# ABSTRACT

## THE BIOCHEMICAL CHARACTERIZATION OF PROTEIN DE AND ITS INTERACTION WITH RAT EPIDIDYMAL SPERM.

Christopher Elliot Tubbs
Committee Chair, Joseph C. Hall, Ph.D.

Using traditional column chromatography, Protein DE has been purified from rat epididymides. Affinity, size exclusion, and ion-exchange chromatography were utilized to purify the protein to homogeneity. Protein DE purity was demonstrated using one and two-dimensional electrophoresis. Using the purified sample, an accurate molecular mass of 27,534 Daltons was determined using electrospray-ionization mass spectrometry. After four chromatographic steps, Protein DE was efficiently separated from all detectable epididymal proteins. This report provides the first rapid and reproducible method for purifying protein DE to homogeneity.

Using western blot analysis and immunofluorescence, protein D is initially detected in rat epididymal tissue and associated with sperm from the distal caput region. In contrast, when sperm were recovered from the female reproductive tract seven hours after mating, protein D was not detected by western blot, but did display faint immunofluorescence. Additionally, using photoactivatible cross-linking, a 120 KD sperm membrane protein that specifically interacts with protein D was identified. A population of membrane bound protein D was released from NaCl washed epididymal sperm when incubated in the presence of phosphatidyl-inositol specific phospholipase C. This report is the first demonstrating that both the secretion and sperm-association of protein D occur in the distal caput region of the rat epididymis. It is the only report showing western blot

analysis and immunolocalization of sperm-associated protein D on sperm deposited in the female reproductive tract after mating. Additionally, this is the first report that: (a) protein D binds specifically to a 120 KD membrane protein on the surface of epididymal sperm, (b) and that protein D is anchored or associated with a protein that is anchored to the sperm plasma membrane through a glycosylphosphatidyl inositol linkage

# THE BIOCHEMICAL CHARACTERIZATION OF PROTEIN DE AND ITS INTERACTION WITH RAT EPIDIDYMAL SPERM

BY

**CHRISTOPHER ELLIOT TUBBS**

A DISSERTATION SUBMITTED TO THE GRADUATE FACULTY OF NORTH CAROLINA STATE UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

**BIOCHEMISTRY**

Raleigh

2000

**APPROVED BY**

_____ _____

_____ _____

_____

CHAIR OF ADVISORY COMMITTEE

# Biography

Christopher Elliott Tubbs was raised in Chicago, Illinois until he was sixteen years old. He then moved to Encino, California and completed his high school education there. He then earned his baccalaureate degree in biology at North Carolina Central University. Christopher then attended North Carolina State University, where he earned his doctorate of biochemistry under the direction of Joseph C. Hall, Ph.D. He now resides in Minneapolis, MN and works as a post-doctoral fellow at the University of Minnesota under the direction of David W. Hamilton.

Christopher is married to Robin Tubbs.

# ACKNOWLEDGEMENTS

I am greatly indebted to my research advisor Dr. Joseph C. Hall, who provided me with a tremendous amount of guidance and motivation. I also wish to express my gratitude to my thesis committee, Drs Paul Wollenzien, Cynthia Hemenway, William Miller, and Thoyd Melton for their advice and help during the course of this work.

My Special thanks go to the GEM foundation, UNCF-Merck Science Initiative, National Science Foundation, and the American Society for Cell Biology for their opportunities and financial support, which made this study possible. Appreciation is extended to Dr. Elvira Doman for her invaluable mentoring and reality checks, to Dr. Barbara Leiting in her assistance in the characterization procedures and the mentoring she provided, Dr. Jerry Bryant for always being available and telling me “exactly how it is”, Mrs. Carlene Hall for always providing me with encouragement, Salman Ashraf, Yuehua Li, Reed Cooper, Tonia Hermon, Clinton Copeland, and Nequita Downling for their timely assistance whenever I asked for it. A special shout-out to my two newest doctor-friends, Christopher “Emil” Whitehead, Ph.D. and Jonathan Hefner, M.D. for supplying me with laughs and additional perspectives by watching them “go through this process” at the same time.

Finally, I want to express a very special appreciation to my loving wife and unconditional supporter, My father Robert Tubbs, Jr. and my mother and step-father Mr. and Mrs. Tim and Daphne Reid for their unwavering support and love.

Table of Contents

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| PI-PLC | phosphatidyl-inositol specific phospholipase C |
| GPI | glycosylphosphatidylinositol |
| CD | circular dichroism |
| ESI-MS | electrospray ionization mass spectrometry |
| PyroGlu | pyroglutamate |
| Sulfo-HSAB | *N*-hydroxysulfosuccinimidyl –1-4-azidobenzoate |
| SDS | sodiumdodecylsulphate |
| β-ME | beta-mercaptoethanol |
| BSA | bovine serum albumin |
| TRIS | tris-(hydroxymethyl) aminomethane |
| DEAE | diethylaminoethyl |
| Con A | concanavalin A |
| PBS | phosphate buffered saline |
| DMSO | dimethylsulfoxide |
| SDS-PAGE | sodiumdodecylsulphate polyacrylamide gel electrophoresis |
| $NaBH_4$ | sodiumborohydride |
| AEG | acidic epididymal glycoprotein |
| SP | sialoprotein |
| CRISP | cysteine rich secretory protein |
| SEP | specific rat epididymal proteins |
| pI | isoelectric point |
| CBBS | Coomassie brilliant blue staining |
| TX-100 | Triton X-100 |
| EDTA | Ethylenediaminetetraacetic acid |
| PVDF | Polyvinylidene fluoride |

# CHAPTER I

## INTRODUCTION

Mammalian sperm from the testis are unable to bind to and fertilize an ovum. Post-testicular development is necessary for fully functional sperm that can be activated to fertilize an egg. The post-testicular development is collectively termed sperm maturation and occurs in the organ adherent to the testes, the epididymis (Figure 1). As sperm traverse the organ, they undergo a series of complex maturational changes, which are necessary for fertilization, such as capacitation (Austin 1985), the acrosome reaction (Oliphant, Reynolds et al. 1985), zona pellucida binding (Primakoff, Hyatt et al. 1985) and fusion to the oolemma (Cuasnicu, Conesa et al. 1990).

## A. THE EPIDIDYMIS: STRUCTURE AND FUNCTION

The epididymis is a crescent shaped highly convoluted organ that is adjacent to the testis (Figure 1a). Spermatozoa move slowly through the lumen of the epididymis, propelled mostly by the peristaltic contractions of smooth muscle cells that surround the tubule (Junqueira, Carneiro et al. 1989), which, in most mammalian species is relatively consistent and takes about ten days (Robb, Amann et al. 1978; Amann 1981; Murakami, Sugita A. et al. 1982).

The epididymis is routinely divided into 3 anatomical segments, caput (head), corpus (body) and the cauda (tail), based on the histology and metabolic function of the secretory epithelium that lines the tubule lumen. (figure 1b**).** The epididymal epithelium

is composed of principal, clear, halo, narrow, and basal cells, which vary in percentages along the epididymal duct. This alteration in cellular makeup reflects the differing functions of the cells and the specialization of each epididymal region (Reid and Cleland 1957; Hamilton 1975). The principal cells are predominant and contain an apical region rich in various coated and uncoated vesicles, large golgi apparatus, endosomes, and lysosomes which is characteristic of cells with active secretory activities (Robaire and Hermo 1988). Conversely, the clear cells are the most active endocytotic cells, reflecting a direct role in changing the luminal fluid components along the length of the organ (Robaire and Hermo 1988). Halo cells are characterized by their clear staining cytoplasm. The narrow cells are found in only the initial segment whereas the principal cells are found throughout the length of the organ and comprise 80% of the total cells in the caput region in the rat (Reid and Cleland 1957). As one observes more distally, the relative number of principal cells decreases while the distribution of clear cells increases to 10%. Additionally, the luminal diameter of the cauda epididymis is substantially increased and the height of the principal cells decreases which is consistent with the storage of sperm in the cauda region.

Dynamic compositional changes occur in the luminal fluid from the caput to the cauda epididymis These changes include alterations in the presence and concentration of small organic solutes (Hinton 1985), ions (Jenkins, Lechene et al. 1980), pH (Levine and Marsh 1971), and protein composition, (Brooks 1981; Brooks 1981; Olson and Hinton 1985). A micropuncture method was adopted for studying the luminal composition of a large

number of ions and organic compounds in different regions of the epididymis (Hinton and Howards 1982; Turner, Jones et al. 1984). Data obtained from using this technique demonstrated that there is a 100 mM decrease in sodium ions but a 30 mM increase in potassium ions between the rat rete testis (Figure 1) and the cauda epididymis. The pH of epididymal fluid decreases from 7.31 in the testicular fluid to 6.48 in the caput and then rises to 6.85 in the cauda (Levine and Marsh 1971).

Some of the proteins found in the lumen of the epididymis are enzymes. These enzymes are thought be important in the process of maturation of sperm as they traverse the epididymis by modifying the sperm plasma membrane proteins. There is evidence that the epididymis synthesizes enzymes which differ in properties from the known enzymes in other tissues (Wong and Uchendu 1990; Ronkko 1992). Some of the active enzymes identified in the lumen include galactosyltransferase (Hamilton 1980), α-lactalbumin (Jones and Brown 1982), α-1-4-glucosidase (Plaquin, Chapdelaine et al. 1984) and angiotensin 1 converting enzyme (Hohlbrugger, Schweisfurth et al. 1982). Other non-enzymatic secreted epididymal proteins that bind to sperm are sperm inhibiting factor (Turner and Giles 1982), Protein DE (Courtens, Rozinek et al. 1982; Wong and Tsang 1982; Brooks and Tiver 1983; Cuasnicu, Gonzalez Echeverria et al. 1984; Hall and Hadley 1990; Hall and Tubbs 1997) and forward motility protein (Acott and Hoskins 1978; Brandt, Acott et al. 1978; Hoskins, Brandt et al. 1978).

The most biologically important events that occur in the epididymis are the acquisition of sperm motility and fertilizing capacity. Spermatozoa require motility for transport through cervical mucus and the egg investments (cumulus oophorus and zona pellucida). In mammals, sperm are incapable of forward, progressive motility or fertilization of an egg until they pass through the caput/corpus regions of the epididymis (Orgebin-Crist and and Olson 1984). In the cauda region, sperm can be stored in a quiescent state, where the temperature is lower than the body core, 33°C vs 37°C, for long periods of time (Brooks 1973).

