62 purification process was repeated under very stringent conditions. Protocols and new materials were adopted to reduce keratin levels and these levels were monitored from the very beginning instead of from part way through the

Fig.23- AntiSLIP1 and Anti-keratin Immunoblotting of Boar Sperm AES Extract and Purified P68/62 Fractions

Panel A: An aliquot of the AES extract ($\sim 1 \mu\text{g}$) was subjected to SDS-PAGE in a 12% polyacrylamide gel with keratin and bovine serum albumin (BSA) standards. The proteins were blotted to nitrocellulose and immunoblotting was performed with antiSLIP1 followed by anti-keratin antibodies. Shown are the immunoblotting results. The left panel is the results of the antiSLIP1 immunoblotting and the right panel is the results of the anti-keratin immunoblotting. Lanes: 1- keratin; 2- BSA; 3- AES extract. Arrowheads indicate the size and position of protein size standards in kilodaltons. **Panel B:** An older blot of P68/62 fractions isolated for the previous P68 sequencing attempt was reprobbed with the anti-keratin antibody to determine if keratin was present in these fractions. The left panel is the original antiSLIP1 results and the right panel is the results of the recent immunoblotting with anti-keratin. Lanes: 1- AES extract ($\sim 560 \text{ ng}$); 2, 3, 4- different purified P68/62 samples. Arrowheads indicate the size and position of protein size standards in kilodaltons.

A)



B)

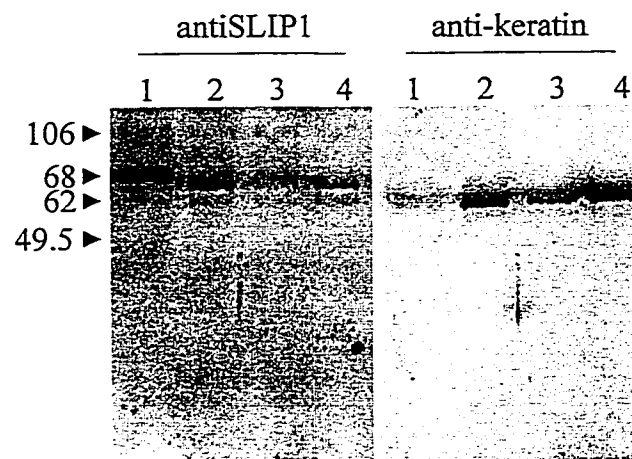


Figure 23

Fig. 24- Wash of the Injection Port and Sample Loop of the FPLC Equipment Used for Chromatofocusing

The injection port and sample loop were washed with a solution of 8 M urea to elute any keratins that may be present. The anti-keratin immunoblot of the eluate is shown. The arrowhead indicates a band approximately 68 kDa in size, based on the migration of co-electrophoresed protein standards.

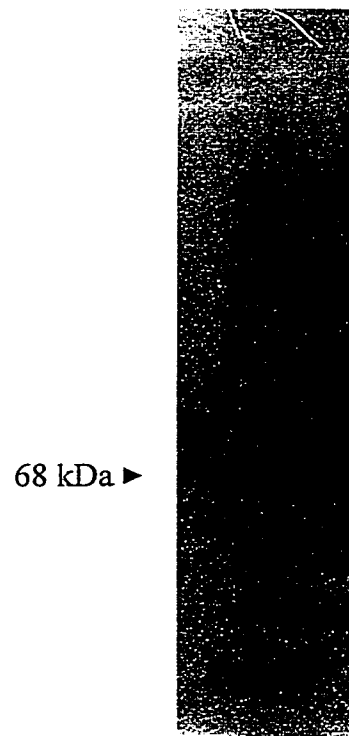


Figure 24

process. New pools of pure P68/62 were collected as follows: I- P68/62 bands on the silver stained gel and only antiSLIP1 cross-reactivity for P68 and P62; II- P68/62 bands on the silver stained gel with a few separable protein bands around them, but only antiSLIP1 cross-reactivity for P68/62; IIIk- P68/62 bands on the silver stained gel that cross-react with antiSLIP1 and only a very small amount of separable (i.e., ~ 66 kDa) keratin; IIIa- P68/62 bands on the silver stained gel that cross-react with antiSLIP1 and only a very small amount of albumin contamination; and, IV- P68/62 antiSLIP1 cross-reactive bands with many other non-separable protein bands on the silver stained gel and some keratin and albumin contamination (Fig. 25). Pools I-III could be used for sequencing, whereas pool IV (~300 ng) was to be used for test trials of the solubilization and blotting procedures. Pools I and II were combined for an estimated total amount of 0.5 μ g of P68/62. The other two pools, IIIk and IIIa, contained approximately 280 ng and 120 ng of P68/62, respectively.

A new protein resolubilization, SDS-PAGE, and P68 band isolation strategy was developed with the lyophilized grade IV protein pool as well as with other P68/62 pools of similar quality (see Methods). The procedures had been improved such that no precipitate was visible following resolubilization in Laemmli's sample buffer, but, as for the first attempt, at the start of SDS-PAGE, the sample moved very slowly through the stacking gel relative to the protein standards and the protein sample widened over the course of electrophoresis. These effects could not be eliminated, but based on improved PVDF membrane staining (by increasing the staining time) and the immunoblotting results, P68 was easily exciseable from the blot.

Fig. 25- Comparison of the Various Grades of P68/62 Purity for the Second Attempt at P68 Microsequencing

P68/62 fractions collected from chromatofocusing of AES boar sperm protein extracts were subjected to SDS-PAGE followed by silver staining or electroblotting to nitrocellulose membrane. The membranes were immunoblotted with antiSLIP1, anti-RSA, and anti-keratin. The purity of the fractions was assessed based on the silver-stained gels and the combination of immunoblotting results. Shown above are the combined results for two fractions from each of the four pools used (Grades I+II, IIIa, IIIk, and IV). The position of the P68 band in each case is indicated by the arrowhead.

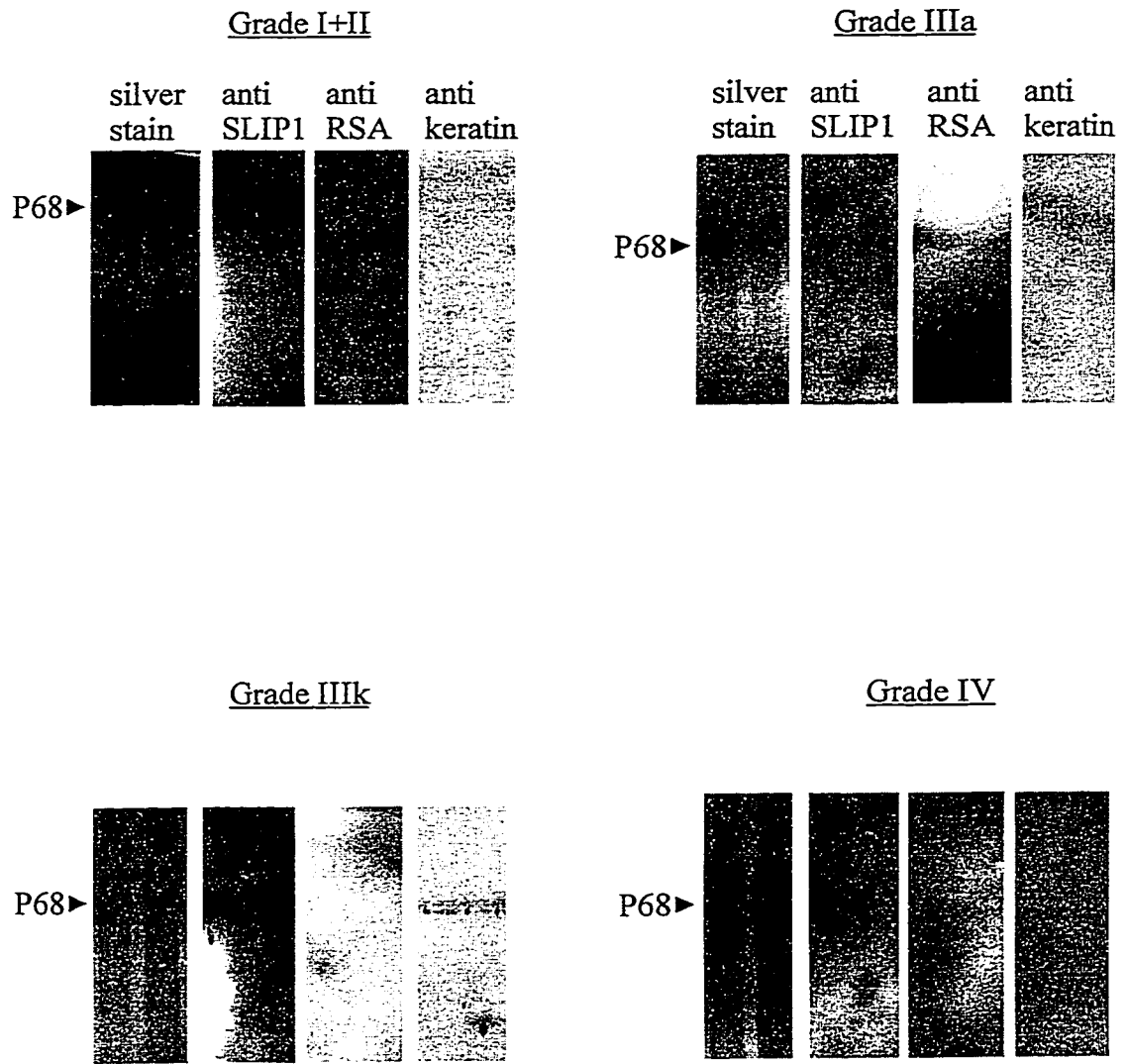


Figure 25

Pools IIIk, IIIa, and finally I+II were resolubilized, subjected to SDS-PAGE, and transferred to PVDF membrane using the optimized protocols. Based on the silver staining results of the SDS-gels after the transfer, electroelution of the P68/62 bands from the gels during the transfer was close to 100% efficient (Fig. 26). The blots were stained with Amido Black to localize and score the position of the potential P68 band using a fresh scalpel blade. The P68/62 bands stained well for pools I+II and IIIa, but did not stain well for IIIk. In all cases (including IIIk), the Amido Black stained the protein bands such that they appeared as clearly smooth, reflective surfaces on the otherwise mottled background of the blot.

Each blot was cut in two such that one third contained the lane of P68/62 for sequencing. The remaining two thirds of the blot included a very small portion of the side of this P68/62 lane, so that it could be used for immunoblotting studies with antiSLIP1, anti-keratin, and antiRSA. After all of the immunoblotting, the blot still retained its Amido Black stain. Therefore, the immunoblotting results could be compared to the blot's stained protein bands. Furthermore, the immunoblotting chemiluminographs could be aligned with the remaining third of the blot. As a result, the bands from the chemiluminographs could be used to identify the portion of the P68 band that had cross-reacted with only antiSLIP1. Only this portion of the P68 band would be excised from the non-immunoblotted blot and sent out for sequencing.

The best results were obtained with the IIIa blot (Fig. 27). Strong signals were visible with all three antibodies and a unique antiSLIP1 P68 band could be identified.

Fig. 26- Silver Stained SDS-Gel Following Protein Electroblothing

Purified P68/62 was subjected to SDS-PAGE in a 12% polyacrylamide gel and electroblotted to PVDF membrane for microsequencing. The gel was silver stained after electroblotting to determine the amount of protein that had remained in the gel. Shown is this silver-stained gel. Lanes: 1- well filled with Laemmli's sample buffer, 2- P68/62 for microsequencing, 3- well filled with Laemmli's sample buffer, 4- partially purified P68/62 control lane, 5- RSA, 100 ng, 6- RSA, 200 ng, 7- keratin sample

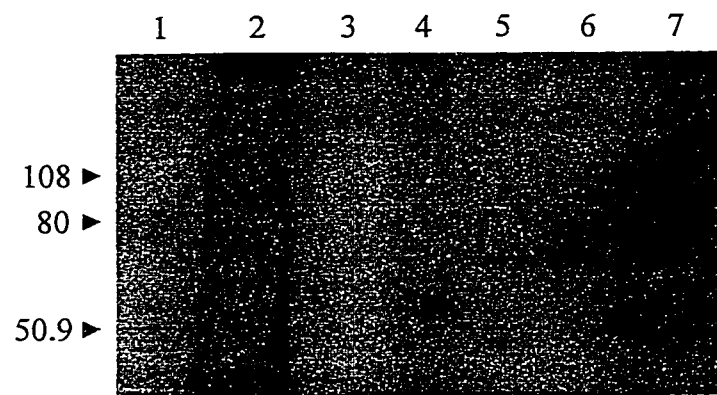


Figure 26

Fig. 27- Comparison of the Immunoblotting Results of the Final P68/62 Pools

P68/62 pools for microsequencing were electrophoresed in 12% polyacrylamide SDS gels and electroblotted to PVDF membrane. P68/62 grade I+II and IIIa pools were probed with antiSLIP1, antiRSA and anti-keratin antibodies to isolate only the antiSLIP1 cross-reactive P68 band. P68/62 grade IIIk was probed with antiSLIP1 and anti-keratin antibodies only. Upper row (A): antiSLIP1 immunoblots; Middle row (B): anti-RSA immunoblots; Lower row (C): anti-keratin immunoblots. Lanes: 1, 6, 11- portion of P68/62 lane for microsequencing; 2, 7, 12- P68/62 control lane (low grade); 3,8,13,- 100 ng RSA; 4,9,14- 200 ng RSA; 5, 10,16- keratin. Arrowheads indicate the position of signals obtained in the P68/62 sample for sequencing.