The plasma membrane undergoes significant changes in the amount and composition of lipids (Aveldano, Rotstein et al. 1992). Different types of lipids are lost and gained by sperm, and they reflect the physical state of the membrane (Hall, Hadley et al. 1991). While some lipid components (phosphatidylcholine and sphingomyelin, cholesterol and phosphatidylethanolamine) decrease as sperm moved from caput to corpus, others (diphosphatidylglycerol and various unsaturated membrane phospholipid-bound fatty acids) increased in the cauda when compared to caput sperm membranes (Hall, Hadley et al. 1991). Lipid compositional changes effected in the epididymis to the sperm plasma membrane appear to enhance the stability of the membrane during sperm storage in the epididymis. These changes include a decrease in cholesterol and an increase in desmosterol and cholesterol sulfate (Legault, Bouthillier et al. 1979; Nikolopoulou, Soucek et al. 1985).

## B. EPIDIDYMAL SECRETED PROTEINS DE BIND TO SPERM

Among the many proteins secreted by the epididymis and determined to be important in fertility are proteins D and E (Martinez, Conesa et al. 1995). Protein D is secreted from the proximal caput to the cauda (Lea, Petrusz et al. 1978; Lea and French 1981; Brooks 1983; Brooks and Tiver 1983; Ensrud, Siiteri et al. 1995) while protein E is secreted only from the distal caput through the corpus (Moore and Bedford 1979; Brooks and Higgins 1980; Brooks 1982; Moore, Ensrud et al. 1994) In the literature, proteins D and E are two distinct molecules, however, they are usually referred to as protein DE because they co-purify. Additionally, most of the studies in the literature utilize purified protein DE together. Thus, in this document, unless they have been separated from each other in the cited experiments, they will be referred to as protein DE..

Once secreted into the lumen of the epididymis, proteins D and E bind to sperm (Lea, Petrusz et al. 1978; Garberi, Kohane et al. 1979; Kohane, Gonzalez Echeverria et al. 1980; Lea and French 1981; Brown, von Glos et al. 1983). Scatchard analysis has shown the D and E binding sites on sperm are saturable, with either a two step binding mechanism, or the presence of two distinct receptor populations (Wong and Tsang 1982). Only two labs have developed monoclonal antibodies that are capable of discerning between the two proteins (Brooks 1981; Moore 1981; Ensrud and Hamilton 1991; Ensrud, Siiteri et al. 1995; Xu and Hamilton 1996). Production of a monoclonal antibody to protein E demonstrated for the first time that protein E bound to a distinctly different

region on sperm (Moore, Ensrud et al. 1994). While protein D is localized to the head region and relocalizes to the equatorial region after capacitation (Cuasnicu, Gonzalez Echeverria et al. 1984; Cameo, Echeverria et al. 1986), protein E binds to the posterior acrosomal region and the tail of sperm (Moore, Ensrud et al. 1994). Importantly, proteins D and E bind to sperm in the region of the epididymis where sperm first gain the capacity to fertilize an oocyte (figure 1B) (Orgebin-Crist and and Olson 1984). Furthermore, scanning electron microscopy (Moore, Ensrud et al. 1994) localizes the proteins in a position that is consistent with a proposed role for D and E in gamete binding and fusion (Cuasnicu, Conesa et al. 1990).

## C) SPERM MATURATION

Sperm are highly differentiated cells whose only function is to bind to and fuse with an oocyte. In order to accomplish this, sperm have developed a polarized morphology (figure 2) as well as highly restrictive plasma membrane domains with specialized protein compositions (Echeverria F.M.G., Cuasnicu P.S. et al. 1982; Rifkin J.M and G.E. 1984; Tezon, Ramella et al. 1985). As described above, some of these proteins are derived from the epididymis while others are present when sperm leave the testis. The plasma membrane domains include the anterior acrosome, the equatorial segment, the postacrosomal region, the midpiece, the principal piece and the end piece (figure 2). The level of sophistication seen with protein domain restriction is illustrated by PH-20, which is a glycosylphosphatidyl inositol (GPI) linked protein. PH-20 is initially found over the

entire sperm cell membrane during spermatogenesis in the testis. By the time sperm are released from the testis, PH-20 is domain restricted to the posterior acrosome (table 1) domain where it is correctly poised for binding to the zona pellucida that surrounds the oocyte (Phelps and Myles 1987; Phelps, Primakoff et al. 1988; Phelps, Koppel et al. 1990).

In addition to the modifications of sperm membrane proteins, further maturational changes occur in the epididymis, which include changes in morphology and plasma membrane lipid composition (Nikolopoulou, Soucek et al. 1985; Dacheux, Chevrier et al. 1990; Hall, Hadley et al. 1991). When sperm enter the epididymis, a cytoplasmic droplet is present at the neck. This droplet migrates down the tail until it reaches the annulus at the end of the midpiece where it is eventually pinched off [Roberts, 1976 #424]. The cytoplasmic droplet is eventually phagocytosed from the luminal fluids by the epididymal clear cells (Robaire and Hermo 1988). Lipid compositional changes effected in the epididymis to the sperm plasma membrane appear to enhance the stability of the membrane during sperm storage in the epididymis. These changes include a decrease in cholesterol and increase in desmosterol and cholesterol sulfate (Legault, Bleau et al. 1979; Nikolopoulou, Soucek et al. 1985)

For fertilization to occur, additional spermatozoan maturational changes must occur after ejaculation and require factors present within the female reproductive tract. Although these changes are not a result of epididymal maturation, they are, however, necessary for

epididymal protein interactions with the oocyte, zona pellucida and plasmalemma. Fluid components from the female fallopian tubes remove or alter some of the sperm bound epididymal proteins that were added in order to stabilize them during caudal storage. This poorly understood process is called the capacitation reaction (Yanagimachi 1981). Incubation of capacitated sperm in the presence of either the intact zona pellucida surrounding the oocyte, or fragmented zona constituents triggers the acrosome reaction, which is the final maturational change necessary for binding and fusion with the oocyte plasmalemma (Yanagimachi 1981). The acrosomal reaction involves multiple fusions between the outer sperm plasma membrane and the outer acrosomal membrane (fig 3a). These fusion events result in the dissolution of the outer plasma membrane with the subsequent exposure of the inner acrosomal membrane and the release of powerful hydrolyzing enzymes from the acrosome into its immediate environment (Barros, Bedford et al. 1967; Ball, Leibfried et al. 1983).

Initial binding of the gametes will occur between the equatorial domain of the sperm (fig 3b) and the vitellus domain of the oocyte (fig 3c) (Gaddum-Rose 1985). The equatorial domain of the sperm must be modified during the acrosomal reaction since the sperm only gains the capacity to bind and fuse with the oocyte plasmalemma after the acrosme reaction (Yanagimachi 1981). Fusion between gametes will continue along the entire length of the tail, which eventually becomes completely incorporated into the new zygote (Bedford and Cooper 1978) (figure 3b).

## D) THE POTENTIAL ROLE OF PROTEINS D AND E IN FERTILIZATION

Recent studies have indicated that proteins D and E may have a role in gamete fusion (Lea, Petrusz et al. 1978; Cuasnicu, Gonzalez Echeverria et al. 1984; Cuasnicu, Conesa et al. 1990; Martinez, Conesa et al. 1995; Ellerman, Brantua et al. 1998). After sperm undergo the acrosome reaction, protein D becomes restricted to the equatorial domain (Rochwerger and Cuasnicu 1992) (see figure 2B and 3B), which is thought to initially bind and fuse with the oocyte (Yanagimachi 1988). Protein E, on the other hand, remains bound to the post acrosomal region, which also participates in the initial fusion event. When sperm are incubated with zona free eggs in the presence of excess proteins D and E, they bind to but do not fuse with the oocyte (Cuasnicu, Conesa et al. 1990). However, if the unbound sperm and excess proteins are washed away, bound sperm then fuse with the oocyte (Cuasnicu, Conesa et al. 1990), implying a role for proteins D and E in gamete fusion. The oocyte plasmalemma has also been determined to have binding sites for proteins D and E (Rochwerger, Cohen et al. 1992); therefore, it appears that the excess proteins D and E act to prevent oocyte receptors from encountering the sperm bound proteins. Additionally, epididymal sperm recovered from DE-immunized animals presented no changes in motility, viability, or their ability to undergo capacitation and the acrosome reaction. Furthermore, they exhibited a significant decrease in their ability to fuse with zona-free eggs, with no effect on their ability to bind to the oolemma. (Ellerman, Brantua et al. 1998). Currently, the data imply a direct role for proteins D and E in either initiating or facilitating gamete fusion.

It is important to note that another sperm surface antigen, fertilin α/β (PH-30α/β) (Blobel, Wolfsberg et al. 1992; Wolfsberg, Bazan et al. 1993), has also been implicated in at least gamete binding and fusion (Blobel, Wolfsberg et al. 1992; Almeida, Huovila et al. 1995). The α6β1 integrin was recently identified on the oocyte plasmalemma, and evidence indicates that it may be the receptor for the sperm bound fertilin α/β (Almeida, Huovila et al. 1995). Based on a portion of fertilin α with sequence similarities to certain viral adhesion/fusion glycoproteins, it has also been proposed that fertilin may function in gamete fusion (Primakoff, Hyatt et al. 1987; Blobel, Myles et al. 1990; Blobel, Wolfsberg et al. 1992; Myles, Kimmel et al. 1994).

When gametes are incubated together either in the presence of excess α6β1 integrin or monoclonal antibody, sperm-egg binding is severely inhibited; however, fusion still occurs between gametes that do bind (Almeida, Huovila et al. 1995). This suggests that similar to other protein-integrin interactions elsewhere in the body, the primary function for fertilin may be tight binding between the gametes. The combined data from fertilin as well as proteins D and E imply their primary functions are uniquely critical for different steps in the gamete binding and fusion reactions, with some potential overlap. Given the importance of gamete fusion even, it becomes imperative to better understand the mechanisms of proteins D and E association to the sperm plasma membrane.

## E) BIOCHEMISTRY OF PROTEINS D AND E

In 1976 Blaquier et al. (Cameo and Blaquier 1976) used native-PAGE to identify four soluble epididymal proteins that were sensitive to androgens in the blood. They named the proteins B, C, D and E based on their relative migration to serum albumin (A). They found that the amount of proteins B-D would decrease with time after rats were castrated and could be increased if the animal was supplemented with testosterone. The authors also noted that proteins C, D and E could be removed from caput sperm by washing. Subsequently, Hamilton and Olson (Olson and Hamilton 1978) identified a 37 kDa protein that was associated with the external face of the sperm membrane in the cauda region but not in the caput region. This protein could also be labeled by galactose oxidase [$^3$H] $NaBH_4$ and sodium metaperiodate [$^3$H] $NaBH_4$, (reactions that label galactose and sialic acid containing glycoproteins, respectively). The authors noted that the presence of the 37 KDa protein in the corpus and not the caput correlated with the completion of sperm maturation. They also hypothesized that the protein might play a role in the development of fertilizing capacity and/or play a role in determining the functional specificity of restricted regions of the sperm surface.

A possible correlation between proteins D and E of Blaquier et al and the 37 kDa protein identified by Olson and Hamilton was published by French et al in 1978 (Lea, Petrusz et al. 1978). They purified what they named acidic epididymal glycoprotein (AEG) from rat epididymis. They found that it was secreted by what appeared to be principal cells of the region distal to the initial segment (Caput, figure 1). They determined, using

immunocytochemical staining, that sperm are coated with this protein as they leave the initial segment and enter the cauda epididymis. After ligating the midcorpus region of the epididymis, they found that the amount of AEG in the cauda would decrease, probably due to the lack of testicular androgens in the cauda. These data also supported the hypothesis that a protein that is secreted in the proximal regions of the epididymis and bound to sperm may be a maturation protein. Another observation that French et al. made was that AEG was highly acidic and could contribute to the increase in negative surface charge on the maturing spermatozoon.

Shortly after French's publication, Blaquier et al. (Garberi, Kohane et al. 1979) isolated and characterized specific rat epididymal proteins (SEP). Using epididymal homogenate they partially purified proteins B, C, D and E and determined some of their biochemical characteristics. The molecular weight of protein DE was 37 kDa. They stained the proteins with periodic acid-Schiff's stain, demonstrating that they were glycoproteins. Proteins D and E had pI's of 5.13 and 4.95, respectively. This report provided further evidence for a maturation protein(s), but there were no correlations made between the various proteins; thus it was not known if they were the same molecules.

In 1982, Wong and Tsang demonstrated a high affinity binding site on rat cauda sperm for a secreted 32K rat epididymal glycoprotein. They partially purified the 32K protein from epididymal homogenate and used it for kinetic studies. Initially, they demonstrated that cauda sperm bound to a reduced number of the $^{125}$I-labelled 32K proteins when

compared to caput and corpus sperm, suggesting that the protein was binding to sperm as they traversed the epididymis to a fixed number of binding sites. They then demonstrated that 30% of the protein could be competed away with 100 molar excess unlabeled 32K protein. Scatchard analysis revealed two binding sites on the sperm membrane. One was a high affinity site ($Kd=2.6x10^{-12}$) with lower capacity and the other was lower affinity ($Kd=2.2\ x10^{-9}$) but higher capacity. This suggested the presence of two populations of receptors for the 32K molecule. When all the data are taken together, they suggest that the 32K protein is binding to the sperm membrane via a membrane linked receptor, and that 70% of it remains reversibly bound.

In an attempt to identify the cell-surface binding molecule for protein DE, Brooks iodinated the entire sperm plasma membrane, solubilized the sperm, immunoprecipitated proteins D and E then resolved the proteins via SDS-PAGE. He compared the banding patterns of native gels with those of a denaturing and reducing gels. Using autoradiography, he identified a 200Kda molecule that was present under native but not reducing and denaturing conditions. Those data suggested that proteins D and E bound to a sperm surface protein via disulfide linkages (Brooks and Tiver 1983).

Brian Setchell (Pholpramool, Lea et al. 1983) measured the effect of acidic epididymal glycoprotein (AEG) on the motility of isolated caput sperm. When compared to gamma globulin and bovine serum albumin (BSA), the addition of AEG to sperm preparations

had no stimulatory effect on motility (Pholpramool, Lea et al. 1983). This was in contrast to Fournier-Delpech's research which showed that rats who were immunized against prealbumin epididymal-specific glycoprotein (PES) maintained only 5% of their fertility when compared to control rats (immunized with saline + Freund's adjuvant without the antigen). The results from this work suggest that PES is involved in sperm motility. However, the protein preparation that was used for the immunizations was not demonstrated to be pure. Thus, the effect could be caused by one or many of the proteins that remained in the preparation and not necessarily PES (Fournier-Delpech, Courot et al. 1985). This work did not verify or contradict Pholpramool's report (Pholpramool, Lea et al. 1983) that AEG, which was presumed to be the same molecule as PES, was not involved in sperm motility.

The cDNA for proten D was isolated by French et al. by screening of a rat epididymal library with antisera. Two full length clones were then isolated from of a lambda gt11 library. Both clones, 1500 and 950 base pairs, were predicted to encode for protein D. One of the clones was shorter in the 3'-UTR region by 538 bp, and by 4 single nucleotide mismatches outside of the coding region. They report that their analysis of individual animals provided evidence that the two mRNA species are the products of allelic genes. They provided data showing that the level of total AEG mRNA is regulated by androgen. And finally, that the predicted sequence of protein D had amino acid sequence homology with various metalloproteins (rubredoxin and aspartate transcarbamoylase), but they do not assay for enzymatic activity. A northern blot of total RNA from various organs and

probed with protein D cDNA demonstrated that it was expressed only in epididymis and ductus deferens. (Charest, Joseph et al. 1988)

Recently, a report published by Cohen et al. (Cohen, Rochwerger et al. 2000) assessed the association of the strongly bound protein DE. Cauda sperm were washed of loosely bound DE by incubation with 0.4M NaCl then subjected to various extraction procedures. Sperm incubated with 2M NaCl still demonstrated the characteristic immunofluorescent staining on the dorsal region. Whereas when sperm were incubated with 2M NaCl then 1% Triton X-100 (TX-100) detergent, which solubilizes integral membrane proteins, there was no observable immunofluorescence, demonstrating that protein DE has been removed from the membrane. To further characterize the interaction, cauda sperm were treated with 2M NaCl and extracted with PBS, 50 mM glycine, pH 3, 100 mM $Na_2CO_3$, 6M Guanidine-HCl, and 1% TX-100 and released protein DE was detected by western blot analysis. Protein DE was not released with PBS, or glycine, but was released with reagents that are known to extract integral membrane proteins, 100 mM $Na^2CO_3$ 6M guanidine-HCL and 1% TX-100. Suggesting that protein DE either is an integral membrane protein or covalently linked to a membrane-anchored protein. To assess whether protein DE integrates into membranes, phosphatidylcholine-liposomes were incubated in the presence of purified protein DE. No protein DE was found to insert into the liposomes. However, the amount of NaCl resistant protein DE increased as the sperm traversed the epididymis, suggesting that the process of “tight” binding was a progressive event. Finally, to assess whether the tightly bound protein DE was the population that

migrates to the equatorial segment and participates in the sperm-egg fusion event, loosely bound DE were removed by NaCl, and the sperm were *in vitro* capacitated, then immunolocalized, or analyzed by western blot. The results demonstrated that the tightly bound protein DE relocalized to the equatorial segment of the sperm head after capacitation, furthermore, when this population is extracted with TX-100 and immunoblotted, it migrates to the same molecular mass as epididymal protein DE. These results suggest that the tightly bound protein DE are involved in sperm-egg fusion and that this population of protein DE is not modified directly, that it either integrates into the plasma membrane or associates covalently to a membrane anchored protein.

## F) Aims of this study

1. To develop a rapid and reversible methodology for purifying protein DE. Previous methods have been used to purify protein DE or molecules that have been called other things, but are assumed to be protein DE Other published purifications have lacked evidence of a purified preparation or simply have used a partially pure preparation and assumed the results could be attributed solely to protein DE.
2. To biochemically characterize the purified protein DE. Determining two and three-dimensional structure, accurate molecular mass and other physical properties will aid in determining how the protein associates to the sperm membrane and facilitates the sperm-egg interaction.

3. Identify what protein DE binds to on the surface of epididymal sperm. Since protein DE appears to be involved in sperm-egg fusion, then it could be used as a male contraceptive target. To further understand how the protein bind to and remains associated with sperm, the binding protein must be identified and biochemically characterized.
4. Determine if protein DE remains bound to epididymal sperm after ejaculation and after sperm have remained in the reproductive tract for various times. An emerging theme is that protein DE is involved in sperm-egg fusion. If that is true, then the protein DE bound to sperm in the epididymis must remain on the surface of ejaculated sperm until they reach the egg plasmalemma.
5. Determine if the tightly bound protein DE is covalently attached via a glycoslyphosphatidylinositol linkage. There are two populations of membrane bound protein D and E, a loosely-bound and irreversibly-bound fraction. Since neither protein D nor E contain hydrophobic stretch that could be used to anchor the proteins in the membrane, I assessed whether the proteins were anchored via lipid attachment.

**FIGURE 1**

**A.** The epididymis is illustrated in its normal position relative to the testis. Spermatogenesis occurs in the seminiferous tubules, which coalesce into the rete testis. The rete testis eventually fuses into a small number of tubules in the ductuli efferens. These tubules then coalesce into a single tubule that is continuous with the epididymis. As shown the single tubule forming the epididymis is highly convoluted until it reaches the vas deferens. Sperm are stored in the cauda of the epididymis prior to ejaculation.

B. Different mammals first gain the ability to bind and fuse with an oocyte at various regions of the epididymis. Illustrated are the epididymis regions where marmoset, rabbits, boar, ram, mouse, rat, hamster and human sperm are first fully functional. As shown sperm from rat and man first become fully functional only after they pass through the corpus of the epididymis where both proteins D and E are synthesized.