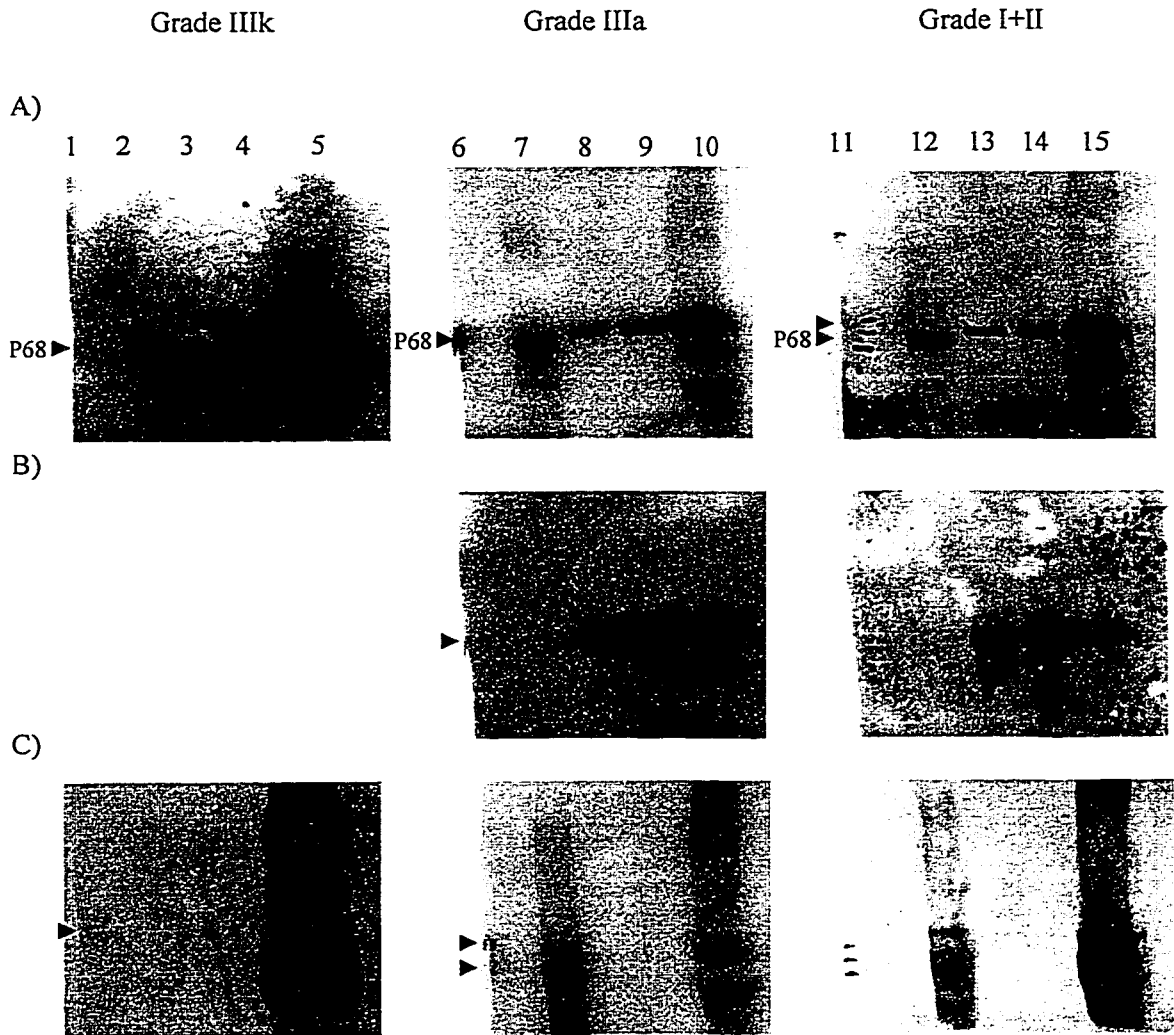


Figure 27

The immunoblotting results and the scores made based on the Amido Black staining were used to cut out P68 for sequencing. For the blot of IIIk (Fig. 27), not enough of the P68/62 lane had been included to enable a strong signal with the antiSLIP1 antibody. All that could be seen was a faint broad dot. The anti-keratin immunoblot yielded a weak but identifiable band slightly below the estimated position of P68. To ensure that as much of the P68 band was excised from the remaining blot as possible, some of the keratin band was included as it was very difficult to determine the SLIP1/keratin border. It should be noted, however, that the amount of keratin in this pool was relatively very low, so it should not dominate the sequencing results. Furthermore, any tryptic peptides from the *in situ* digestion that matched the expected molecular masses of tryptic keratin peptides would be excluded from peptide sequencing.

The immunoblotting results for the I+II pool was the most ambiguous of the three pools (Fig. 27). The protein bands had widened to almost double the normal width during the course of electrophoresis. Therefore, the protein bands were very thin. There were only faint antiSLIP1 cross-reactive bands around the expected P68 position, but they matched the scores made from the blot staining. Interestingly, there were strong antiSLIP1 bands of much lower molecular weight forming a ladder-like pattern all the way down the lane. It is not clear what these bands were. Any anti-keratin bands were only faintly visible after a long film exposure, indicating that the preparation was significantly free of keratin. Furthermore, the faint bands were below the expected position of P68. No albumin band was detected. To help with the excision of the P68 band on the remaining blot, a trace was made of the silver stained bands remaining in the

transfer gel. The trace matched the bands from Amido Black staining of the blot and the immunoblotting results. P68 was excised using the trace as a guide.

3.5.2 P68 tryptic peptide sequencing

Since the last P68 sequencing attempt, the microsequencing facility had improved the sensitivity of its equipment such that it was possible to sequence 7-10 pmol of protein. Although the amount of protein for pool I+II was around this minimum amount, it was felt that trying for peptide sequences from the purest P68 preparation was worth the risk. Therefore, the excised band from pool I+II was digested with trypsin separately from a combined digest of the IIIa and IIIk P68 bands.

The trypsin digest chromatograms of the two sets of samples were not quite the same (Fig. 28). The difference in digest profiles could be a result of the difference in the amount of protein present in each sample. This difference may have led to a difference in the extent of digestion. Also, IIIa+IIIk contained some albumin and keratin, which are probably responsible for some of the different peaks. For IIIa+IIIk, there were very few major peaks that could be sequenced. For this reason, and because this sample was not as pure as I+II, sequencing was performed only for peptides from pool I+II.

There were fifteen main peaks in the trypsin digest chromatogram of pool I+II (Fig. 29). From the profile and the mass spectrometry data of the peaks, it was determined that P68 from pool I+II did not contain a significant amount of either keratin or albumin. None of the major peaks, for which mass spectrometry data could be obtained, were of the expected size for a keratin or albumin peptide.

Fig.28- Comparison of the Tryptic Peptide Chromatograms of Different Grades of P68

In situ digestion of blotted P68 with trypsin was performed. The peptides were eluted from the blot and separated by capillary HPLC (Worcester Foundation for Biomedical Research, Shrewsbury, MA). The upper panel shows the chromatogram resulting from the digest of 400 ng of a P68 protein pool containing trace amounts of albumin and keratin (Pool IIIk+IIIa). The lower panel shows the chromatogram resulting from the digest of 0.5 μ g of the Grade I+II P68 pool (Pool I+II).

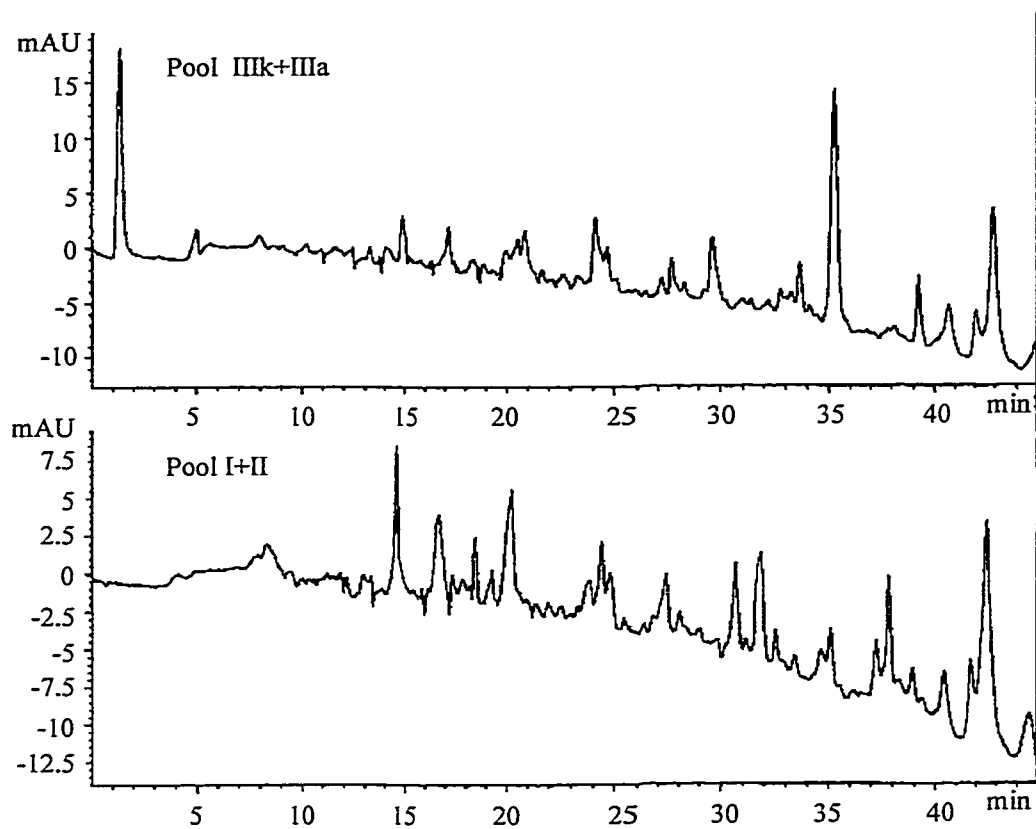


Figure 28

Fig. 29- Tryptic Peptide Chromatogram from the Second Attempt at P68 Microsequencing

In situ digestion of blotted P68 with trypsin was performed. The peptides were eluted from the blot and separated by capillary HPLC (Worcester Foundation for Biomedical Research, Shrewsbury, MA). Shown is the chromatogram resulting from the digest. Major peptide peaks have been numbered. The mass of some of these numbered peptides were given: 1- 829; 6- 1422, 1870; 9- 1620; 13- 2190.

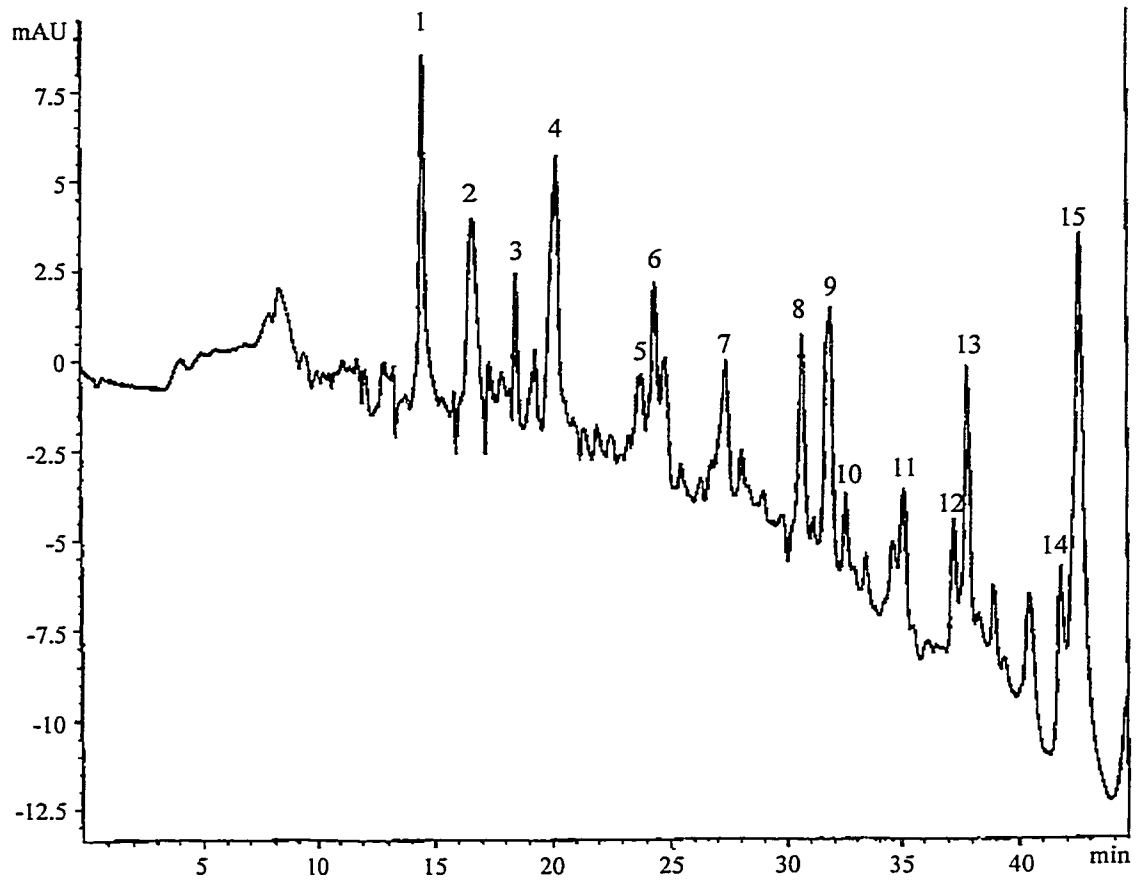


Figure 29

The first peak sequenced, #13, produced a 19 amino acid sequence (mass = 1618.8, initial yield= ~0.8 pmols) in which the first two sequencing cycles were not clean enough for the amino acids to be determined. The sequence was: XXPIQ AFLLY QEPVL GVP(R). The brackets around the last amino acid indicates that its identity is questionable, but most likely what is written. A protein database search revealed that this entire sequence was a 100% match to bovine beta casein precursor protein (Fig. 30). Due to this result, and because the casein sequence is known, all the protein peaks obtained from the P68 digest were compared to the expected peptides for a casein tryptic digest. Only peak #13 was a match to an expected casein peptide (John Leszyk, personal communication).

Three more peptides were sequenced (peaks # 7, 8, and 9 on the chromatogram). These peaks were selected because: the peptide peak areas indicated that there was enough protein for sequencing, the position of the peaks indicated that they were long enough to provide enough sequence information for future cloning work, and they were isolated enough from other peaks to obtain clean sequences. Peak #6 was not sequenced because the mass spectrometry data indicated that the peak was not pure. Peak #15 was not sequenced because it was a trypsin autolytic fragment.

Peak #9 (mass = 1619.18, initial yield= ~800 fmols) was sequenceable over 13 amino acids. The sequence, TLFFY PAYPD EVR, was 84% identical and 100% similar to amino acids 372 to 384 of human testis lysosomal arylsulfatase-A (AS-A) (Fig. 30). The sequence obtained is supported by the fact that the mass spectrometry data

Fig.30- Amino Acid Sequence Comparisons of P68 Tryptic Peptides to Bovine Casein and Human Testis AS-A

The amino acid sequences of P68 tryptic peptides (based on the digest chromatogram- Fig.28) are shown with the percentage identity to the known protein's sequence. The bracketed amino acids indicate that their identity is questionable, but based on mass spectrometry data for the peptide, they are most likely what is written. The full amino acid sequences of bovine casein and human testis AS-A are shown for comparison. The homologous regions are underlined.

1. P68 Peptide Sequence (Peak # 13): PIQ AFLLY QEPVL GPV(R) 100% identity

Bovine Casein Sequence:

(SWISS-PROT Protein Sequence Database; Accession number P02666)

MKVLI	LACLV	ALALA	RELEE	LNVPG	EIVES	LSSSE	ESITR
INKKI	EKFQS	EEQQQ	TEDEL	QDKIH	PFAQT	QSLVY	PFPGP
IPNSL	PQNIP	PLTQT	PVVVP	PFLQP	EVMGV	SKVKE	AMAPK
HKEMP	FPKYP	VEPFT	ESQSL	TLTDV	ENLHL	PLPLL	QSWMH
QPHQP	LPPTV	MFPPQ	SVLSL	SQSKV	LPVPQ	KAVPY	PQRDM
<u>PIQAF</u>	<u>LLYQE</u>	<u>PVLGP</u>	<u>VRGPF</u>	PIIV			

2. P68 Peptide Sequence (Peak # 9): TLFFY PAYPD EVR 84% identity

3. P68 Peptide Sequence (Peak # 8): F(T)D(F)Y V(P)VSL X(T)P 100% identity

4. P68 Peptide Sequence (Peak #7): AQFDA AVTFS PSQIA R 81% identity

Human Testis AS-A Sequence:

(SWISS-PROT Protein Sequence Database; Accession number P15289)

MGAPRSLLLA	LAAGLAVARP	PNIVLIFADD	LGYGDLGCYG
HPSSTTPNLD	<u>QLAAGGLRFT</u>	<u>DFYVPVSLCT</u>	<u>PSRAALLTGR</u>
LPVRMGMYPG	VLVPSSRGGL	PLEEVTVAEV	LAARGYLTGM
AGKWHLGVGP	EGAFLPPHQG	FHRFLGIPYS	HDQGPCQNLT
CFPPATPCDG	GCDQGLVPIP	LLANLSVEAQ	PPWLPGLEAR
YMAFAHDLMA	DAQRQDRPFF	LYYASHHthy	PQFSGQSFAE
RSGRGPFGDS	LMELDAAVGT	LMTAIGDLGL	LEETLVIFTA
DNGPETMRMS	RGGCSGLLRC	GKGTTYEGGV	REPALAFWPG
HIAPGVTHEL	ASSDLLPTL	AALAGAPLPN	VTLDGFDLSP
LLLGTGKSPR	<u>QSLFFYPSYP</u>	<u>DEVRGVFAVR</u>	TGKYKAHFFT
QGSAHSDTTA	DPACHASSSL	TAHEPPLLYD	LSKDPGENYN
LLGGVAGATP	EVLQALKQLQ	<u>LLKAQLDAAV</u>	<u>TEGPSQVARG</u>
EDPALQICCH	PGCTPRPACC	HCPDPHA	

Figure 30

corroborates the calculation of the expected peptide mass based on the amino acid sequence.

Peak #8 actually contained two peptides: one was a partial cleavage fragment of peak #9 (with an extra R at the N-terminus), and the other peptide's sequence was F(T)D(F)Y V(P) VSL X(T)P (Fig. 30). The mass of the latter peptide was 1733.47. Although the identity of the amino acids in parentheses was not certain, amino acid assignment based on the known sequence of human testis AS-A provided a calculated mass of 1732. This value is in agreement with the mass data obtained and, therefore, the amino acids selected are most likely correct. With these amino acid assignments, the peptide sequence is a 100% match to amino acids 59 to 71 of human AS-A.

Peak #7 (mass= 1709.61, initial yield= ~900 fmols) yielded a 16 amino acid sequence, AQFDA AVTFS PSQIA R, that has an 81% sequence identity and 87% sequence similarity to amino acids 464 to 479 of the carboxy terminal end of human AS-A (Fig. 30). Again, the sequence data is in agreement with the mass spectrometry data. Therefore, of four tryptic peptides sequenced, three were highly homologous to various regions of human AS-A and one was identical to bovine beta casein precursor protein.

An analysis of the human AS-A amino acid sequence was performed whereby the sequence was separated into the expected peptides for a tryptic digest. It was found that the number and size of the expected AS-A peptides are similar to results of the P68 chromatogram and that the P68 peptide sequences could be located in specific AS-A tryptic peptides (Fig. 31). Analysis of the AS-A amino acid sequence also revealed that 10% of the total number of amino acids are negatively charged (i.e., amino acids D and

Figure 31- Expected Tryptic Peptides from the Digestion of Human Testis AS-A

The human testis AS-A sequence was cut after all arginine and lysine residues, as expected for a trypsin digest. The number of different sizes of peptides expected was determined, as was the number of different peptides longer than 7 amino acids (to compare with the P68 digest chromatogram whose first marked peptide peak had a molecular mass of 829 Da). The peptide sequences obtained from P68 microsequencing are underlined.

Human Testis AS-A Sequence:
(SWISS-PROT Protein Sequence Database; Accession number P15289)

MGAPR/ SLLALAAGLAVAR/
PPNIVLIFADDLGYGDLGICYGHPSSTTPNLDQLAAGGLR/ FTDFYVPVSLCTPSR/
AALLTGR/ LPVR/ MGMYPGVLVPSSR/ GGLPLEEVTVAEVLAAR/
GYLTGMAGK/ WHLGVGPEGAFPPHQGFHR/
FLGIPYSHDQGPCQNLTCPATPCDGGCDQGLVPILLANLSVEAQPPWLPGLEA
R/ YMAFAHDLMADAQR/ QDR/ PFFLYYASHHTHYPQFSGQSFAER/ SGR/
GPFGDSLMELDAAVGTLMTAIGDLGLLEETLVIFTADNGPETMR/ MSR/
GGCSGLLR/ CGK/ GTTYEGGVR/
EPALAFWPGHIAPGVTHELASSDLLPTLAALAGAPLPNVTLDGFDLSPLLLGTG
K/ SPR/ QSLFFYPSYPDEVR/ GVFAVR/ TGK/ YK/
AHFFTQGSAHSDTTADPACHASSSLTAHEPPLLYDLSK/
DPGENYNLLGGVAGATPEVLQALK/ QLQLLK/ AQLDAAVTFGPSQVAR/
GEDPALQICCHPGCTPR/ PACCHCPDPHA

- 20 different sizes of peptides expected
- 14 of these peptide sizes are longer than 7 amino acids. They are:
9, 11, 13, 14, 15, 16, 17, 20, 39, 24, 38, 39, 44, 56, and 57 amino acids long

Figure 31

E), whereas 20% are positively charged (i.e., amino acids L, R, and H). The larger proportion of positive amino acids is unexpected because this would suggest that the protein is basic, but the chromatofocusing results demonstrated that the protein is very acidic. The apparent conflict can be resolved by the extent of protein glycosylation. Phosphorylated sugar residues on the protein would be sufficient for the protein to become acidic.

The amino acid sequence was also searched for any matches to common protein motifs that have been described for SLIP1/P68/62, including SGG binding and ATP binding motifs (Lingwood and Nuttika, 1991; Law *et al.*, 1988; Tanphaichitr *et al.*, 1998). A match of only 20% and 29% identity was found for these two motifs, respectively (Fig. 32).

3.6 Preliminary results of a comparative study of AS-A and P68

3.6.1 Immunoblotting of P68 and human recombinant AS-A

Preparations of boar sperm AES extract, P68/62, and human recombinant AS-A were blotted to nitrocellulose and probed with a polyclonal antibody against human recombinant AS-A. Results revealed a common ~68 kDa band in all three samples (Appendix F). When the antiSLIP1 antibody was used, 68 kDa bands were visible only in the negative control samples (i.e., keratin and RSA). Therefore, the antiSLIP1 antibody was most likely exhausted. Recently, a polyclonal antibody directed against boar sperm P68 was produced in our lab by intrasplenic immunization. Immunoblotting with this antibody detected a common ~68 kDa band in all three samples, but a higher

Figure 32- Homologous Domains of the Human Testis AS-A Amino Acid Sequence to Known Protein Motifs

The human testis AS-A amino acid sequence was searched for known protein motifs of relevance to known characteristics of P68/62. The following two motifs were found at the AS-A sequence locations identified. The percent identity between the AS-A sequence and the typical motif sequence was also calculated.

MOTIF	TYPICAL MOTIF SEQUENCE	AS-A MATCH LOCATION	HOMOLOGY
ATP Binding Site	GDFIALDLGGSSFR	47-60	29% identity
SGG Binding Domain	CSVTCGXGXXRXR	485-498	20% identity

Figure 32

molecular weight band was also present in the P68/62 sample. (Appendix F). Therefore, P68 may contain antiSLIP1 epitopes that are not present in human recombinant AS-A (see Discussion).

3.6.2 Localization of AS-A on mouse and human sperm by indirect

immunofluorescence

In the mouse system, AS-A and P68/62 were localized on live sperm using the anti-AS-A and antiSLIP1 antibodies, respectively. The staining patterns were similar (Appendix G). Various sperm head AS-A staining patterns were observed: at the convex ridge (panel a, panel b-bottom sperm); at the convex ridge, within the acrosomal region, and at the postacrosomal region (panel b- upper sperm); and over the entire sperm head (panel c). Midpiece AS-A staining was also observed in a number of sperm (panel a, panel b-bottom sperm, and panel c). In live human sperm, AS-A was detected mainly at the postacrosomal region of the sperm head and at the midpiece (panel a). These IIF staining patterns were similar to those observed in mouse and human sperm using antiSLIP1 (Tanphaichitr *et al.*, 1993; Rattanachaiyanont *et al.*, submitted).

3.6.3 Inhibition of sperm-ZP binding in mature mouse eggs by egg pretreatment with AS-A

Zona-intact mouse eggs were pretreated with differing concentrations of human recombinant AS-A before co-incubation with capacitated mouse sperm. It was found that the AS-A pretreatment of the eggs inhibited sperm-ZP binding in a dose-dependent

manner (Appendix H). This finding supports the possibility that AS-A is present on the sperm and that it is involved in sperm-ZP binding. However, the concentrations of AS-A needed for this inhibition were higher than those required in a similar experiment in which eggs were pretreated with SLIP1 (Tanphaichitr *et al.*, 1993). Therefore, there are probably other SLIP1 components that are important for sperm-ZP binding.

CHAPTER 4

DISCUSSION

The involvement of P68/62 in the initial stage of sperm-egg interaction has been supported by *in vitro* studies in both the mouse and human systems (Tanphaichitr *et al.*, 1993, 1998; Rattanachaiyanont *et al.*, submitted), as well as by *in vivo* studies in the mouse (Tanphaichitr *et al.*, 1992). Clearly, P68/62 is the ZP binding component of SLIP1. However, we still lack an understanding of the P68/62 protein biochemistry that is responsible for this binding. Consequently, current research is focused on the isolation and structural characterization of P68/62.

The fact that P68/62 is present in very low amounts on the sperm head (Tanphaichitr *et al.*, 1993) is a barrier to such work. Therefore, production of P68/62 by recombinant gene expression would be time-saving, if not necessary. As a result, the original goal of this research project was to clone the cDNA sequence of boar testis P68/62 for recombinant protein production.

4.1 cDNA Cloning of P68/62

For any gene cloning strategy, one must base the cloning tools on either nucleotide sequences of a homolog or amino acid sequences from the gene of interest. If the nucleotide sequence of a gene homolog is known, PCR primers or oligonucleotide probes can be constructed from the regions most likely to be conserved. If only amino acid sequences are known, the PCR primers and oligonucleotide probes must be designed according to the theoretical nucleotide sequence. The nucleotide sequence is considered

“theoretical” because a particular amino acid can be defined by more than one codon, due to the degeneracy of the DNA code. Therefore, an oligonucleotide based on a protein sequence is either a “guessmer”, which is the most likely nucleotide sequence based on probabilities, or degenerate, a mixture of oligonucleotides representing all possible nucleotide combinations.

As no P68/62 or homolog nucleotide sequences are known, P68/62 cloning work was based on previously obtained P68 amino acid sequences. Previous P68 purification and sequencing work from our lab had generated three peptides (see Appendix I). These amino acid sequences were used for various cloning strategies. These strategies included oligonucleotide and antibody screening of testis cDNA libraries, as well as this work, whereby degenerate primers based on two of the P68 peptides were used in a reverse transcription-polymerase chain reaction (RT-PCR) of boar testis RNA.

Although cloning successes have been achieved by oligonucleotide or antibody screening of cDNA libraries (Kinloch *et al.*, 1988; Young and Davis, 1983), the RT-PCR approach was also initiated because it is a very sensitive technique and it can be performed relatively quickly. In addition, an RT-PCR strategy had been successful in obtaining the cDNA sequence of a novel protein (Takahashi and Shirasawa, 1994; Matsuoka *et al.*, 1993).

The success of the PCR depends largely on the primers, since the amplification of a template sequence is controlled by specific primer binding. However, other reaction conditions must be optimized because they can also affect the outcome. The most important of these conditions include: MgCl₂ concentration, the amount of template DNA

(and for RT-PCR, the amount and type of RNA), the amount of polymerase, and the number and length of the PCR cycles.

The $MgCl_2$ concentration is an important factor to consider because it affects the action of the polymerase. The Taq polymerase enzyme requires $MgCl_2$ in order to function. However, if the concentration of $MgCl_2$ is too high, it may result in the amplification of non-target sequences (Saiki, 1989). Conversely, if it is too low, the efficiency of the enzyme is reduced.

The amount of template DNA should be high enough that PCR products can be visualized after the standard 30 cycle program. RT-PCR generally requires more template RNA to compensate for the potential loss incurred during the additional cDNA synthesis step. The extra handling during this step and/or inefficient reverse transcription may cause the loss of target cDNA sequences for the PCR that follows.

Furthermore, mRNA is often used as the reverse transcription starting material, instead of total RNA. In this way, the opportunity to amplify only coding sequences is increased by eliminating cDNA synthesis of the very abundant rRNA species. However, the process of mRNA isolation adds another step where the RNA message of interest could be lost. Therefore, for RT-PCR, a balance must be made between the amount and type of starting RNA.

The amount of DNA polymerase in the PCR can be adjusted to improve the likelihood of success. The polymerase is partially inactivated during the high temperature denaturation steps of the PCR program. Therefore, enough enzyme must be added to ensure that there is still some active enzyme for the last amplification cycle of

the program. However, it is being wasted if the amount of enzyme is too high.

Finally, each step of the PCR must be long enough to carry out its function, and the number of PCR cycles must be high enough that the PCR products can be visualized. For example, for maximum product amplification, the PCR extension step must be long enough to synthesize the complete sequence between the two primers. However, should the cycles take too long to complete, or should there be too many PCR cycles, the active life-span of the enzyme is unnecessarily shortened, and the amplification of a specific product is compromised.

Eight trial polymerase chain reactions were performed to determine the optimal RT-PCR conditions for potential amplification of P68 cDNA. Since the expected size of the correct amplicon was not known, valid results were defined as those where amplified products were different from the negative control's amplified products and where the non-specific background amplification (often seen as a long smear on stained gels) was as low as possible.

Total RNA and mRNA were prepared from boar testis tissue as described in Chapter 2. The quality of the total RNA was determined by the $A_{260/280}$ and its appearance after agarose gel electrophoresis and ethidium bromide staining (Fig. 1). The $A_{260/280}$ of 1.8 indicated that it had been isolated with little protein contamination. Since proteins absorb at 280 nm, a lower value would indicate an increase in protein contamination (Ausubel *et al.*, 1992).

The absorbance reading estimated the purity of the preparation, but it gave no indication of the sizes or state of the RNA. Agarose gel electrophoresis of the RNA

followed by ethidium bromide staining revealed if the range of native RNA species had been extracted and if the RNA was still intact. On the ethidium bromide-stained gel, the boar testis total RNA was visible as a long, continuous streak of RNA species with strong 28S and 18S rRNA bands (Fig.1). The sizes of the 28S and 18S rRNA species are 4.4 kb and 2 kb, respectively. Therefore, the presence of RNA species above, in between, and below these bands on the gel demonstrated a good size representation of the total RNA. As 28S and 18S rRNA are very abundant RNA species, the appearance of strong 28S and 18S rRNA bands indicated that little RNA had been degraded. The absence of low molecular weight RNA spots also suggested that there had been no degradation during the extraction process. Therefore, the extraction procedure had collected a wide range of intact RNA species for reverse transcription.

To save all the mRNA for reverse transcription, its quality was assumed to be similar to that of the total RNA preparation. Therefore, only the yield was assessed. The percentage yield was used to approximate the amount of isolated mRNA. The relative amount of mRNA present in eukaryotic cells is approximately 3-5% of the total RNA (Stryer, 1988; Alberts *et al.*, 1989). Therefore, the 3.4% mRNA yield suggested that an average amount of mRNA had been isolated. Analysis of the cDNA synthesis products following reverse transcription would be used to indicate whether mRNA of a variety of sizes had been isolated (and transcribed).

From the autoradiograph of the cDNA products (Fig. 2), it was clear that transcription of a range of mRNA species had occurred. The control lane, containing cDNA transcribed from mouse spleen total RNA, was used to assess the sizes of the

cDNA products from the boar testis mRNA. The 28S and 18S rRNA bands were visible in the mouse control lane. Comparison of the boar testis cDNA products to these size markers suggested that transcripts greater than 4.4 kb to transcripts smaller than 2 kb had been reverse transcribed from the boar testis mRNA. Based on the protein size of P68 (~68 kDa), the full-length P68 cDNA may be as large as approximately 2000 bp. The actual size of P68 cDNA may be much smaller because P68/62 is known to be glycosylated (Tanphaichitr, unpublished results), but the extent is not known. In any case, the P68 mRNA size was clearly represented by the range of cDNA sizes visualized on the autoradiograph.