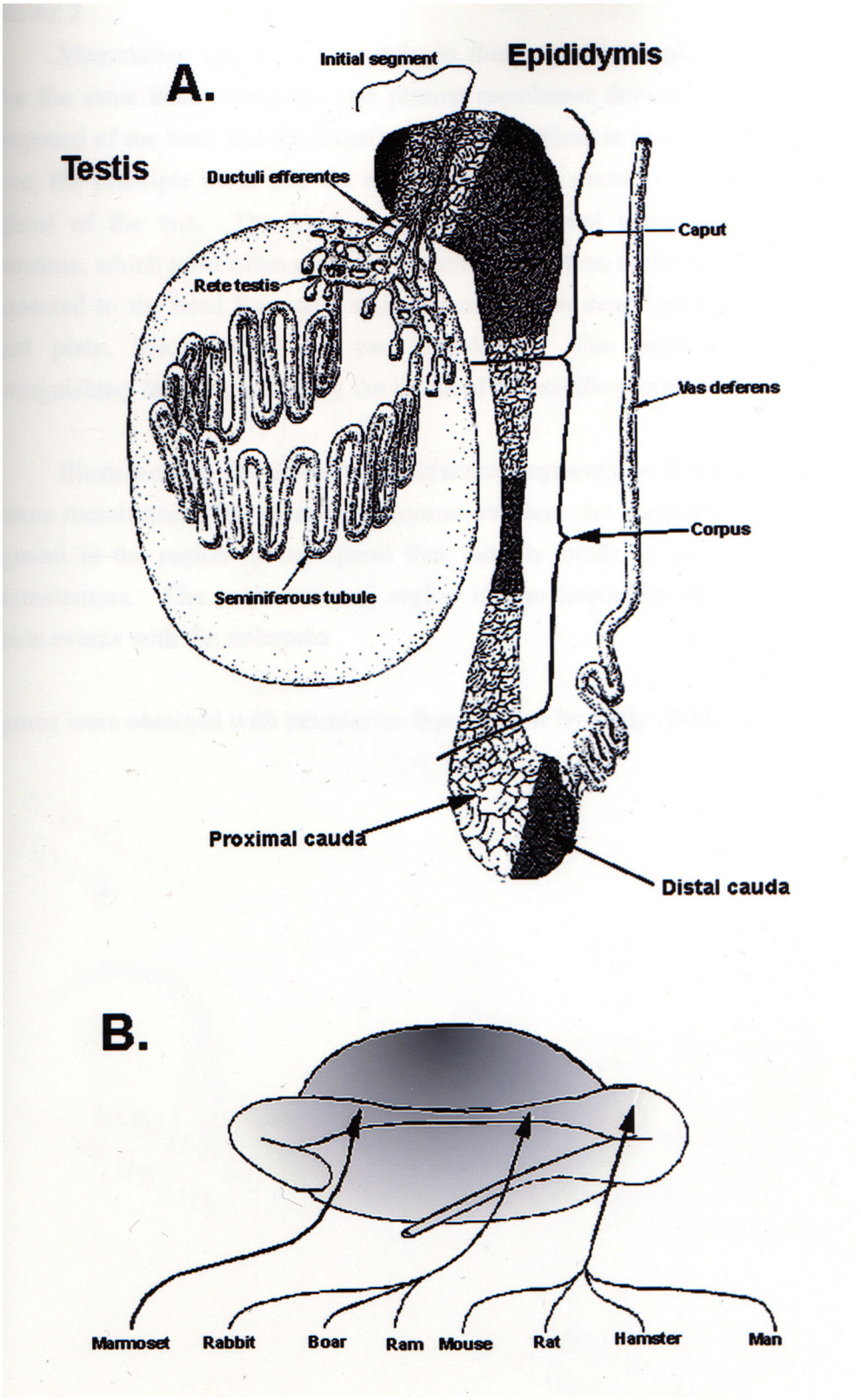

A.
Initial segment
Epididymis
Testis
Ductuli efferentes
Caput
Rete testis
Vas deferens
Corpus
Seminiferous tubule
Proximal cauda
Distal cauda
B.
Marmoset
Rabbit
Boar
Ram
Mouse
Rat
Hamster
Man

**FIGURE 2**

**A.** Mammalian sperm vary slightly in their overall morphology; however, they all have the same basic structures and plasma membrane domains or regions. Sperm are composed of the head and the flagellum. The flagellum is further divided into the middle piece, the principal piece, and the end piece. Cross sections are illustrated for different regions of the tail. The head contains the paternal complement of DNA and the acrosome, which most often overlays the anterior portion of the nucleus. The flagellum is connected to the head through a region containing a dense network of fibers called the basal plate, below which lie two centrioles. The mitochondrial sheath is the distinguishing factor determining the limits of the middle piece.

**B.** Illustrated are the distinct membranous segments or domains of the sperm head plasma membrane. The anterior acrosome overlays the acrosomal cap. The equatorial segment is the region of the sperm that initially binds to and fuses with the oocyte plasmalemma. The postacrosomal region is also important in the initial binding and fusion events with the oolemma.

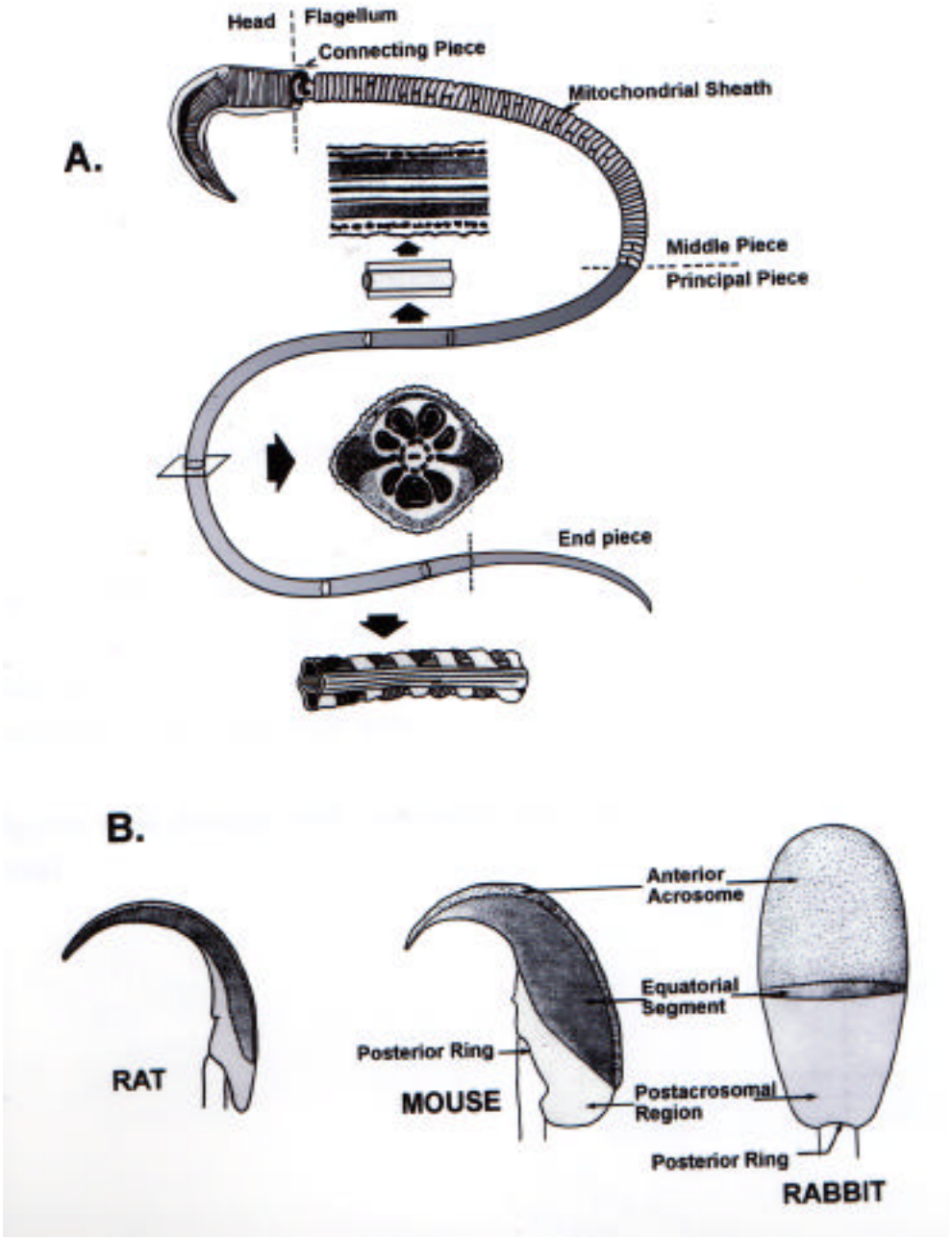



Image taken from The Physiology of Reproduction. E. Knobil, J.D. Neill, G.S. Greenwald, C. L. M. and and D. W. Pfaff. New York, Raven Press**:** 999-1080**.**

**FIGURE 3**

**A.** The acrosome reaction results in multiple fusions between the outer acrosomal membrane and the sperm plasma membrane. The equatorial domain is illustrated; it is this domain that is critical in initial gamete binding and fusion as shown in B.

**B.** Illustrated is a cartoon showing the initial binding and fusion between gametes. (a.) Binding and fusion is thought to first occur through the equatorial segment, (b-d.) then to continue along the entire length of the tail. As illustrated in A-d, after the acrosomal reaction the inner acrosomal membrane becomes exposed and this region does not fuse with the oocyte, but rather forms a vacuole within the new zygote. The paternal DNA can now be seen to decondense, which is the criterion used to ascertain that fusion has occurred between the gametes.

Binding is illustrated between the vitellus domain or ruffled region of the oocyte. A smooth cap is seen that overlays the mitotic spindle, which is arrested in meiosis II. Binding of the sperm to the oolemma activates the completion of meiosis and the expulsion of the second polar body.

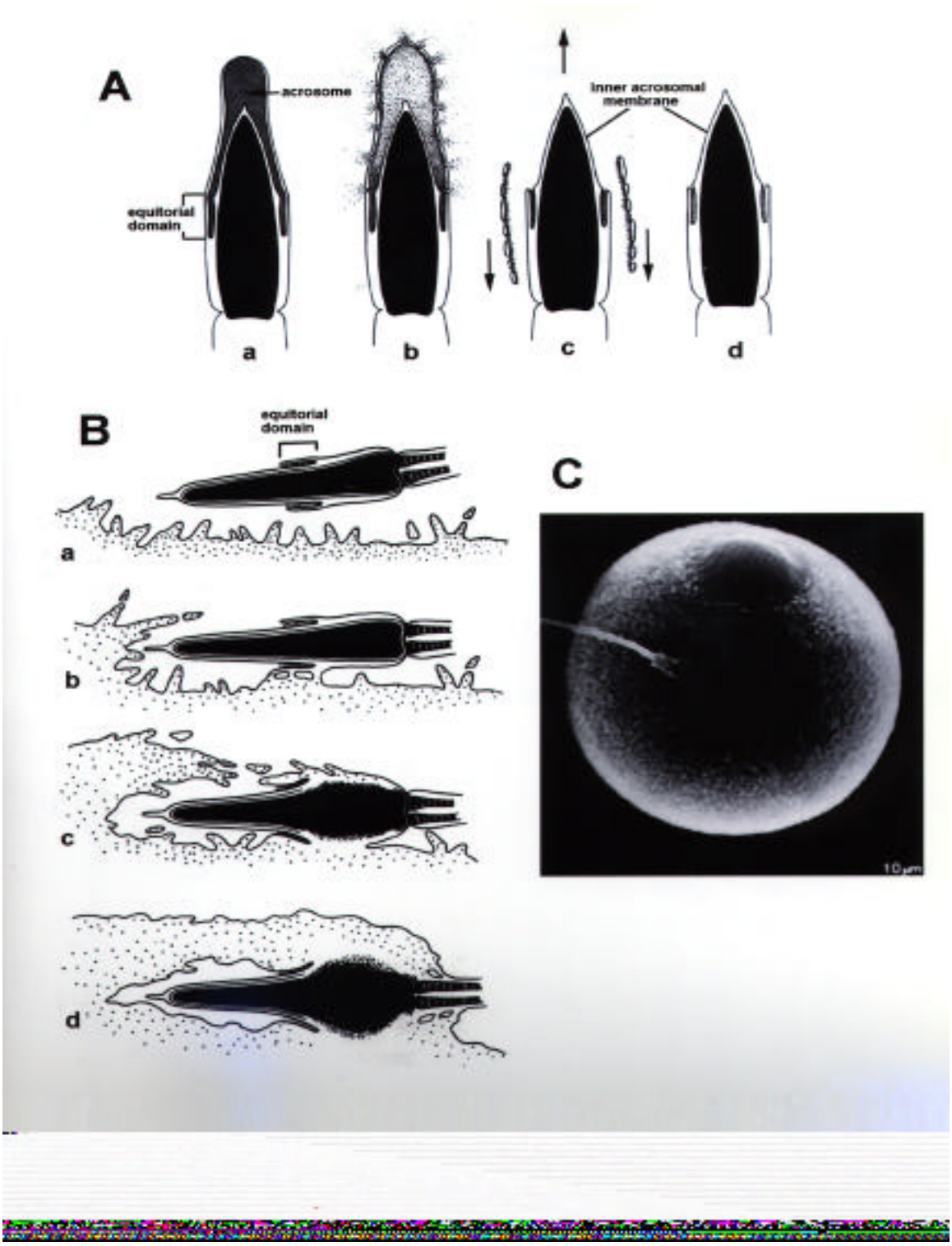



Image taken from The Physiology of Reproduction. E. Knobil, J.D. Neill, G.S. Greenwald, C. L. M. and and D. W. Pfaff. New York, Raven Press**:** 999-1080**.**

# CHAPTER II

## DEVELOPMENT OF A PURIFICATION METHOD FOR PROTEIN DE.

International Journal of Bio-Chromatography (2000) “A Rapid Method For Purification Of Protein D/E From The Rat Epididymis: Biochemical Characterization Of A Protein Involved In The Fertilization Process”

## INTRODUCTION

Many proteins are secreted by the epididymal epithelium and are involved in sperm maturation. However, there are only two, that are androgen dependent and have been shown to be directly involved in sperm and egg binding and membrane fusion. These proteins have been identified and given different names by various labs including, proteins D and E (Cameo and Blaquier 1976), AEG (Lea, Petrusz et al. 1978), sialoprotein (Faye, Duguet et al. 1980), protein IV (Jones, Fournier-Delpech et al. 1982), 32K rat epididymal protein REP (Wong and Tsang 1982). As discussed in the introduction to chapter 1, these proteins have been partially purified or purified to homogeneity, but the method was not efficient enough to use in functional assays. For example, proteins D and E were purified to homogeneity by Hamilton (Xu and Hamilton 1996) using chromatofocusing. The separation of proteins D and E using this technique involved elution with ampholines. The removal of ampholines from a sample requires HPLC or extensive dialysis, which results in low yields of purified protein.

To clarify published data obtained using crude preparations of protein DE and to establish a unified method for the isolation of these proteins in high yield, a rapid purification protocol was developed.

## MATERIALS AND METHODS

Sexually mature (>60 days of age) male Harlan Sprague-Dawley rats, weighing 300-400 grams were used. The animals were terminated by $CO_2$ asphyxiation; the attached testes and epididymides were excised from the scrotal sac. Each epididymis was trimmed of extraneous adipose tissue. The epdidiymides were homogenized in 25 mM Tris/HCl, pH 7.5 (four times the volume of the wet tissue weight) in a Waring blender for two minutes on the high setting, two minutes on the low setting, and then two minutes on the high setting. The homogenate was allowed to cool on ice for five minutes between each homogenization step and spun for 30 minutes at ~80,000 x g at 4°C in a Beckman L-50 centrifuge using a Ti-70 rotor. The supernatants were decanted from the pellet, pooled, filtered using a Whatman #2 filter paper, and used immediately to purify protein DE.

*Purification of Protein DE*

Protein DE was purified from an epididymal homogenate using the procedure described by Wong and Tsang (Wong and Tsang 1982), with some modifications. The “crude” protein homogenate was dialyzed overnight at 4°C against 25 mM Tris/HCl, pH 7.5 (running buffer) containing the divalent cations $Mg^{2+}$, $Mn^{2+}$, and $Ca^{2+}$ at a concentration of 1 mM. The sample was concentrated under $N_2$ pressure by Amicon filtration using a PM-10 filter (molecular mass exclusion limit = 10,000 daltons) and applied to a Con-A column (1.0 x 5 cm) equilibrated with running buffer. Bound proteins were eluted with 0.2 M methyl-α-D-mannopyranoside in running buffer without the divalent cations. Fractions containing protein DE were pooled, concentrated by Amicon filtration and

made to be 150 mM NaCl and further purified on a size-exclusion column (Sephacryl-200 HR, 2.5 x 225 cm). The column was equilibrated with running buffer containing 150 mM NaCl, and fractions were collected at a flow rate of 0.5 mL/min. Fractions containing protein DE were pooled, concentrated by Amicon filtration, dialyzed against running buffer, and applied to a DEAE A-25 column (2.5 x 15 cm). The column was equilibrated with running buffer, and fractions were collected at a flow rate of 1 mL/min. Bound proteins were eluted from the column by using running buffer containing a linear gradient of NaCl (150 to 500 mM). Fractions containing protein DE were pooled, concentrated by Amicon filtration, dialyzed against running buffer, and applied to a smaller DEAE-A25 column (0.9 x 15 cm). Protein DE was eluted from the column by using running buffer containing a linear gradient of NaCl (150 to 300 mM), then a step gradient using running buffer containing 500 mM NaCl. Fractions containing protein DE sample were pooled, concentrated to ~1 mL by Amicon filtration (Centricon-10), and the protein content estimated. The protein content of each fraction obtained from the various chromatographic steps was monitored at an absorbance of 280 nm. The purity of protein DE at various steps of the purification procedure was monitored by SDS-PAGE under denaturing and reducing conditions followed by western blotting. The homogeneity of the protein sample at the final stage of purification was assessed by two-dimensional (2D) SDS-PAGE under denaturing and reducing conditions.

*Two-Dimensional (2D) SDS-PAGE*

Two-dimensional (2D) SDS-PAGE analysis of the protein sample was performed using a modification of O'Farrell's method (Hall and Killian 1989). Briefly, the pH gradient in the first dimension gel was established using an ampholine mixture consisting of pH3.0 to 10.0 and pH 3.0 to 7.0 (0.3 mL each) (Serva Fine Chemicals, Garden City Park, NY). To minimize cathodic drift, a one-cm Teflon plug was inserted into the bottom of each gel and sealed with dialysis membrane prior to casting the isoelectric focusing (IEF) gel. The protein sample was focused at 400 constant volts for 12 hrs followed by one hour at 800 volts (5600 volt-hours). The second dimension separation utilized a 10% uniform polyacrylamide slab gel containing 0.1% SDS at pH 8.5.

The internal protein standards (Sigma Chemical Co., St. Louis, MO) that were routinely used for IEF and molecular mass calibration of gels in the second dimension were urease, pI = 5.0; 120,000 daltons/subunit; rat serum albumin (RSA), pI = 4.8, 68,000 daltons; ovalbumin (egg), pI 4.6, 45,000 daltons; carbonic anhydrase, pI = 5.0, 29,000 daltons; hemoglobin (bovine), pI 6.8, 16,000 daltons; myoglobin (sperm whale), pI = 8.3, 17,000 daltons; and α-lactalbumin, pI 4.4, 14,000 daltons. Significant polypeptide spots were visualized by staining with Coomassie Brilliant Blue dye and/or silver.

*Preparation of Chicken Anti-Protein DE polyclonal Antibodies*

Polyclonal antibodies (IgY Abs) against purified protein DE were raised (Strategic BioSolutions, Inc. Ramona, CA) in two chickens (hens), after pre-immunizing egg

collection. The purified protein (~3.36 mg total) was dissolved in sterile phosphate buffer, pH 7.2 and suspended at a final concentration of 0.84 mg/mL. Each chicken was given four immunizations, followed by post-immunization egg collection for 15 days. The post-immunized eggs were then pooled into three batches; those collected on days 1-5, 6-10, and 11-15. IgY Abs were isolated from each of the three batches plus the pre-immune batch and then titered by Strategic BioSolutions (Ramona, CA) using an ELISA. The final titer for the chicken anti-protein DE IY Abs was ~1:8,800 and ~1:8,500 for chicken one and two, respectively. The Abs were determined to be monospecific, as assessed by a western immunoblot competition assay. Briefly, protein samples were subjected to electrophoresis on 10% SDS-PAGE slab gels, transferred to PVDF membrane, and immunoblotted with chicken anti-protein DE IgY Abs. The immunoblotted PVDF membrane was incubated with the chicken anti-protein DE IgY Abs that had been preabsorbed with 100 to 1000-fold protein DE purified. The major 32,000 dalton protein band identified using the chicken anti-protein DE IgY Abs was abolished, indicating that the chicken anti-protein DE IgY Abs were specific for protein DE.

*Enhanced Chemiluminescence Western Blot Analysis*

SDS-PAGE analysis of protein samples was accomplished using a Hoefer SE 600 Dual cooled Vertical Slab Unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA). Protein samples were incubated with SDS sample treatment buffer (STB, made 0.1% SDS, 5mM

β-mercaptoethanol, 30% glycerol, and 0.05% bromophenol blue tracking dye) and boiled for two minutes. Proteins were electrophoretically resolved by SDS-PAGE using a 10% uniform polyacrylamide slab gel at a constant current of 15 mA per gel until the tracking dye reached the separating gel. The current was increased to 30 mA per gel until the tracking dye was approximately one cm from the bottom of the slab gel. The proteins were transferred onto nitrocellulose for 40 minutes at 24 volts using a Genie Blotter (Idea-Scientific, Minneapolis, MN). To assess the efficiency of protein transfer, the gel was stained with Coomassie Brilliant Blue dye and/or silver. The nitrocellulose membrane was locked with commercially available 5% nonfat dry milk dissolved in 10 mM Tris/HCl, pH 8.0 containing 150 mM NaCl, 0.05% Tween 20, and 0.02% sodium azide (membrane blot wash buffer). Goat-anti-rabbit HRP (GAR-HRP) or Rabbit-anti-chicken-HRP (RAC-HRP) (Pierce, Rockford, IL) was diluted 1:25,000 and incubated for one hr at room temperature and washed three times (15 minutes each) with membrane blot wash buffer. To detect the biotinylated molecular mass ($M_r$) protein markers, NeutraAvidin-HRP was diluted 1:20,000 and co-incubated with GAR/RAC-HRP for one hour. After the one-hour incubation, the nitrocellulose blot was washed three times (15 minutes each) with membrane wash buffer. To visualize protein banding, the nitrocellulose blot was incubated for 30 sec to one minute with SuperSignal chemiluminescent substrate (Pierce, Rockford, IL). Positive immune protein bands were detected by exposing the nitrocellulose blot (covered in plastic wrap) to Kodak BioMax x-ray film at room temperature for 30 sec to one minute, longer if necessary.