The results of a standard PCR using the boar testis cDNA under optimized conditions (see Methods 2.5a) are shown in Figure 3. A size comparison of the PCR products from boar testis with those of the mouse spleen negative control revealed that none of the former products were unique. Therefore, it was likely that the boar testis PCR products were a result of non-specific amplification. Some non-specific amplification is expected since the PCR primers were highly degenerate (as high as 512-fold) and may hybridize to a variety of DNA sequences. However, size identity is not conclusive proof that two amplified products are the same. To be sure, DNA sequencing would have to be performed on all the PCR products. This would be very time consuming and may not uncover a PCR product of interest. Instead, the PCR was repeated, but using the touch-down technique (Don *et al.*, 1991).

Using this technique, the likelihood of amplifying the most primer-specific product is increased by optimizing specific primer annealing (Don *et al.*, 1991; Ault *et al.*,

1994; Osterrieder *et al.*, 1994 ; Hecker and Roux, 1996). The boar testis cDNA as well as an aliquot from an unamplified UNIZAP mouse testis cDNA library were used as the template DNA. PCR products were obtained in both cases (Figs. 4 and 9). However, PCR products were also detected for the negative control reaction using mouse spleen cDNA, suggesting at the time that non-specific primer annealing had not been completely eliminated. Approximately 25 differently-sized boar testis PCR products and 5 mouse testis PCR products could be identified from the ethidium bromide-stained agarose gel. Some of these products were visibly unique from the negative control PCR products. Therefore, all the mouse testis PCR products and as many as possible of the boar testis PCR products were subcloned and sequenced.

Based on partial or complete DNA sequencing, none of the clones contained an open reading frame. Even the most interesting clone, boar testis clone A, contained both P68 peptide sequences on one strand, but in different reading frames. One of these reading frames contained many stop codons. The clone was re-sequenced to confirm these results and the sequence was still the same.

What makes clone A interesting is that the antisense strand to the one containing the P68 sequences had a very high homology to human cleavage signal 1 (HCS1) protein. Like P68/62, HCS1 protein is a protein doublet and a sperm surface protein that is evolutionally conserved (Javed and Naz, 1992; Naz, 1992). HCS1 protein is believed to be involved in early cleavage of the fertilized oocyte (Javed and Naz, 1992; Naz, 1992). Clone A is not believed to be a P68 clone and may be the pig homolog of HCS1.

Due to the high homology of the clone A and HCS1 DNA sequences, a PCR of

boar testis and human lung genomic DNA was performed to confirm that clone A was not the result of PCR amplification of human DNA contamination. The primers designed for this PCR, and the sequence they were to amplify, targeted a region of the clone A sequence that differed the most from the HCS1 sequence. Sequencing of the boar testis PCR products revealed that they matched the targeted region of clone A. However, clone A's exact sequence was also amplified from the human lung DNA (Fig. 7). Therefore, the primer annealing conditions may not have been stringent enough for the subtle sequence differences between the two species. However, even if the PCR primers were able to bind to a human DNA sequence, the sequence between the primers should match the HCS1 sequence and not clone A. Since it did not, no conclusion can be drawn regarding the origin of the clone A sequence. Clone A could still be used for further study of the role of HCS-1 protein in the pig.

The TD-PCR method used to obtain clone A, or the antibody and oligonucleotide library screening methods, consistently yielded a large number of clones. However, none of the clones were promising ones (i.e., containing an open reading frame; having relevant homologies to known proteins; or lacking any protein homologies, which would indicate it was a novel protein). The high degeneracy of the P68 nucleotide sequences and the unavailability of other cloning tools made cloning success difficult and unpromising.

In addition, 5 out of 12 amino acids from one of the P68 peptides were identical to amino acids found in a 12 amino acid peptide from RSA (Appendix I). Despite this finding, P68/62 is not albumin for the following reasons. During P68/62 protein

purification, most salt and acid chromatofocusing fractions containing only 68 and 62 kDa bands cross-react with the antiSLIP1 antibody, but not with antiRSA (Fig. 13). Also, SLIP1 (of which P68 is a component) is N-terminal blocked (Lingwood's and our unpublished results), unlike albumin. And, P68/62 inhibits sperm-egg binding, whereas albumin does not (Tanphaichitr *et al.*, 1993).

Due to the P68 cloning difficulties regardless of the approach, and because one of the P68 peptides had partial homology to albumin, the project was refocused on obtaining more P68 peptide sequences. The new peptide sequences could yield less degenerate nucleotide sequences and would offer alternative cloning tools.

4.2 First Attempt at P68/62 Protein Purification and P68 Microsequencing

Even though previous P68 microsequencing had resulted in poor sequences for cloning work, P68 was selected again for sequencing. P68 was selected because it cross-reacts more strongly with the antiSLIP1 antibody, one of the original characteristics used to define SLIP1. Furthermore, P68 electrophoreses as a sharper protein band compared to P62. This trait would be useful when identifying the protein on silver-stained gels, immunoblotting results, and when a sharp band had to be excised from a blot for microsequencing. Finally, P68 is the slightly more abundant protein of the doublet. Therefore, fewer rounds of protein purification would be necessary.

P68/62 purification was begun using a procedure that had already been developed in the lab (Tanphaichitr *et al.*, 1998). This procedure involved the extraction of P68/62 from boar sperm with a solution containing a low concentration of ATP, EDTA, and

sucrose (AES), followed by dialysis, protein concentration by lyophilization, and chromatofocusing. From AES extraction to chromatofocusing, the procedure required approximately two weeks. Only ~100 ng of pure P68/62 was obtained from one chromatofocusing run because only 1/5th of the boar sperm protein extract could be processed at a time.

In order to increase the efficiency of the procedure, a new chromatofocusing method was developed that employed FPLC. Using FPLC, the time required for each chromatofocusing run was reduced from 2 days to 4½ hours. In addition, a larger chromatofocusing column could be used. With these changes, the amount of purified P68/62 was doubled to ~200 ng for the two week period.

Chromatofocused fractions were initially characterized by silver-stained SDS-PAGE and immunoblotting analyses using antiSLIP1 and antiRSA (Fig. 13). The silver-stained SDS-PAGE results quickly identified the fractions that contained only a P68/62 doublet. Comparison of the silver stained gels of fractions from different chromatofocusing runs revealed that fractions containing a P68/62 doublet could be isolated successfully from a very large number of proteins in the AES extract. Furthermore, the doublet-containing fractions were eluted predictably over the course of a run.

The P68/62 doublet visualized by silver stained SDS-PAGE could be composed of proteins other than the P68/62 component of SLIP1. Therefore, immunoblotting studies were used to identify fractions that contained this protein component of SLIP1. Immunoblotting with the antiSLIP1 antibody was performed for all potentially pure

P68/62 fractions as well as for other selected fractions (Fig. 13). As this antibody defines the original SLIP1 protein, which includes the SGG binding (Lingwood, 1985) and ZP binding (Tanphaichitr *et al.*, 1993) component, antibody cross-reactivity to the purified P68/62 suggested that the P68/62 doublet was a component of SLIP1.

However, the antiSLIP1 antibody cross-reacts with albumin, also ~68-69 kDa, and some albumin-like sequences were found in one of the previously obtained P68 peptide sequences. For both of these reasons, antiRSA immunoblotting was performed following antiSLIP1 immunoblotting to exclude antiSLIP1 cross-reactive fractions that may also contain albumin. P68/62 fractions were identified that cross-reacted with antiSLIP1 and not with antiRSA (Fig. 13). And as with the silver staining results, comparison of the immunoblotting results from different runs revealed that the pure P68/62 fractions were eluted consistently.

Based on P68/62's consistent elution pattern, it was predicted that the protein is acidic. The purest P68/62 fractions (i.e., with little or no other co-eluted proteins) consistently eluted in the high salt wash (~ 1.4-1.7 M) and the 1 M acid fractions, suggesting that the protein must be interacting strongly with the resin. Since the resin is positively charged, P68/62 should be highly negatively charged or acidic. Furthermore, serum albumin, also an acidic protein, was eluted in most fractions of the pH gradient (~ pH 5 to 4.3). P68/62 must be more acidic than albumin because the pH gradient alone was not enough to elute P68/62. However, P68/62's ionic interaction with the resin may interfere with its elution based on the pH change. Therefore, although the exact pI cannot be determined by the elution results of this chromatofocusing method, what can be

concluded is that P68/62 is probably a highly acidic protein that is separable from albumin.

Mid-way through the purification process, additional immunoblotting of the fractions with anti-keratin was added. It was discovered that, besides albumin, an additional 68/62 kDa protein, keratin, may be present in the “pure” fractions. Keratin is a common contaminant of protein work (B. Bérubé, personal communication; Golaz *et al.*, 1996) through the shedding of skin and hair cells. New immunoblotting results indicated that antiSLIP1 antibodies cross-reacted with the 68 kDa keratin, but that the anti-keratin antibody did not cross-react with P68 (Fig.14). From these immunoblotting results, P68/62 fractions that did and did not contain keratin were identified.

Consequently, sequencing-grade purity for the P68/62 fractions was re-evaluated. Fractions were characterized based on the results of silver-stained SDS-PAGE and immunoblotting with antiSLIP1, antiRSA, and anti-keratin antibodies. P68/62 purity was defined and fractions were pooled according to four grades (Fig. 15), whereby grade I was the purest preparation and grade IV was the least pure.

These evaluations were based on physical characteristics of the protein, but purification of P68/62 was also evaluated at a functional level. As expected, the *in vitro* addition of biotinylated P68/62 to the isolated zona pellucidae of various mammals resulted in significant binding to the entire surface of all ZP's tested. In addition, when P68/62 was added to *in vitro* gamete coinoculates, it inhibited sperm-egg binding in a dose-dependent manner (Tanphaichitr *et al.*, 1998). Therefore, the protein that had been purified was functionally active in zona binding. Furthermore, the purified P68/62

maintained its SGG binding ability, as demonstrated by its ability to bind to an SGG affinity matrix (Tanphaichitr *et al.*, 1998). This functionally active, purified P68/62 was used as a standard for characterizing human sperm P68 in later studies (Rattanachaiyanont *et al.*, submitted).

The amount of pure P68/62 was estimated from comparative densitometric analyses of the silver stained SDS-PAGE of P68/62 fractions and known amounts of co-electrophoresed RSA. Due to the high sensitivity of silver stain, only nanogram amounts of P68/62 was required. Although the binding mechanism of silver molecules to proteins is unknown, and therefore the staining may not be proportional to the protein loaded, with such low amounts of protein, there was visibly a linear range where the amount of protein loaded correlated directly with the staining density (Fig.10). The only minor drawback was that the P68/62 sample could not be salvaged.

The protein sample can be retrieved after A_{280} readings, but this method could not be used in this case. An unidentified component of the protein sample was skewing the readings such that they were consistently an overestimate of the amount of protein present. The overestimation (~ 1000 x) was obvious when the sample was subjected to SDS-PAGE and silver staining and/or immunoblotting. No explanation has been found to explain the overestimate.

Quantitation methods based on the Bradford assay, such as the Bio-Rad microassay used to quantitate the AES protein extract, were also inappropriate in this case. Like the silver stain method, the dye irreversibly affects the protein sample such that it could not be used for sequencing. However, the Bradford-based methods require

much larger amounts of protein than the silver stain method.

Based on the estimates from silver-staining analyses, approximately 900 ng of grade I P68/62 had been purified. For a 68 kDa protein, the minimum amount of protein suggested for microsequencing was 20 pmol (John Leszyk, personal communication) or 1.36 μg . Therefore, the 424 ng of grade II P68/62 was added to the grade I pool for sequencing. The only difference between the grade I and II pools was that the grade II pool contained other higher or lower molecular weight protein bands on the silver stained gels. These other proteins were significantly larger or smaller than P68, so they would not interfere with the isolation and sequencing of the P68 protein band. The combined grade I and II P68/62 pool was still slightly under (1.35 μg) the suggested amount, but in all questionable cases during P68 quantitation, the quantity of protein was presumably underestimated.

Before blotting and sequencing the grade I+II P68/62 pool, the lower grade pools were used to optimize the conditions for P68/62 in all the required procedures. Only the resolubilization in Laemmli's SDS-PAGE sample buffer proved difficult. An insoluble precipitate formed when the lyophilized protein was mixed with the sample buffer. This may have been due in part to the presumably low pI of P68/62, since increasing the pH of the sample buffer facilitated the solubilization. However, the precipitate could never be completely resolubilized.

The precipitate may have been protein that had aggregated irreversibly or it may have been a contaminant. To determine if the precipitate was aggregated P68/62, the supernatant of the protein suspension was electrophoresed and silver stained. Heavy

protein staining was visible, indicating that a large amount of protein was present in the supernatant. This suggested that P68/62 had been resuspended in the sample buffer and that the precipitate might be a contaminant. If this is the case, the levels of the contaminant may have been too low to be detected in the analyses of individual P68/62 fractions. It was only once the fractions were pooled that the contaminant was present in high enough amounts to affect the overall solubility of the protein solution. Since there seemed to be a large amount of P68/62 that had been solubilized in the sample buffer, subsequent lyophilized protein samples were resolubilized as much as possible, centrifuged, and only the protein in the supernatant was used for microsequencing.

Before sequencing, P68 was digested with trypsin and from the digest profile, good candidate peptides were selected for sequencing. Good candidates were peptides that were present in high enough amounts for sequencing and whose peak profiles suggested that they were pure, single peptides. Also, since albumin sequence had been obtained previously, the peptides selected were ones that were not of an expected size for an albumin tryptic digest.

Eighteen amino acids were obtained from the first peptide selected. Comparison of the amino acid sequence of this peptide to the protein databases revealed that it was a 100% match to the tail region of human keratin 9 (Fig. 21). The sequence of the tail region of all keratins is very unique to each keratin. Therefore, despite the precautions that had been taken during the preparation of P68, it was very unlikely that the sequenced protein was not keratin.

This postulation was confirmed by the fact that the complete P68 digest profile

was almost an identical match to the profile of a previously sequenced keratin (Fig. 22), as well as by the following observations. First, no positive fluorescence was observed on mouse and pig sperm for indirect immunofluorescence studies performed in our lab with anti-keratin antibodies. Therefore, unlike P68, it did not appear that keratin was found on the sperm head. Second, no anti-keratin cross-reactive proteins were detected in the AES protein extract or on old P68 blots from the lab's first sequencing attempt of P68. Third, a wash of the FPLC injection port contained keratin (Fig. 24). In addition, keratin contamination could be caused by the re-use of gloves, the outsides of which could have come in contact with the bare hand.

4.3 Second Attempt at P68/62 Protein Purification and Microsequencing

A second attempt at P68 sequencing was carried out with extensive precautions to avoid keratin contamination (as described in Methods). The most important changes were that a cap, gown, and only new gloves were worn at all times; materials that came in direct contact with the purified protein (such as dialysis tubing) were handled as little as possible; new materials and equipment were dedicated for P68/62 purification; and 12% (instead of 10%) acrylamide gels were used in conjunction with a longer electrophoresis time for increased separation of P68 from other similar-sized proteins like albumin and keratin.

P68/62 fractions were analyzed and pooled as before except the three final pools were: I+II- approximately 0.5 μg of pure P68/62, IIIa- approximately 120 ng of P68/62 with relatively very little albumin, and IIIk- approximately 280 ng of P68/62 with

relatively very little keratin contamination. For this protein sequencing attempt, the microsequencing facility had been improved such that it was able to digest and sequence 7-10 pmol of protein. Therefore, pool I+II was sequenced separately from IIIa and IIIk.

As described in the Results section, the digest chromatogram for pool I+II did not contain any peptides expected from a keratin or albumin tryptic digest. Therefore, the purification had been successful in eliminating both albumin and keratin from P68. Sequencing of four major peptides revealed that one was identical to the carboxyl region of bovine casein precursor protein and the remaining three had high homologies to various regions of human testis arylsulfatase-A (AS-A) (Fig. 30).

The casein sequencing result suggests that casein is a component or part of P68. P68 is not casein because the casein peptide was present in relatively large amounts compared to the other P68 digest products. If P68 was casein, other casein peptides would be expected in similarly high amounts. Furthermore, the masses of the remaining P68 peptides were checked for possible matches to expected casein digest peptides and none were found. Therefore, the digest results suggest that only the one casein region is part of P68.

Alternatively, this sequencing result may have been obtained because casein had contaminated the P68 sample (with milk being the most likely source). However, this is unlikely because the sequenced P68 never had the opportunity to come in contact with milk. In addition, other casein peptides would be expected in the digest profile. Since none were detected, again the more plausible conclusion is that P68 contains a casein domain.

Since dissimilar proteins often share similar functional domains (i.e., a region with a specific functional activity) (Gmachl and Kreil, 1993; Blobel *et al.*, 1992; Blaschuk *et al.*, 1990), the casein sequence in P68 may indicate an important functional region that may be found in other proteins as well. However, based on a database search of known protein sequences, this casein sequence was not found in other proteins. Therefore, it may still be a functionally important region of P68, but it is unique to casein and P68.

For P68, this casein sequence may function as an SGG binding domain. The casein peptide has an angiotensin and opioid factor activity (Fiat and Jollès, 1989) and SGG is known to be a receptor for an opioid factor (Wu *et al.*, 1986). Furthermore, the casein sequence is very hydrophobic (50%) and would be able to interact with the lipid.

The idea that P68 contains a casein domain that interacts with SGG is a provocative one, but further experimentation is still required to confirm that the casein domain is part of P68. Indirect immunofluorescence of boar sperm with anti-casein antibody and anti-P68 antibody could be used to verify whether casein colocalizes with P68/62 on the sperm head. A positive result would provide indirect evidence that casein is a part of P68/62. However, casein may still be a part of P68/62 if a negative result is obtained. A lack of sperm head labelling may indicate that the domain is not accessible to the sperm surface, not that the casein domain is not there. Immunoblotting of the AES extract and P68/62 using an antibody against this casein sequence could also confirm the presence of a casein domain in P68.

Since the remaining three peptides had matched human testis AS-A sequences, the

complete AS-A sequence was analysed to determine if a casein-like sequence could have come from a region of the AS-A sequence. No homologous regions were found. Therefore, the casein-like P68 sequence was distinct from the other sequences.

Based on the analysis that human testis AS-A (Swiss Protein # P15289) has a total number of 507 amino acids with a derived molecular weight of 53 588 Da and the 19 amino acid casein peptide has a derived molecular weight of 2510 Da, the expected size of a polypeptide containing both of these components is 56 098 Da. Since the size of P68 is approximately 68 kDa including glycosylation, it is possible that these two components are parts of a single polypeptide sequence. Glycosylation could account for the difference in the expected polypeptide's and actual protein's molecular weight.

The three P68 peptides matching the AS-A sequences presented a strong case regarding the general identity of P68. The AS-A regions matching the P68 sequences were spread out over the AS-A protein: one at the carboxy terminal end, one in the middle of the protein, and one at the amino terminal end. The sequences were obtained from the most abundant peptides produced by the digest. The overall P68 digest chromatogram was consistent with an AS-A tryptic digest chromatogram (John Leszyk, personal communication). Therefore, all of these results suggest that AS-A is a major component of P68.

This suggestion is supported by preliminary sequence and immunoblotting analyses. From the human testis AS-A sequence, the number and size of expected tryptic peptides was calculated (Fig. 31). Based on the assumption that trypsin cleaves on the carboxy terminal side of arginine and lysine amino acids (Stryer, 1988), the human AS-A

sequence predicts 20 differently sized peptides with 14 larger than 7 amino acids. This prediction correlated with the P68 chromatogram, which contained 14 major peaks for peptides larger than 7 amino acids (note: peak number 15 is an autolytic tryptic peptide). Furthermore, the size of the P68 peptides (Fig. 29) matched the expected peptide sizes of a human AS-A digest (Fig. 31). Therefore, analysis of the digest chromatogram strongly suggests that AS-A is a major component of P68.

Preliminary immunoblotting studies were also completed to support the sequencing results. Blotted preparations of boar sperm AES extract, P68/62, and human recombinant AS-A were probed with anti-AS-A, antiSLIP1, and antiP68. The anti-AS-A results show that a ~68 kDa protein band was present in all samples (Appendix F). Although the anti-AS-A results demonstrate that there is a common component in AS-A and P68, the antiSLIP1 antibody was used to select the protein fractions for sequencing. Therefore, to confirm that AS-A is a component of P68 (and SLIP1), it is necessary to show that the antiSLIP1 antibody cross reacts with the same 68 kDa AS-A protein band.

When the same blot was re-probed with the antiSLIP1 antibody, cross-reactive bands were detected for keratin and RSA negative control samples only (not shown). Therefore, the SLIP1-specific antibodies may have been used up and the antibody preparation had been exhausted from frequent use. There was no more antiSLIP1 available at the time, so immunoblotting was repeated later with antiP68, an antibody prepared specifically against P68. With this antibody, a common cross-reactive band was present in all samples (Appendix F). However, despite the success with the antiP68 immunoblotting, the antiSLIP1 immunoblotting should be repeated to strengthen the

results.

The antiSLIP1 results may still be negative for the AS-A sample since a recombinant AS-A protein was used. This recombinant form may be missing epitopes present in the native protein (i.e., post-translational modifications, such as the addition of sugar residues) that antiSLIP1 may recognize. Or, because antibody cross-reactivity depends heavily on the 3-D structure of an epitope, the correct sugar residues may be present but in a different orientation. Therefore, the immunoblotting should be performed with native boar sperm surface AS-A and P68/62.

In addition to these preliminary studies, many known properties common to the two proteins support that AS-A is at least a major component of P68. P68 and AS-A: are glycosylated, are of similar size, are localized on the surface of the sperm head, are expressed at the premeiotic sperm cell stage, and bind to SGG. It is also possible that P68 and AS-A have the same tissue distribution and that they both bind to the ZP, but more studies are required to confirm these possibilities. Therefore, they share not only physical but also functional characteristics.

Both proteins are glycosylated and are of similar molecular weights. From the amino acid sequence, the expected size of AS-A is 57 kDa. Multiple glycosylation and phosphorylation increase its size to 61-63 kDa, compared to ~68 kDa for P68. Despite the apparent 5-7 kDa difference in size, it is possible that P68 and AS-A are actually the same size. The size of P68/62 was calculated from migration distances after SDS-PAGE. The size of P68/62 is most likely overestimated because the P68 band migrates a little further than the 68 kDa RSA electrophoresis protein size standard (Fig. 27). This P68

band is also visualized at approximately the same position as an AS-A band on an immunoblot containing both proteins (Appendix F). Gel migration of glycosylated proteins during SDS-PAGE could also be disproportionate to their molecular weights (Lääs, 1989). Therefore, the small difference in P68 and AS-A sizes could be attributed to the inherent inaccuracies of size measurements by SDS-PAGE.

In addition to being of similar molecular weights, both proteins have been localized to the surface of the sperm head. Indirect immunofluorescence (IIF) studies have shown that SLIP1 (and presumably P68) is present in the convex ridge of the mouse sperm head (Tanphaichitr *et al.*, 1993). Using anti-AS-A antibodies, convex ridge staining was also observed for mouse sperm IIF studies, although a small percentage of sperm were stained in the post acrosomal region as well as the convex ridge of the sperm head (unpublished results). In the rabbit system, AS-A has been localized by IIF to the apical and postacrosomal region of the sperm head, and in fixed rabbit sperm, AS-A is also in the acrosome (Nikolajczyk and O'Rand, 1992). Therefore, an acrosomal form of AS-A may also exist. In any case, P68/62 and at least one form of AS-A are documented to be in the same region of the sperm head.

The expression of both P68 and AS-A appears to be similar spatially as well as temporally. *In situ* localization by immunocytochemistry within the rat seminiferous tubules revealed that SLIP1, and therefore P68, is present in spermatogenic cells, possibly starting from the spermatogonia stage (Lingwood, 1985). SLIP1 expression in spermatogenic cells was also demonstrated by immunoblotting (Lingwood and Nutikka, 1991). Immunoblotting for the detection of AS-A expression has not been performed, but

low activity levels of AS-A are found in mouse germ cells as early as the late pachytene and secondary spermatocyte stage (Kreysing *et al.*, 1994). Furthermore, the respective mRNA levels are unexplicably high and increase twenty fold in late pachytene and secondary spermatocytes, unlike in other tissues tested (Kreysing *et al.*, 1994). Therefore, P68 and AS-A are both expressed in the premeiotic spermatogenic cell stage during spermatogenesis.

The two proteins may also have similar tissue expression. Immunoblotting of tissue homogenates indicate that SLIP1/P68 is expressed in germ cells and to a lesser extent in the brain (Law *et al.*, 1988). Although AS-A protein expression studies have not been performed, the AS-A transcript is present in the testis and in many somatic tissues, but the transcriptional level is 30% higher in the testis (Kreysing *et al.*, 1994). This is countered by the finding that a testis-specific factor prevents polysome formation on AS-A mRNA (Kreysing *et al.*, 1994). Thus, AS-A activity appears to be the same in all tissues. Despite this finding, it is still possible that a special cell surface variant form of AS-A is restricted to the germ cells and the brain. Alternatively, testis-specific protein expression may be achieved through a restrictive cell surface transport mechanism. Therefore, AS-A immunoblotting studies of cell surface and cytoplasmic proteins should be carried out in order to prove or disprove a similar pattern of protein expression between AS-A and P68.

Both proteins bind SGG. SLIP1 was originally isolated based on this characteristic and P68/62 was shown to maintain this property (Lingwood, 1985; Tanphaichitr *et al.*, 1998). SGG has been identified as a substrate for AS-A (Fluharty *et*

al., 1974) and, more specifically, AS-A recognizes and binds to the sulfate group of SGG. The exact binding location of P68 has not been determined.

Furthermore, recent AS-A studies from our lab suggest that AS-A, like P68, may also bind to the ZP. Many studies have been performed to show that P68/62 binds to the ZP. In indirect studies, pretreatment of eggs with SLIP1 or P68/62 significantly reduced the eggs' ability to be bound by sperm. The minimum concentration of exogenous SLIP1 and P68/62 needed for inhibition of sperm-ZP binding to 20% of the control level was 0.4 nM and 10 nM, respectively (Tanphaichitr *et al.*, 1993, 1998). Similar results were obtained when eggs were pretreated with human recombinant AS-A (Appendix H). The results clearly showed a drastic reduction in sperm-ZP binding in the presence of AS-A, but the minimum concentration required for reduction to control levels was 0.4 μ M (White *et al.*, in preparation). A higher inhibitory protein concentration for AS-A is required probably because AS-A is only a component of the ZP binding activity of P68/62. Alternatively, because a recombinant AS-A was used, the binding domains in the recombinant form may have contained differences compared to the native protein. Therefore, the study should be repeated using native sperm surface AS-A.

These inhibition studies only suggest that P68 and AS-A bind to the egg ZP.

Another study has been performed whereby fluorescently labelled P68 was incubated with eggs (Tanphaichitr *et al.*, 1998). In this case, a direct demonstration of P68 binding to the ZP was shown. A similar study should be performed with labelled AS-A.

Furthermore, AS-A's activator may have been generated from the precursor protein during the sperm-egg interaction, which would lead to the activation of AS-A's enzyme

activity. And, providing that the ZP sugar residues included a substrate for AS-A, AS-A inhibition results may have been caused by its sulfatase activity instead of simple steric hindrance due to AS-A binding. Therefore, the presence of its activator needs to be assessed or whether the ZP's sulfated sugar residues were desulfated. Nonetheless, the studies to date provide preliminary evidence that both proteins share not only physical qualities, but also functional ones.

Many of P68/62's characteristics (Tanphaichitr *et al.*, 1998) remain to be studied in AS-A. For example, it remains to be shown that like P68, this form of AS-A: is testis-specific; is expressed in species across the evolutionary scale (so far it has been found in sea urchin testis (Hoshi and Moriya, 1980)); is present on the egg plasma membrane; is unable to induce the acrosome reaction when aggregated; and, binds to the zona pellucida and to fertilized eggs. Confirmation that AS-A possesses these P68 characteristics would strengthen the notion that AS-A is a component of P68 and SLIP1.

Once the porcine AS-A cDNA is cloned using the P68 peptides, the presence or absence of both the casein and AS-A sequences will be confirmed. Therefore, ongoing work in the lab entails using the P68 peptide sequences to isolate the complete porcine AS-A cDNA sequence. A human AS-A cDNA clone is also being used concurrently to help isolate the pig homolog. With the cDNA in hand, the presence of the casein domain and an ATP binding site would be assessed. In addition, it would be possible to generate recombinant AS-A protein to complete AS-A/P68 comparative functional studies, such as the direct ZP binding experiments.

If the results of all studies confirm that AS-A is a component of P68, recombinant

proteins based on the whole, or parts of, the cDNA could be produced to determine the exact ZP binding domain(s) of AS-A/P68. These proteins would contain deletions of portions of the polypeptide chain. Using these recombinant proteins in the established sperm-ZP binding assays, positive and negative binding would identify the active domain(s). Due to the recent acceptance that sugar residues play an important role in the interaction (Macek and Shur, 1988; Wassarman and Litscher, 1995; Tulsiani, 1997; Benoff, 1997; Chapman and Barratt, 1996), experiments using native protein to assess the effect of modifying P68 sugar residues should also be considered.

Furthermore, if AS-A is the major component of P68, it would be interesting to investigate whether ZP binding by AS-A/P68 occurs synergistically with SGG, which is also present on the sperm surface. Both sperm AS-A/P68 and SGG have already been shown to bind to each other and to the egg ZP. In working as a sperm surface complex, AS-A may interact with the sulfated sugar residues of the ZP proteins, and sperm SGG would strengthen the interaction by binding to the ZP polypeptide via its sulfate group.

Despite the fact that SGG is a substrate for AS-A (as mentioned previously), SGG is able to co-exist with AS-A on the sperm surface without SGG desulfation (Tanphaichitr *et al.*, 1990; Kornblatt, 1979). *In vitro*, purified AS-A can desulfate SGG, but only when the sulfoglycolipid is treated with detergent (Gadella *et al.*, 1992), or in the presence of a co-activator protein, saposin B (Louis and Fluharty, 1991), which presents the sulfolipid to the enzyme in the correct physical orientation (Fischer *et al.*, 1978). Saposin B is produced by the digestion of its precursor, prosaposin. A rat prosaposin homolog, SGP-1, is produced by Sertoli cells and binds transiently to the surface of

testicular sperm (Collard *et al.*, 1988; Morales *et al.*, 1996). Caput epididymal sperm lack SGP1 on the cell surface (Morales *et al.*, 1996), but prosaposin (and therefore possibly SGP-1) is present at a high level in the seminal plasma (Hiraiwa *et al.*, 1993).

Furthermore, recent studies from our lab indicate that prosaposin is present on live caudal epididymal and uterine mouse sperm and ejaculated human sperm (unpublished results).