*Other Methods*

Soluble protein content was measured using the method of Lowry et al. (Lowry, Rosebrough et al. 1951), with bovine serum albumin (BSA) as the standard protein. Samples were assayed in duplicate with the concentration of BSA ranging from 10 to 80 μg/μL in 10 μg increments.

*Results and Discussion*

The first step in the purification of protein DE was the homogenization of rat epididymal tissue (Figure 1); 1,462.5 mg of protein were obtained and applied to a Concanavalin-A Sepharose affinity column. The bound peak (Figure 2a), was determined to contain protein DE. 240.4 mg of total protein were recovered in this peak representing a 6.1% degree of purification. No protein DE was detected in the first peak or flow-through fraction, demonstrating that all of the protein DE was bound to the column. The bound peak was further resolved into three distinct peaks using a Sephacryl S-200Hr column (Figure 2b). Using western blot analysis, protein DE was detected in the last peak eluting from the column. Forty-five mg of protein were recovered, a 32.5% purification from the initial homogenate. The pooled S-200Hr fractions were applied to an anion-exchange column for further fractionation (Figure 3a). Protein DE was identified in the second peak of the bound fraction eluted from this column. Protein DE began eluting at approximately

0.175M NaCl and was completely eluted with 1M NaCl. Fourteen mg were recovered from this column representing 104-fold purification. The final purification step involved a smaller anion exchange column utilizing the same resin and a shallower NaCl gradient (Figure 3b). There was no bound fraction, as expected, and protein DE began to elute at ~1.75M NaCl and was completely eluted with the addition of 0.5M NaCl. The eluted protein DE from this column represents the purified protein. There were 3.2 mg recovered for a total of a 457-fold purification and a 0.2% protein recovery when compared to the initial protein amount (Table 1).

While protein DE has been one of the most studied members of the CRISP protein family, a variety of different isolation procedures have been used to purify the protein. Previous studies (Lea, Petrusz et al. 1978; Kohane, Garberi et al. 1979; Faye, Duguet et al. 1980; Wong and Tsang 1982; Fournier-Delpech, Courot et al. 1985) describing the purification of protein DE used magnesium sulfate ($Mg_2SO_4$) salt fractionation as the initial step in the purification of protein DE, with subsequent steps including ion exchange, gel permeation and affinity chromatography. Here is described the first published rapid, reproducible, and efficient purification of protein DE. The novel features of this approach toward purifying protein DE from epididymal tissue included: (a) an omission of the salt fractionation step, (b) application of the epididymal tissue protein homogenate to a Con-A affinity column as an initial step in the purification procedure, and (c) employment of an additional exclusion column with a diameter of 2.5 cm and a height of 225 cm. As

shown in Figure 2a, a significant amount (>85%) of the contaminating epididymal tissue proteins was removed by Con-A affinity chromatography as an initial purification step. The percentage of protein removed by this initial step was assessed by estimating and comparing the protein content of the epididymal homogenate to the pooled, bound fractions (Fig. 2a, hatched area) prior to and after application on the Con-A column. SDS-PAGE and western blot analysis of the pooled, bound protein fractions revealed more than 12 distinct protein bands. The second step in the purification procedure utilized an exclusion column, which eliminated the need to dialyze the protein sample and removed ~80% of the contaminating proteins (Figure 2b). The third and fourth steps (Figures 2c and d) of the purification procedure utilized two separate ion-exchange columns to resolve weakly and strongly charged protein contaminants.

**TABLE 1** Efficiency of the purification protocol for protein DE.

| | Purification Stage | Protein, (mg) | Recovery (%) | Degree of Purification (Fold) |
|---|---|---|---|---|
| I | Homogenate | 1, 462.5 | 100.0 | - |
| II | Concanavalin A | 240.4 | 16.4 | 6.1 |
| III | Size-Exclusion | 45.0 | 3.1 | 32.5 |
| IV | Ion Exchange | 14.0 | 1.0 | 104.5 |
| V | Ion-Exchange | 3.2 | 0.2 | 457.0 |

Purification of p10 [b]

| | Step | Protein, (mg) | Activity, (units) | Specific activity, (units/mg) | Purification Fold | Yield, (%) |
|---|---|---|---|---|---|---|
| 1. | 146,000 x g supernatant | 2213 | 118,000 | 53 | — | 100 |
| 2. | DE-52 | 1408 | 461,660 | 329 | 6 | 391 |
| 3. | EDTA/25% $(NH_4)_2SO_4$ | 1392 | 377,660 | 271 | 5 | 319 |
| 4. | Octyl-Sepharose | 40 | 171,600 | 4,290 | 81 | 145 |
| 5. | Phosphocellulose | 30 | 153,900 | 5,130 | 97 | 130 |
| 6. | Q-Sepharose | 2.7 | 84,270 | 31,211 | 589 | 71 |
| 7. | Octyl-Sepharose | 0.6 | 38,940 | 64,900 | 1225 | 33 |
| 8. | Superdex-75 | 0.063 | 13,563 | 215,300 | 4062 | 11 |

[a]Purification based on protein content (see materials and methods)

[b]Purification of p10 *Moore and Blobel, 1994*

**FIGURE 1** Schematic illustration of rat testis and epididymis. The rat epididymis wsa used as tissue source for protein DE.

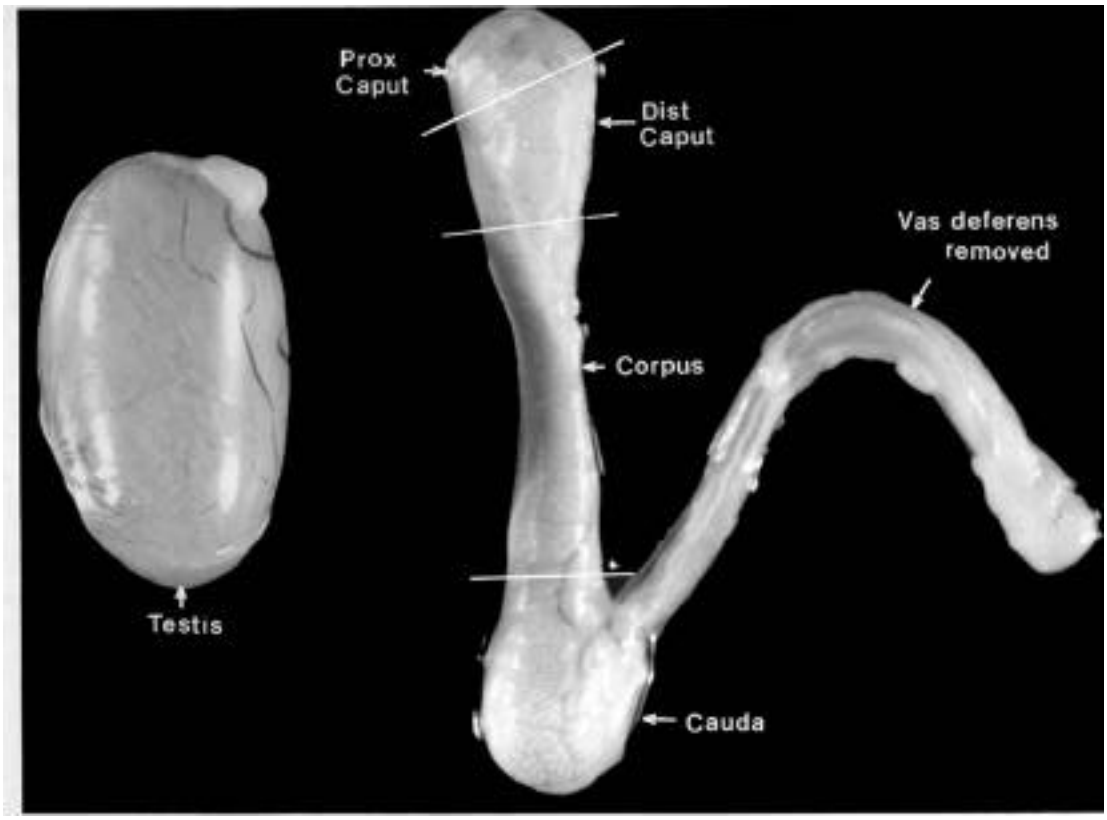

Prox
Caput
Dist
Caput
Vas deferens
removed
Corpus
Testis
Cauda

**FIGURE 2** Chromatographic purification of protein DE from rat epididymal tissue. **(A)** Con-A affinity chromatography. "Crude" protein homogenate was dialyzed overnight at 4°C against 25 mM Tris/HCl, pH 7.5 (running buffer) containing the divalent cations $Mg^{2+}$, $Mn^{2+}$, and $Ca^{2+}$ at a concentration of 1mM and applied to the Con-A column. Bound proteins were eluted with 0.2 M methyl-α-D-mannopyranoside in running buffer without the divalent cations.

**(B)** Sephacryl-S200-HR size-exclusion chromatography. Pooled Con-A fractions containing protein DE were applied to a size-exclusion column (Sephacryl-200HR). The columnn was equilibrated with running buffer containing 150 mM NaCl.

The shaded regions of each chromatograph denote pooled fractions containing protein DE. Proteins in pooled fractions were resolved by SDS-PAGE (Coomassie stained, reducing and denaturing 10% gels) followed by western blot analysis (insets).