Therefore, prosaposin (and possibly SGP-1) is present on the mature sperm surface, but until it is processed to saposin B, AS-A is not active. The presence of saposin B has not been documented, but it is presumed that it is not generated on sperm of these stages.

This proposed mechanism, requiring the generation of saposin B, would also explain why sperm SGG, despite the presence of AS-A, would not be desulfated during spermatogenesis and the early steps of capacitation (Kornblatt, 1979; Lingwood, 1985; Lingwood, 1986; Tanphaichitr *et al.*, 1990).

4.4 The Possible Role of Arylsulfatase-A on the Sperm Head

ZP binding probably involves the co-ordination of SGG and many of the documented sperm proteins. P68/62 is one such documented sperm protein. Therefore, firstly, it is possible that AS-A is responsible for P68's ZP binding activity. A model has been developed to summarize AS-A's potential involvement in the initial steps of fertilization (Fig. 33) if it is, in fact, a component of P68. P68/62's known characteristics were taken into account when developing the model.

Briefly, there exists a sperm surface form of AS-A that is extractable using the AES solution. Similar to galactosyltransferase, there may be cytoplasmic and a plasma

Figure 33- A Hypothetical Model for AS-A's Participation in the Initial Sperm-Egg Binding Event

A hypothetical model was developed to address the need to show how AS-A might be a component of P68, and, more specifically, of P68's ZP binding function. The model describes in general how AS-A might interact with the ZP and also how it may interact with SGG, a sperm surface lipid to which P68/62 has been shown to bind.

MODEL:

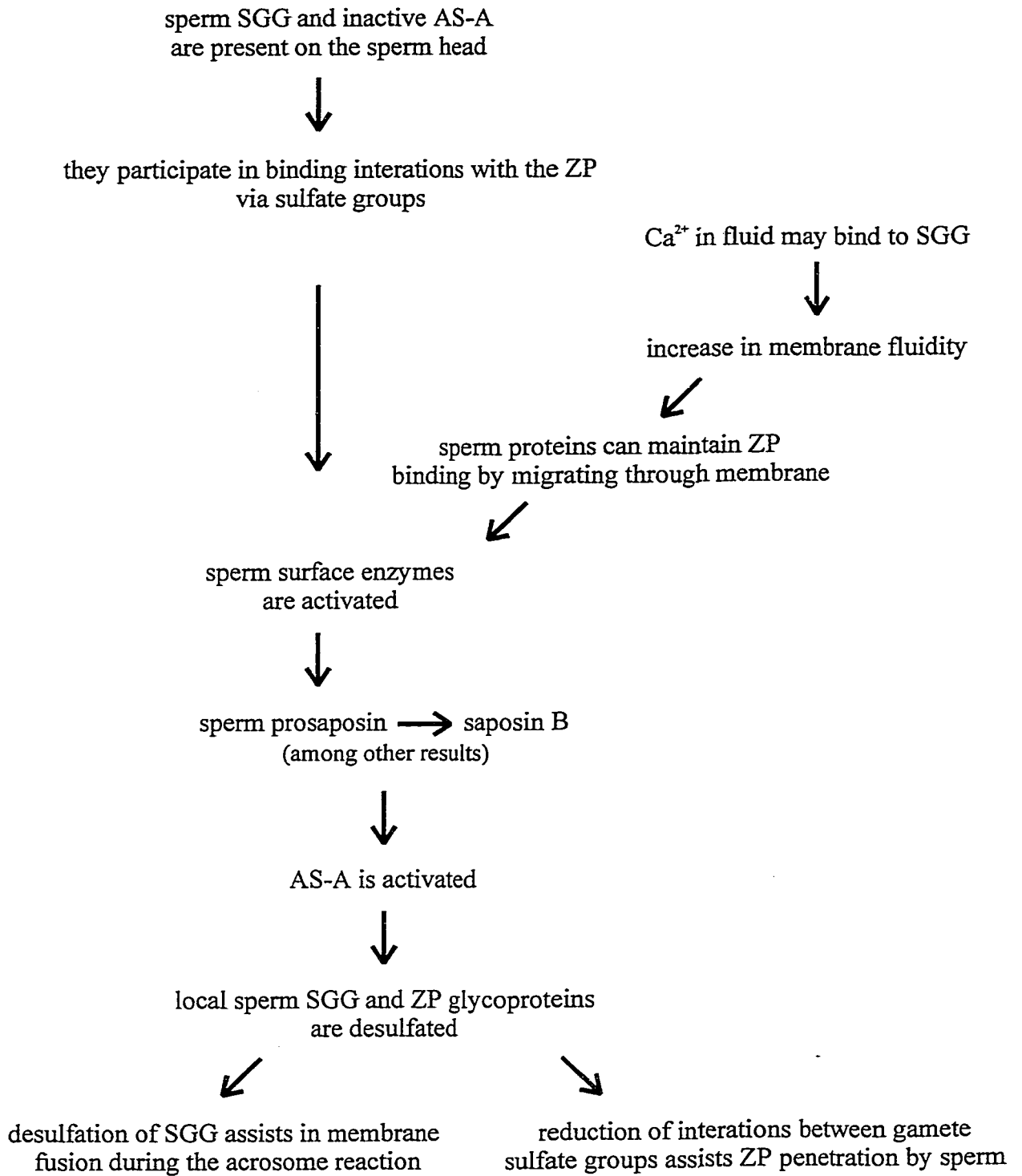


Figure 33

membrane form found in somatic cells, but sperm cells contain only the plasma membrane form. In the case of galactosyltransferase, the sperm cell surface form contains an extra domain as compared to its cytoplasmic counterpart (Miller *et al.*, 1992; Gong *et al.*, 1995). It has not yet been determined whether or not there are any differences between somatic and sperm-surface AS-A. Alternatively, there may be only one form of AS-A, but the testis, ovary, and brain (where SLIP1 expression has been detected) have a specific transport mechanism that targets AS-A to the plasma membrane surface.

Like SGG, a known substrate for AS-A, the sugar residues of the ZP glycoproteins have a high sulfate content (Nakano *et al.*, 1990; Chapman and Barratt, 1996). Therefore, on the sperm surface, AS-A may bind to the sulfate groups on the ZP oligosaccharides in a similar fashion to its seminolipid interaction. When other sperm-surface enzymes such as β -galactosidase, fucosyltransferase, mannosidase, and galtransferase are bound to carbohydrate, they may function as either hydrolases or lectins (Goldstein *et al.*, 1980), depending on the availability of substrates and/or cofactors. It has been suggested that the same may be the case for sperm surface AS-A (Farooqui and Srivastava, 1979; Dudkiewicz, 1984). Therefore, during initial sperm-ZP binding, AS-A would be acting as a lectin in the absence of its activator.

Acting initially as a lectin, AS-A, located in the post-acrosomal region of the sperm head, may complex with SGG to strengthen its binding to the ZP. AS-A may interact with the sulfated sugar residues of the ZP glycoproteins. Ca^{2+} present in the female reproductive tract fluid (Yanagimachi, 1994) may bind to SGG on the sperm head,

leading to a change in the membrane potential and an increase in membrane fluidity (Tupper *et al.*, 1994). This increase in membrane fluidity would facilitate AS-A movement. AS-A's ability to move around the sperm head may be useful/necessary to maintain contact with the ZP. Initially, the ZP proteins interact with AS-A in the post-acrosomal region, but once bound to the ZP, AS-A would be able to move and maintain its binding. It could be "pulled" to the convex ridge, as is observed for antibody immunoaggregation of sperm surface proteins (Wolf *et al.*, 1992; Moase *et al.*, 1997).

Following the initial sperm-ZP interaction, sperm surface AS-A may become a functional sulfatase. AS-A's activity would depend on the generation of its activator following ZP binding. The presumed precursor of AS-A's activator, SGP-1/prosaposin, is still present on the uterine sperm surface (unpublished results), and it may not be processed before the sperm bind to the ZP. However, upon ZP binding, sperm surface proteases identified on the plasma membrane (Lee and Wei, 1994; Saling, 1981; Boettger-Tong *et al.*, 1992, 1993) may be activated. They may be responsible for the processing of SGP1/prosaposin to saposin B (and A, C, and D). β -galactosidase, a similarly-regulated sperm surface enzyme implicated in ZP binding (Sosa *et al.*, 1991), can be activated by saposin A or C (O'Brien and Kishimoto, 1991). We postulate that the generation of saposin B by membrane proteases activates AS-A.

AS-A's enzyme activity may be required for membrane fusion during the acrosome reaction. Approximately 25% of the total sperm lipids is found in the apical sperm head plasma membrane (Gadella, personal communication) and SGG accounts for 8-10% of total sperm lipids (Ishizuka and Yamakawa, 1985). Therefore, with the

presence of such a large concentration of SGG in this region, it would be expected that SGG's negatively charged and bulky galactosyl group would be an impediment to membrane fusion during the acrosome reaction. Desulfation of SGG by AS-A followed by degalactosylation of GG by β -galactosidase would reduce this impediment. The observation that 15% of mouse sperm head SGG is desulfated after more than 1 hour capacitation *in vitro* suggests that SGG desulfation had most likely occurred in the proportion of sperm that would have spontaneously acrosome reacted in this time (Tanphaichitr *et al.*, 1990).

Furthermore, not only would desulfation of sperm SGG assist in sperm membrane fusion, AS-A may have a secondary function assisting in sperm penetration of the ZP. With the release of hydrolytic enzymes during the acrosome reaction, sperm would begin to penetrate the ZP (Yanagimachi, 1994). The desulfation of sperm SGG by AS-A would also locally eliminate SGG-mediated sperm-ZP binding, and thus assist sperm penetration through the ZP layer (White *et al.*, in preparation). Furthermore, AS-A's hydrolytic activity may also desulfate the sugar residues of the ZP proteins. Thus, removing ligands on the ZP as well as on the sperm. In this manner, sperm could detach from the ZP in the region of AS-A and penetrate further into the ZP. Secondary binding of sperm to ZP2 by other sperm proteins such as PH-20 would maintain sperm contact with the ZP (Primakoff *et al.*, 1985, Myles *et al.*, 1987).

Therefore, if AS-A is a component of P68, one can create a provocative model of its interaction with other molecules during the early stages of fertilization. However, a lot of information regarding AS-A's characteristics, as well as the mechanisms of many of

the steps, is still unknown. Therefore, this model remains quite speculative. The P68 peptides obtained from my study will be useful for cloning the full porcine AS-A cDNA sequence. From that, recombinant protein studies should substantiate many of the outstanding assumptions. The model will be useful for the direction of this future research, which hopes to elucidate the role of one component of a very complex system.

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Appendix A- Diagram of a Mammalian Sperm Head During the Acrosome Reaction

During the acrosome reaction, there is fusion of the outer acrosomal membrane and the anterior head plasma membrane of the sperm head as shown. Sperm must be capacitated and Ca^{2+} is required in order for the reaction to occur. An ionophore, A23187, plus calcium can also induce the reaction. Hybrid membrane vesicles are released along with the acrosomal contents. The inner acrosomal membrane is exposed and proteins contained on its surface can now assist any remaining plasma membrane proteins in ZP binding. A= acrosome, N= nucleus (ref.: Myles and Primakoff, 1984).

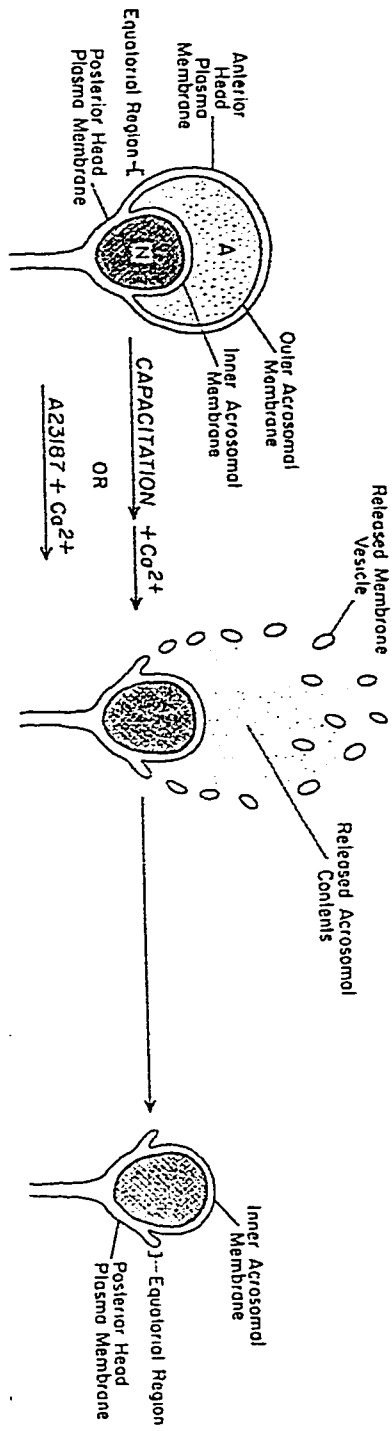


Table 1 - PCR Clones and Their Major Nucleotide and Amino Acid Homologies

The table includes fully and partially sequenced clones from the TD-PCR of boar testis cDNA and from an unamplified mouse testis UNIZAP cDNA library. The clone name, size in base pairs (bp) and the major homologies and/or other comments are provided.

Boar testis TD-PCR products		
Clone name¹	Size² (bp)	Major Homologies³/Comments
<i>Isolated by agarose gel electrophoresis followed by direct excision from the gel</i>		
A	(600), 453	- Homology of the antisense strand to human cleavage signal 1 (HCS1) protein- at the nucleotide (nt) and amino acid (aa) level (86% and 85% positives, respectively) - 10 stop codons in the sense strand
B	(350)	- Was not able to sequence the insert (repeatedly long streaky lanes on the sequencing films)
C	(240)	- Has not been sequenced
D	(200), 214	- Sequenced only once, therefore must still be re-sequenced to confirm the results
<i>Identified by double filter hybridization to labelled PCR primers (34 positives) (PCR9-1->13: could detect a signal from a 45 hour film exposure, PCR9-14->34: required longer exposure: 5 days)</i>		
PCR9-1, 2, 3	(700)	Not sequenced because approximately the same size as PCR9-4
PCR9-4	(700)	DNA sequence contained only the A1 primer sequence

¹Clones in **bold** indicate those whose DNA sequencing has been completed

²based on completed DNA sequencing; "()" = approximation based on EcoRI digestion of the plasmid containing the PCR product

³homologies are to the sense strand unless indicated otherwise; sequencing was carried out in both directions: "forward" = using the M13(-20) forward primer, "reverse" = using the M13 reverse primer

Clone Name ⁴	Size ⁵ (bp)	Major Homologies ⁶ /Comments
PCR9-5	(420), 325	- nt homologies: Human mRNA for LAR protein (cell adhesion molecule), rat nuclear protein - aa homologies: Polycystic kidney disease 1 protein, Yes-associated protein, a <i>C.elegans</i> protein similar to LAR protein, Influenza virus lipopolysaccharide core - Primer sequences in different reading frames, or in the same reading frame but with 1 stop codon
PCR9-6	(390), 209	- nt homologies: <i>C.elegans</i> cosmid R07E5, <i>S. cerevisiae</i> chromosome IX - aa homologies: cystic fibrosis transmembrane conductance regulator, fibrillin precursor, chloramphenicol <i>o</i> -acetyltransferase - One primer sequence is split over two reading frames. The other primer sequence is in one of these frames. 2 stop codons
PCR9-7, 8	(390)	Not sequenced because approximately the same size as PCR9-6
PCR9-9, 10, 11, 12, 13	no insert	
PCR9-14	(600)	Sequenced 142 bases in the forward direction: missing complete primer aa sequence Sequenced 109 bases in the reverse direction: a large number of non-coding triplets of nt sequence (ie., X)

⁴Clones in **bold** indicate those whose DNA sequencing has been completed

⁵based on completed DNA sequencing; "()" = approximation based on EcoRI digestion of the plasmid containing the PCR product

⁶homologies are to the sense strand unless indicated otherwise; "forward" = using the M13(-20) forward primer, "reverse" = using the M13 reverse primer

Clone Name ⁷	Size ⁸ (bp)	Major Homologies ⁹ /Comments
PCR9-15	(600)	Not sequenced because it is approximately the same size as PCR9-14
PCR9-16	(750)	- Sequenced 231 bases in the forward direction: nt homologies- HIV-1 proviral genes, cloning vectors - Sequenced 202 bases in the reverse direction: nt homologies- cloning vectors
PCR9-17	No band, long smear	Transformant colony was white with a blue center, so it is possible that this is not a true positive clone
PCR9-18	(550, 600)	- Sequenced 167 bases in the forward direction: 2 stop codons - Sequenced 128 bases in the reverse direction: 2 stop codons - Possible EcoRI site in insert
PCR9-19	(600)	- Sequenced 239 bases in the forward direction: 1 stop codon - Sequenced 192 bases in the reverse direction: 1 stop codon
PCR9-20	(550)	- Sequenced 155 bases in the forward direction: 3 stop codons - Sequence 144 bases in the reverse direction: 2 stop codons
PCR9-21, 23	No insert	The transformant colony colour was white with a blue center for PCR 9-23
PCR9-22	(550)	Not sequenced because it is approximately the same size as PCR9-20

⁷Clones in **bold** indicate those whose DNA sequencing has been completed

⁸based on completed DNA sequencing; "()" = approximation based on EcoRI digestion of the plasmid containing the PCR product

⁹homologies are to the sense strand unless indicated otherwise; "forward" = using the M13(-20) forward primer, "reverse" = using the M13 reverse primer

Clone Name ¹⁰	Size ¹¹ (bp)	Major Homologies ¹² /Comments
PCR9-24	(500)	A truncated A1 primer sequence was immediately next to the A2 primer sequence (i.e., no insert)
PCR9-25	No insert	May not be a true positive because the transformant colony was white with a blue center
PCR9-26	(400)	nt homologies: antisense strand homology to uncharacterized clones from a human retina and a liver/spleen cDNA library. No matches to the sense strand. aa homologies: no matches to the sense strand -Primer sequences are in the same reading frame, but it contains 3 stop codons
PCR9-27, 28, 29, 30, 31	No insert	All were white transformant colonies with blue centers except for PCR9-31 (the position of the colony didn't line up well with the corresponding signal on the film)
PCR9-32	Very faint band, ~700	Unable to sequence (may not be a true positive for the same reason as PCR9-31)
PCR9-33	550, 700	Sequenced 171 bases in the forward direction: 6 stop codons Sequenced 154 bases in the reverse direction: 1 stop codon
PCR9-34	No insert	May not be a true positive because the transformant colony was white with a blue center

¹⁰Clones in **bold** indicate those whose DNA sequencing has been completed

¹¹based on completed DNA sequencing; "()" = approximation based on EcoRI digestion of the plasmid containing the PCR product

¹²homologies are to the sense strand unless indicated otherwise; "forward" = using the M13(-20) forward primer, "reverse" = using the M13 reverse primer

TD-PCR products from an unamplified mouse testis cDNA library		
Clone Name¹³	Size¹⁴ (bp)	Major Homologies¹⁵/Comments
<i>Isolated by agarose gel electrophoresis followed by direct excision from the gel</i>		
PCR15-E	305	Primer sequences are in different reading frames, one of which contains 7 stop codons
PCR15-F	263	Primer sequences are in different reading frames: one has 7 and the other has 3 stop codons
PCR15-G	206	Primer sequences are in different reading frames: one has 7 and the other has 3 stop codons
PCR15-H	150	Primer sequences are in different reading frames, but neither reading frame contains stop codons
PCR15-I	130	Primer sequences are in different reading frames, one of which contains 2 stop codons

N.B.- The sequencing films for clones PCR9-5, 6, 19, and 33 were re-read by Dr. N. Kamolvarin, a visiting research associate, to confirm the sequences recorded.

¹³Clones in **bold** indicate those whose DNA sequencing has been completed

¹⁴based on completed DNA sequencing; "()" = approximation based on EcoRI digestion of the plasmid containing the PCR product

¹⁵homologies are to the sense strand unless indicated otherwise; "forward" = using the M13(-20) forward primer, "reverse" = using the M13 reverse primer

Appendix B- Primer Stocks

The DNA sequences are shown for the degenerate PCR primers for P68 PCR cloning and the specialized PCR primers used to amplify a region of TD-PCR clone A. For the degenerate PCR primers, information on the primers as well as the two amino acid sequences they are based on are also shown. Since the relative orientation of the two amino acid sequences are unknown, four PCR primers were necessary. The least degenerate regions of the protein sequences (underlined) were used.

The TD-PCR primers, A-1 and A-2, amplify a 130 bp region of the clone A DNA sequence (see Appendix D). Information on the primers is also given.

DEGENERATE P68 PRIMERS FOR PCR CLONING

#1: **A1**: based on the P68 tryptic peptide sequence: EYTYDFDDYSDA

- All possible codons were included in the oligo
- Degeneracy: $2^7 \times 4 = 512$ -fold
- 26-mer + BamHI linker (GGG/GATCC)-> 34-mer
- Lowest $T_m \approx 59.8^\circ\text{C}$ (Using the nearest neighbour method- Oligo Primer Analysis Software, version 4.0)

<u>Peptide:</u>	E	Y	T	Y	D	F	D	D	Y
<u>Codon:</u>	GAG	UAC	ACC	UAU	GAC	UUU	GAU	GAC	UAU
	A	U	A	C	U	C	C	U	C
			G						
			U						
<u>Oligo:</u>	GAG	TAC	ACI	TAT	GAC	TTT	GAT	GAC	TA
	A	T		C	T	C	C	T	

#2: **B1**: Antisense primer of A1 primer

- All possible codons included in the oligo
- Degeneracy: 512-fold
- 26-mer + BamHI linker -> 34-mer
- Lowest $T_m \approx 57.7^\circ\text{C}$ (Using nearest neighbour method- Oligo Primer Analysis Software, version 4.0)

<u>Oligo:</u>	TAG	TCA	TCA	AAG	TCA	TAI	GTG	TAC	TC
	A	G	G	A	G		A	T	

GENOMIC DNA PCR PRIMERS

#1: A-1:

- 26-mer
- Bases 69-94 of the complete clone A sequence:
5' CCC ATC TGA TAC CAA GCA AGA AAG TA 3'
- $T_m \cong 59^\circ\text{C}$

#2: A-2:

- 25-mer
- Bases 175-199 of the complete sequence:
5' CTC CTC AAC TGC TCC AAC TTC CTC A 3'
- $T_m \cong 58^\circ\text{C}$

Appendix C- Complete Nucleotide Sequence and Amino Acid Translations for PCR Clone A

The nucleotide sequence of both strands of PCR clone A are shown from the complete DNA sequencing of both strands. The DNA sequence of each strand was translated in each of the three possible reading frames. The amino acids are indicated by their one-letter code. The underlined amino acid sequences are the sequences of the PCR primers used. Asterisks denote stop codons. The amino acid reading frame highlighted in bold identifies the sequence that is highly homologous to a region of human cleavage signal 1 protein (Genbank accession number M61199).

N S A P R R T G E L Q Y X L V T Q W A N
E F S S Q A N R R T A V X F S D S V G K
G I Q L P G E Q E N C S T L * * L S G Q
5' GGAATTCAGCTCCCAGGCGAACAGGAGAAGTGCAGTACSCCTTAGTGACTCAGTGGGCAA
10 20 30 40 50 60
3' CCTTAAGTCGAGGGTCCGCTTGTCTCTTGACGTCATGSGAAATCACTGAGTCACCCGTT
S N L E W A F L L V A T X K L S E T P L
F E A G L R V P S S C Y X K T V * H A F
I * S G P S C S F Q L V X * H S L P C V

P R P I * Y Q A R K Y S E H R W P * H Q
P K A H L I P S K K V F R A S V A L T P
T Q G P S D T K Q E S I P S I G G P N T
ACCCAAGGCCCATCTGATACCAAGCAAGAAAGTATTCGAGCATCGGTGGCCCTAACACC
70 80 90 100 110 120
TGGGTTCCGGGTAGACTATGGTTCGTTCTTTTCATAAGGCTCGTAGCCACCGGGATTGTGG
G L A W R I G L L F T N R A D T A R V G
G L G M Q Y W A L F Y E S C R H G * C W
W P G D S V L C S L I G L M P P G L V L

L L H L E Q A L C R H L Q K * K V L R K
T A P S R T G S V Q T P P E V E S S E E
N C S I * N R L C A D T S R S R K F * G
AACTGCTCCATCTAGAACAGGCTCTGTGCAGACACCTCCAGAAGTAGAAAGTTCTGAGGA
130 140 150 160 170 180
TTGACGAGGTAGATCTTGTCCGAGACACGTCTGTGGAGGTCTTCATCTTTCAAGACTCCT
V A G D L V P E T C V G G S T S L E S S
S S W R S C A R H L C R W F Y F T R L F
Q E M * F L S Q A S V E L L L F N Q P L

L E Q L R R P * R L * D L N L K W R K S
V G A V E E A L E A V G P K S E V E K E
S W S S * G G L R G C R T * I * S G E R
AGTTGGAGCAGTTGAGGAGGCCTTAGAGGCTGTAGGACCTAAATCTGAAGTGGAGAAAGA
190 200 210 220 230 240
TCAACCTCGTCAACTCCTCCGGAATCTCCGACATCTGGATTTAGACTTCACCTCTTCT
T P A T S S A K S A T P G L D S T S F S
N S C N L L G * L S Y S R F R F H L F L
Q L L Q P P R L P Q L V * I Q L P S L A

M E K S H Y C Q L L R K Y I K M W G R M
H G K I S L L P A A E E V H K N V G Q D
A W K N L I I A S C * G S T * K C G A G
GCATGGAAAAATCTCATTATTGCCAGCTGCTGAGGAAGTACATAAAAAATGTGGGGCAGGA
250 260 270 280 290 300

250 260 270 280 290 300
CGTACCTTTTTAGAGTAATAACGGTCGACGACTCCTTCATGTATTTTTACACCCCGTCCT
C P F I E N N G A A S S T C L F T P C S
M S F D * * Q W S S L F Y M F I H P L I
H F F R M I A L Q Q P L V Y F H P A P H

S S S K S * G R L K S L L L G R S D G K
E F Q Q V I R E I K E S I V G E I R R E
* V P A S H K G D * R V Y C W G D Q T G
TGAGTCCAGCAAGTCATAAGGGAGATTAAGAGTCTATTGTTGGGGAGATCAGACGGGA
310 320 330 340 350 360
ACTCAAGGTCGTTCAAGTATCCCTCTAATTTCTCAGATAACAACCCCTCTAGTCTGCCT
S N W C T M L S I L S D I T P S I L R S
L E L L D Y P L N F L R N N P L D S P F
T G A L * L P S * L T * Q Q P S * V P F

L * V D S W Q Q Y L Q V K H L I L G K I
I V S G L L A A V S S S K A S N S R Q D
N C K W T L G S S I F K * S I * F * A R
AATTGTAAGTGGACTCTTGGCAGCAGTATCTTCAAGTAAAGCATCTAATTCTAGGCAAGA
370 380 390 400 410 420
TTAACATTCACCTGAGAACCGTCGTCATAGAAGTTCATTTTCGTAGATTAAGATCCGTTCT
I T L P S K A A T D E L L A D L E L C S
N Y T S E Q C C Y R * T F C R I R P L I
Q L H V R P L L I K L Y L M * N * A L Y

V V E I I R I F G S
S R R N H T Y I R I
* S S K S Y V Y S D P
TAGTCGTCGAAATCATACGTATATTCGGATCCC 3'
430 440 450
ATCAGCAGCTTTAGTATGCATATAAGCCTAGGG 5'
L R R F * V Y I R I G
T T S I M R I N P D
D D F D Y T Y E S G

Appendix D- Nucleotide Sequence Alignment of PCR Clone A Antisense Strand and Human Cleavage Signal 1 (HCS1) Protein

Identical sequences have been bolded. Underlined sequences refer to the plasmid vector linker sequences and double underlined sequences indicate the sequences selected for the PCR primers A-1 and A-2, used to amplify clone A sequence from human and boar genomic DNA. The asterisk indicates the location where 6 extra nucleotides were removed from the HCS1 sequence in order to continue the matches between the two sequences. A: PCR clone A DNA sequence, H: Human cleavage signal 1 DNA sequence.

A: GGAATTCAGC TCCCAGGCGA ACAGGAGAAC TGCAGTACCC
H: C TCTCATGCTA ACAGAAGAAC TGGAGTACCT

A: TTTAGTGACT CAGTGGGCAA ACCCAAGGCC CATCTGATAC
H: TCTACTGCCT CAGTGGGCAA ATCCAAAACC CCATTAGTGG

A: CAAGCAAGAA AGTATTCCGA GCATCGGTGG CCCTAACACC
H: CAAGGAAGAA AGTGTTCCGA GCATCGGTGG CTCTAACGCC

A: AACTGCTCCATCTAGAACAG GCTCTGTGCA GACACCTCCA
H: AACAGCTCCTTCTAGAACAG GCTCTGTGCA GACACCTCCA

A: GAAGTAGAAA GTTCTGAGGA AGTTGGAGCA GTTGAGGAGG
H: GATTGGAAA GTTCTGAGGA AGTTGATGCA GCTGAAGGAG
*

A: CCTTAGAGGC TGTAGGACCT AAATCTGAAG TGGAGAAAGA
H: CCCCAGAAGT TGTAGGATCT GAATCTGAAG TGGAAGAAGG

A: GCATGGAAAA ATCTCATTAT TGCCAGCTGC TGAGGAAGTA
H: GCATGGAAAA CTCCCATCAA TGCCAGCTGC TGAGGAAATG

A: CATAAAAATG TGGGGCAGGA TGAGTTCCAG CAAGTCATAA
H: CATAAAAATG TGGAGCAAGA TGAGTTGCAG CAAGTCATAC

A: GGGAGATTAA AGAGTCTATT GTTGGGGAGA TCAGACGGGA
H: GGGAGATTAA AGAGTCTATT GTTGGGGAAA TCAGACGGGA

A: AATTGTAAGTGGACTCTTGG CAGCAGTATC TTCAAGTAAA
H: AATTGTAAGTGGACTTTTGG CAGCAGTATC TTCAAGTAAA

A: GCATCTAATTCTAGGCAAGA TAGTCGTCGA AATCATACGT
H: GCGTCTAATTCTAAGCAAGA TTATCATTA A

A: ATATTCGGAT CCC

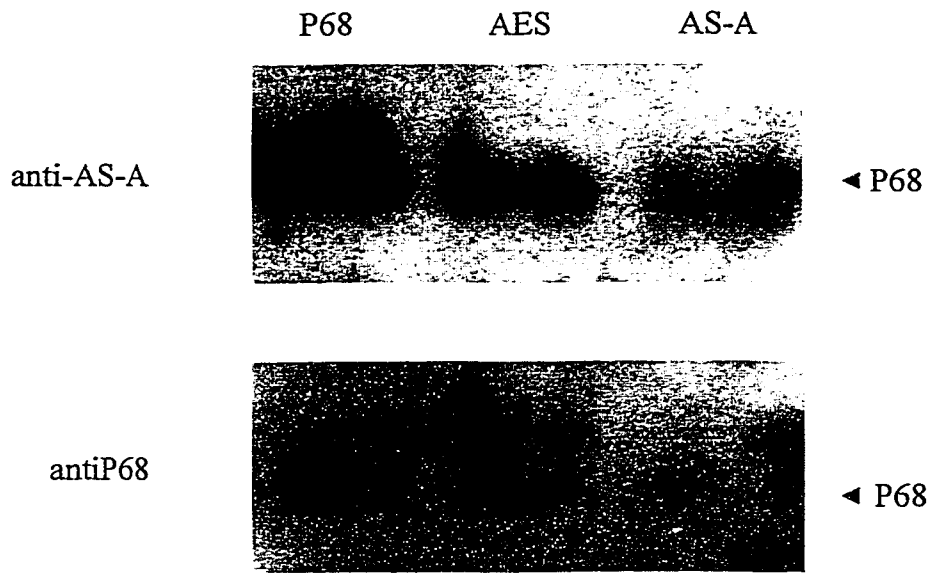
Appendix E- Amino Acid Sequence Alignment of PCR Clone A Antisense Strand and Human Cleavage Signal 1 (HCS1) Protein

Identical sequences have been bolded. Underlined sequences indicate similar amino acids between the two sequences. The asterisk indicates the location where 6 extra nucleotides (2 amino acids) were removed from the HCS1 sequence in order to continue the matches between the two sequences. The same 6 nucleotides were removed as for the nucleotide match (Appendix D). A: PCR clone A DNA sequence, H: Human cleavage signal 1 DNA sequence.