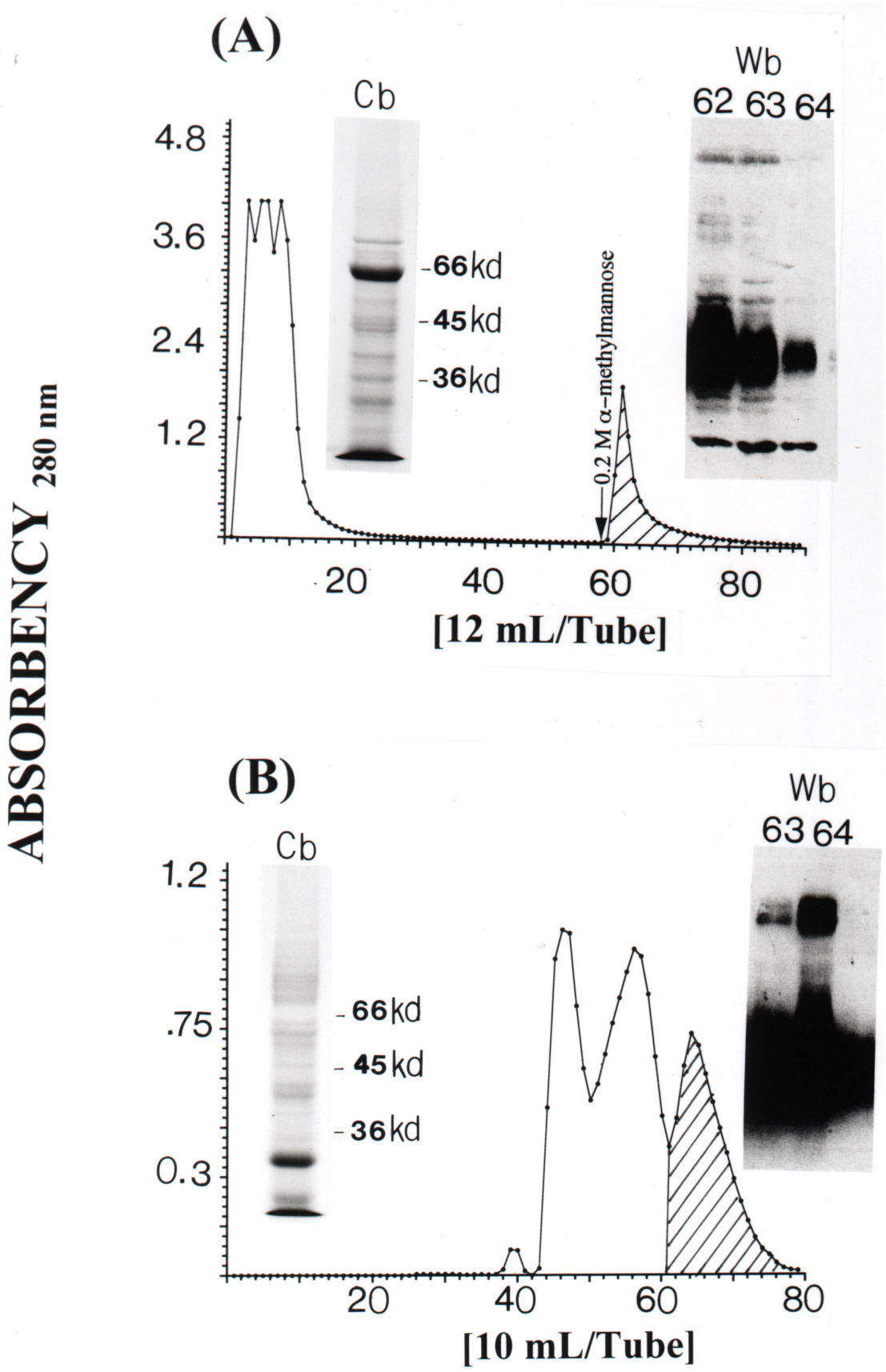

(A)
Cb
Wb
62 63 64
4.8
3.6
2.4
1.2
-66kd
-45kd
-36kd
0.2 M α-methylmannose
20
40
60
80
[12 mL/Tube]
ABSORBENCY 280 nm
(B)
Cb
Wb
63 64
1.2
.75
0.3
-66kd
-45kd
-36kd
20
40
60
80
[10 mL/Tube]

**FIGURE** 3 Chromatographic purification of protein DE from rat epididymal tissue. **(A)** Ion-exchange (DEAE-A25; 2.5 x 15 cm) chromatography. Pooled size-exclusion fractions containing protein DE were applied to the first ion-exchange column. The column was equilibrated with running buffer. Bound proteins were eluted from the column by using running buffer containing a linear gradient of NaCl (150 to 500 mM).

**(B)** Ion-exchange (DEAE-A25; 0.9 x 15 cm) chromatography. Fractions, containing protein DE, from the first ion-exchange column were pooled and applied to the second ion-exchange column. The column was equilibrated in running buffer. Bound proteins were eluted with running buffer containing a linear gradient of NaCl (150 to 300 mM) and then 500 mM.

The shaded regions of each chromatograph denote pooled fractions containing protein DE. Proteins in pooled fractions were resolved by SDS-PAGE (Coomassie stained, reducing and denaturing 10% gels) followed by western blot analysis (inserts).

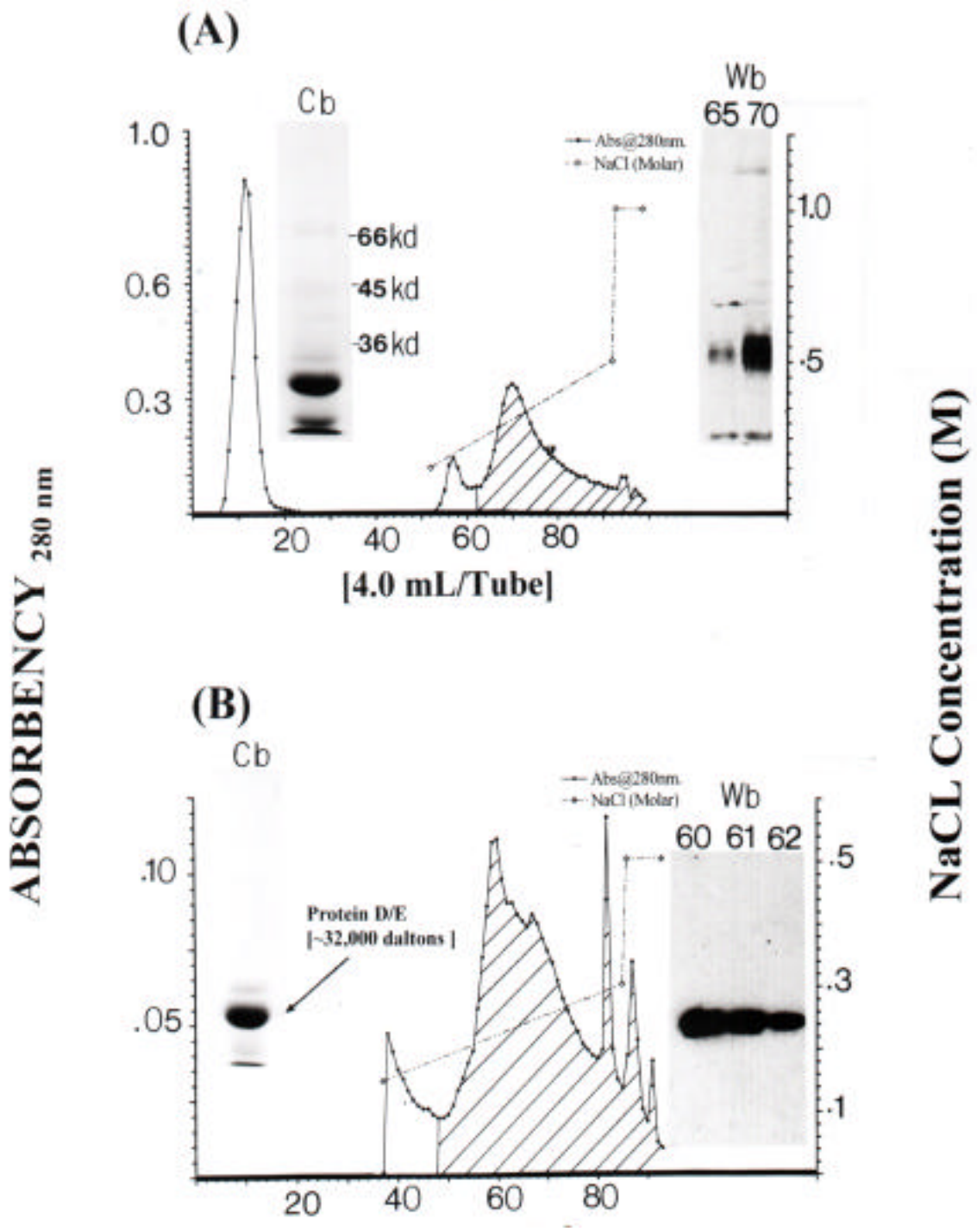
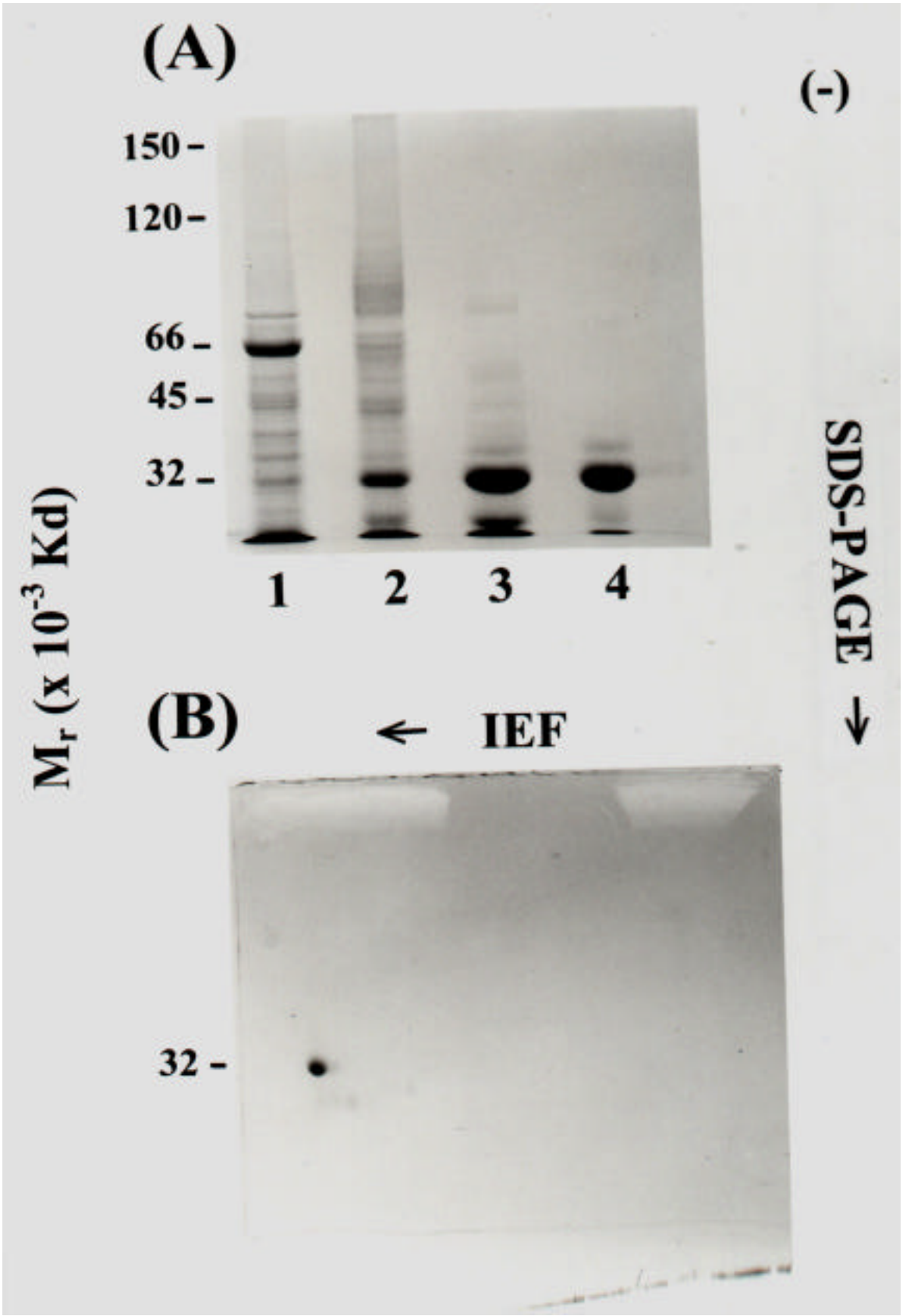