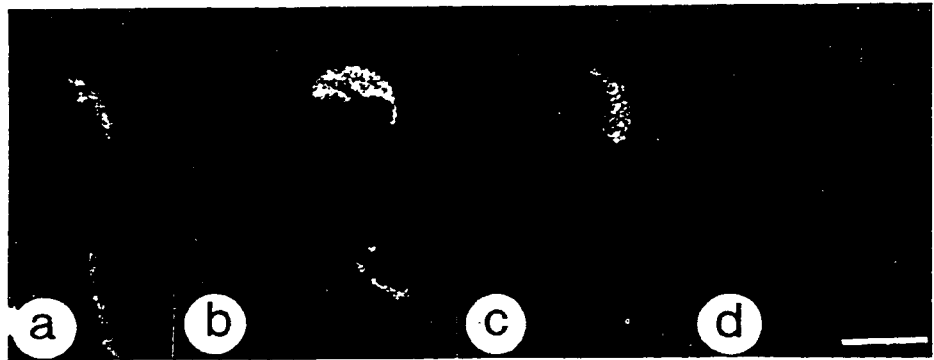
A:	SSQAN	RRTAV	PFSDS	VGKPK	AHLIP	SKKVF	RASVA
H:	SHAN	RRTGV	PSTAS	VGKSK	TPLVA	RKKVF	RASVA
A:	LTPTA	PSRTG	SVQTP	PEVES	SEEVG	AVEEA	LEAVG
H:	LTPTA	PSRTG	SVQTP	PDLES	SEEVD	AAEGA	PEVVG
A:	*PKSEV	EKEHG	KISLL	PAAEE	VHKNV	GQDEF	QQVIR
H:	SESEV	EEGHG	KLPSM	PAAEE	MHKNV	EQDEL	QQVIR
A:	EIKES	IVGEI	RREIV	SGLLA	AVSSS	KASNS	RQDSR
H:	EIKES	IVGEI	RREIV	SGLLA	AVSSS	KASNS	KQD
A:	RNHTY	IRI					

Appendix F- Immunoblotting of P68, Boar Sperm AES Extract, and Human Recombinant AS-A with Anti-AS-A and AntiP68

Protein samples were subjected to SDS-PAGE and electroblotted to nitrocellulose membrane. The membrane was probed with the anti-human recombinant AS-A, stripped and re-probed with an antibody developed against P68. The blotted protein sample is indicated at the top of each lane. The upper panel shows the results of immunoblotting with anti-AS-A, whereas the lower panel reveals the results of antiP68 immunoblotting. The relative position of the P68 band is indicated by the arrowhead in both cases.



A)

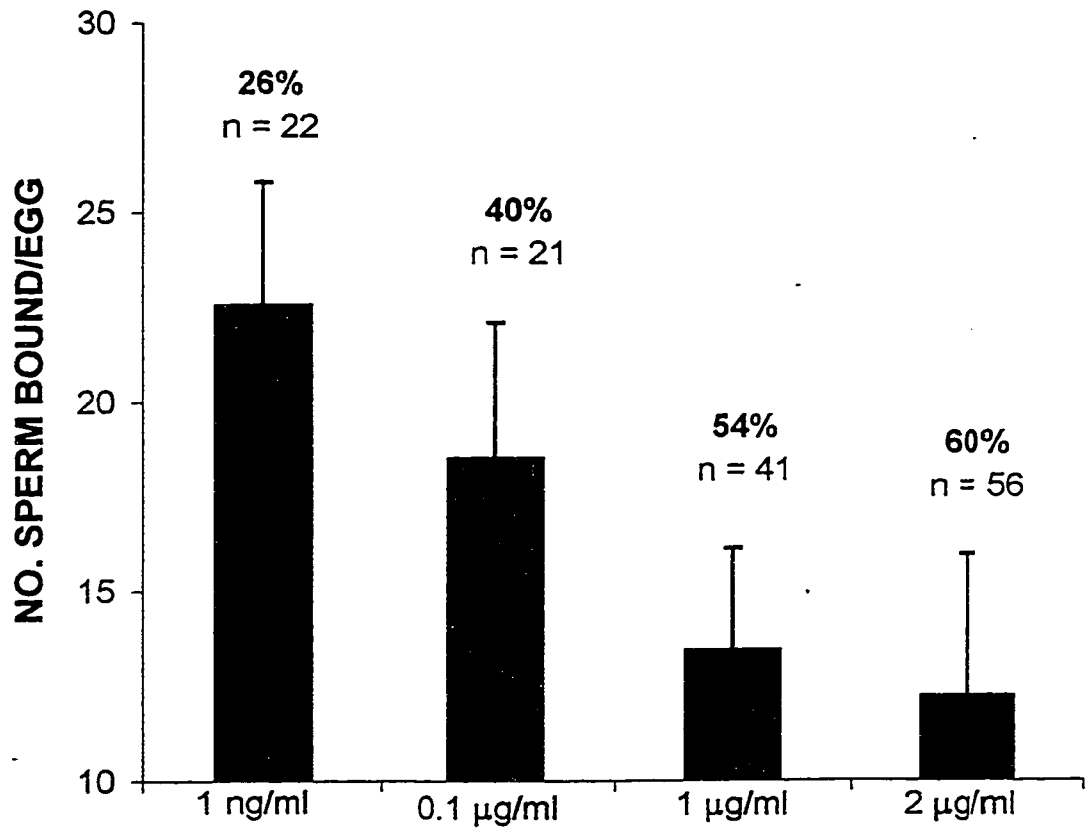


B)



Appendix H- Inhibition of Murine Sperm-ZP Binding Using Recombinant Human AS-A Treated Eggs

Eggs were exposed to recombinant AS-A or ovalbumin (control) at different concentrations (x-axis) before co-incubation with sperm. The graph demonstrates the increase in binding inhibition with an increase in AS-A dosage. "N" represents the total number of eggs counted in three separate experiments. The percentages represent the number of sperm whose binding to the egg was inhibited, as a percentage of control egg binding levels. Control eggs bound 31 ± 5 sperm.



Appendix I- P68 Peptide Sequences Obtained from a Previous Attempt to Sequence P68 Peptides

Shown are two amino acid sequences obtained from a tryptic digest of P68 (1. and 2.) and one amino acid sequence obtained from a P68 digest using V-8 protease (3.). The underlined sequences from two of the peptides were used to design degenerate PCR primers for touchdown PCR cloning of P68 cDNA. Shown below peptide 1 is a homologous region of rat serum albumin (R). Of the 12 amino acids, 5 are identical to peptide 1 (bold).

1. EYTYDFDDYSDA
- (R: EYSRRHPDYSVS)
2. HSTEIGA
3. SIVLIFAWEL

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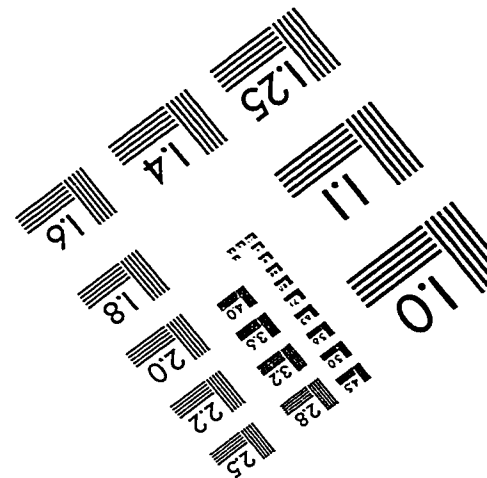
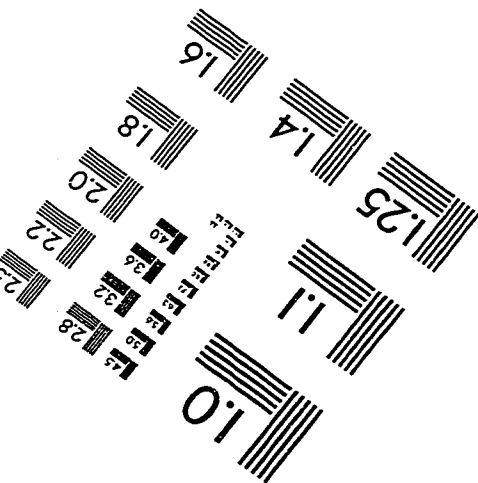
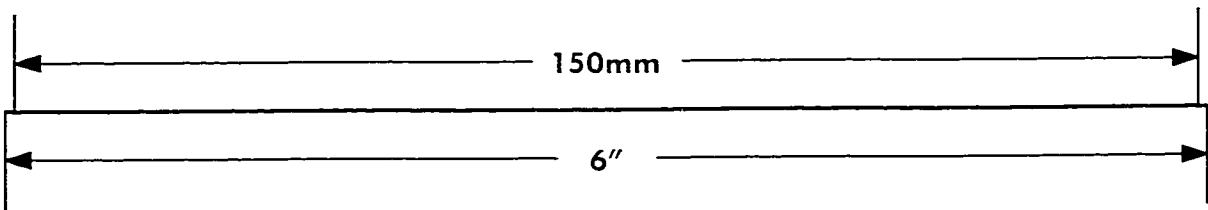
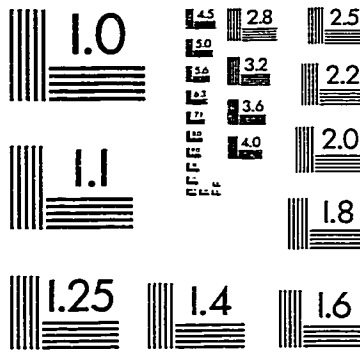
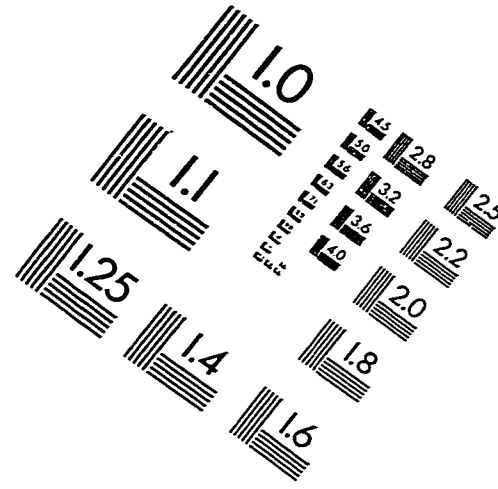
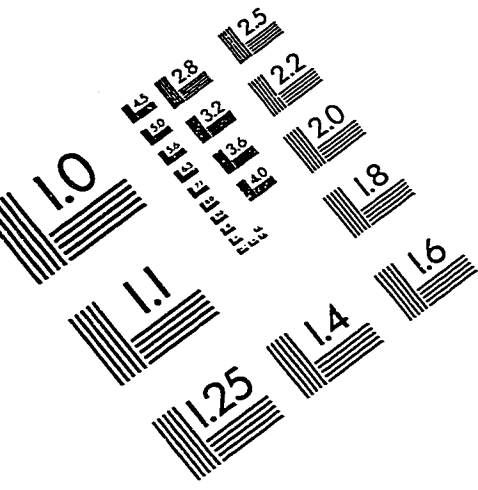
(1) Full Papers:

1. Tanphaichitr N, Moase C, Taylor T, Surewicz K, Hansen C, Namking M, Bérubé B, Kamolvarin N, Lingwood C, Sullivan R, Rattanachaiyanont M, White D. (1998) Isolation of AntiSLIP1-Reactive Boar Sperm P68/62 and Its Binding to Mammalian Zona Pellucida. *Molecular Reproduction and Development*, 49:203-216.
2. Rattanachaiyanont M, Léveillé MC, Taylor T, Rivers D, Leader A, Tanphaichitr N. (Submitted) Sulfolipid Immobilizing Protein 1 (SLIP1) is Involved in Human Sperm-Zona Pellucida Binding. *Molecular Human Reproduction*
3. Tanphaichitr N, White D, Taylor T, Attar M, Rattanachaiyanont M, Kates M. (1998) Role of male germ-cell specific sulfogalactosylglycerolipid (SGG) and its binding protein, SLIP1, in mammalian sperm-egg interaction. In: Gagnon C, ed. *The Male Gamete: From Basic Knowledge to Clinical Applications*. Cache Press, Vienna, IL. (In press).

(2) Abstracts:

1. Arylsulfatase-A is a component of boar sperm SLIP1 (P68) and may be involved in sperm-zona pellucida binding. N. Tanphaichitr, T. Taylor, D. White, M. Rattanachaiyanont, C. Moase. Abstract to be presented at the Society for the Study of Reproduction (SSR) Annual Meeting to be held in College Station, Texas in August 1998.
2. T. Taylor, C. Moase, B. Berube, N. Kamolvarin, D. Lobsinger, N. Tanphaichitr. Isolation and Characterization of Sulfolipid Immobilizing Protein 1 (SLIP1) from Boar Sperm. Presented at the Annual SSR meeting in London, Ontario, July 1996. Biol. Reprod. 54 Suppl 1:72.
3. Taylor T., Moase C., Hodgkinson b., Ahnonkitpanit V., Tanphaichitr N. Characterization of a SLIP1 tryptic peptide and the effect of its antibody on sperm functions. Presented at the Annual SSR meeting in Davis, California, July 1995. Biol. Reprod. 52 Suppl 1:123 and the Gordon Research Conference, Plymouth, New Hampshire, August 1995.
4. C.E. Moase, T. Taylor, G. Bleau, N. Tanphaichitr. Cloning of SLIP1: A Sperm Surface Protein Involved in Sperm-Zona Binding. Presented at the Montreal Reproductive Biology Research Day in Montreal, Quebec, November 1994.
5. Moase C., Surewicz K., Taylor T., Tanphaichitr N. Molecular characterization of sulfolipid immobilizing protein 1 (SLIP1), a novel sperm membrane protein, and its interaction with mammalian zona glycoproteins. Presented at the Annual SSR meeting in Ann Arbor, Michigan, July 1994. Biol. Reprod. 50 Suppl 1:147a.

IMAGE EVALUATION TEST TARGET (QA-3)



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