(A)
Cb
Wb
65 70
Abs@280nm
NaCl (Molar)
1.0
0.6
0.3
66kd
45kd
36kd
1.0
.5
20
40
60
80
[4.0 mL/Tube]
ABSORBENCY 280 nm
NaCL Concentration (M)
(B)
Cb
Abs@280nm
NaCl (Molar)
Wb
60 61 62
.10
.05
Protein D/E
[~32,000 daltons ]
.5
.3
.1
20
40
60
80

FIGURE 4 One and two dimensional SDS-PAGE analysis of purified protein DE (A) Pooled fractions from each chromatographic purification were analyzed b SDS-PAGE under denaturing and reducing conditions. (1) Con-A; (2) size-exclusion; (3) first ion-exchange; and (4) second ion-exchange column. The samples were stained with Coomassie Brilliant Blue R450.

(B) The purified protein DE sample from the second ion-exchange column was analyzed by two-dimensional SDS-PAGE and stained with Coomassie Brilliant Blue R450. The presence of a single spot demonstrates the purity of the fraction eluted from the second ion-exchange column.


(A)
(-)
150 –
120 –
66 –
45 –
32 –
1
2
3
4
SDS-PAGE →
$M_r$ (x $10^{-3}$ Kd)
(B)
← IEF
32 –

# Chapter III

## BIOCHEMICAL CHARACTERIZATION OF PROTEIN DE: A PROTEIN INVOLVED IN THE FERTILIZATION PROCESS.

The data presented in this chapter has been published in *The International Journal of Biochromatography (2000).*

## INTRODUCTION

Except for a molecular mass estimation by SDS-PAGE analysis, a thorough biochemical characterization of protein DE has been limited. This may account for a) the wide variation in the nomenclature of proteins D and E (Cameo and Blaquier 1976), AEG (Lea, Petrusz et al. 1978), sialoprotein (Faye, Duguet et al. 1980), protein IV (Jones, Fournier-Delpech et al. 1982), 32K rat epididymal protein REP (Wong and Tsang 1982); and b) the different reported molecular mass values of 37 kDa (Olson and Hamilton 1978; Garberi, Kohane et al. 1979; Faye, Duguet et al. 1980), 32 kDa (Lea and French 1981; Wong and Tsang 1982; Hall and Hadley 1990), 28 kDa (Brooks and Tiver 1983).

A comparison of the amino acid sequence homology, as deduced from the cDNA nucleotide sequence (Brooks, Means et al. 1986; Charest, Joseph et al. 1988), of protein DE with the metal-binding domains of several proteins suggests that protein DE is a metalloprotein. However, a careful review of the literature indicates that there have been no published reports of protein DE exhibiting any type of enzymatic activity. Amino acid homology of protein DE with other known protein sequences has revealed that it belongs to the CRISP (for Cysteine RIch Secretory Protein) protein family, named because of the presence of 16 conserved cysteinyl residues, 14 of which are in the carboxyl half of the proteins. CRISP proteins have been identified in mouse (Schwidetzky, Haendler et al. 1995; Haendler, Toda et al. 1999), rat (Klemme, Roberts et al. 1999) human (Kratzschmar, Haendler et al. 1996), horse, and monkey (Sivashanmugam, Richardson et al. 1999), suggesting that they are conserved across species and, thus, serve important

functions. Mammalian CRISP proteins have a ~45% identity to helothermine, a ryanodine receptor (RyR) antagonist ((Morrissette, Kratzschmar et al. 1995), as well as to venom allergens in a variety of species, many of which can regulate ion channels.

Indirect methods have been used to attempt to elucidate the function(s) of protein DE (Cuasnicu, Gonzalez Echeverria et al. 1984; Fournier-Delpech, Courot et al. 1985; Cohen, Munuce et al. 1996; Hall and Tubbs 1997; Hall, Tubbs et al. 1997; Ellerman, Brantua et al. 1998), which have suggested that it is involved in sperm-egg fusion, zona pellucida binding and sperm egg-binding, and acquisition of motility. However, the primary structure of protein DE is insufficient to provide possible functional domains and/or catalytic sites within the three dimensional structure of the protein. The biochemical characterization of protein DE was designed to provide structural information that would provide 1) exact molecular mass, 2) the percent carbohydrate by mass, 3) *N*-terminal sequence, 4) the secondary structural domains in the protein and 5) aid in the elucidation of the three dimensional structure when the molecule is crystallized.

This chapter describes experiments and result that provide a more thorough biochemical characterization of protein DE. With the exception of an earlier study by Xu and Hamilton (Xu and Hamilton 1996), in which proteins D and E were purified to apparent homogeneity and biochemically characterized, 2D-SDS-PAGE analysis and/or additional experimental procedures that have been routinely used to assess the purity of a protein preparation, were frequently omitted.

## MATERIALS AND METHODS

The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO): Coomassie Brilliant Blue R250, dithioerythretol (DTE), and ethylaminediaminetetraacetic acid (EDTA, disodium calcium salt). Tris-Base, Tris-HCL, and sodium phosphate (dibasic) were obtained from Fisher Scientific (Fair Lawn, NJ). Pyroglutamate aminopeptidase and trypsin (sequencing grade) were purchased from Roche Molecular Biochemicals (Indianapolis, IN). PVDF membrane was purchased from Bio-RAD (Hercules, CA) and reagent grade urea was obtained from EM Scientific Co. (Cherry Hill, NJ).

### *Removal of N-terminal Pyroglutamate and Amino Acid Sequencing*

Previous studies by Brooks et al. and Xu and Hamilton (Brooks, Means et al. 1986; Xu and Hamilton 1996) demonstrated that the *N*-terminus of the protein D was blocked, thereby preventing the determination of the amino acid sequence of the protein from the *N*-terminus by direct Edman degredation sequencing. Since the predicted first amino acid of the mature, secreted protein, as deduced from the cDNA sequence (Brooks, Means et al. 1986; Charest, Joseph et al. 1988), was predicted to be glutamine. To remove the *N*-terminal pyroglutamate, which forms from glutamine *in vivo*, 34μg of purified protein DE were incubated in 0.1 M sodium phosphate buffer, pH 8.0 containing 10 mM $Na_2EDTA$, 5 mM DTE, 1.0 M urea, and 5% glycerol (v/v). The protein DE sample was

placed in a boiling water bath (100°C) for one minute. After the one minute incubation period, the protein DE sample was allowed to cool to room temperature, and 3.4 µg pyroglutamate aminopeptidase were added to the protein sample, which was then incubated at 4°C for 42 hr (Podell and Abraham 1978).

After 42 hr incubation, 50 µL of SDS sample treatment buffer (SDS STB, made with 5 mM Tris, 0.5% SDS, 10% β-mercaptoethanol, and 30% glycerol) were added to the protein sample. The protein sample was placed in a boiling water bath (100°C) for two minutes, resolved using a 10% uniform SDS-PAGE slab gel, and then transferred to the PVDF membrane. The PVDF membrane was washed with deionized water, and the protein was visualized by staining for one minute in 50% methanol (v/v), 7% acetic acid (v/v), and 0.1% Coomassie Brilliant Blue dye, and then destained in the same solution containing no Coomassie dye. The band was excised using a single-edge razor blade and sequenced by Edman degradation (The Pennsylvania State University's Protein Sequencing Facility, Hershey, PA).

*Mass Spectral Analysis of Purified Protein DE*

Mass spectral analysis of purified protein DE was performed using a Finnigan MAT TSQ7000 triple quadrupole mass spectrometer equipped with an Applied Biosystems 130 high pressure liquid chromatography (HPLC) system. Samples (9µg) were applied onto a

1.0 x 100 mm reverse phase octyl column and eluted directly into the mass spectrometer using a linear gradient of acetonitrile in aqueous 0.05% trifluroacetic acid (TFA) and analyzed by electrospray ionization mass spectrometry (LC-ESI-MS).

*Circular Dichroism Analysis of Purified Protein DE*

A purified protein DE sample at a concentration of 55.6 μM was analyzed by circular dichroism (CD). CD was performed using a Jasco J-715 spectropolarimeter and a water-bath to adjust the temperature in a cylindrical water-jacketed quartz cuvette with 0.05-cm path-length. The temperature was changed at a rate of one grad per minute; however at each temperature where an entire spectrum was recorded, the temperature was placed on hold for five minutes.

## RESULTS AND DISCUSSION

Protein DE has been shown to be secreted by the epididymis and binds to the surface of spermatozoa during maturation within the epididymis (Kohane, Cameo et al. 1980; Kohane, Gonzalez Echeverria et al. 1980; Gonzalez Echeverria, Cuasnicu et al. 1982; Eddy, Vernon et al. 1985; Fournier-Delpech, Courot et al. 1985; Hall and Killian 1989; Vreeburg, Holland et al. 1992; Hall, Tubbs et al. 1997). It is becoming apparent that the x-ray crystal structure of the protein would provide some insight into how the protein interacts with the sperm surface. When attempting crystallization of an isolated protein

domain, ultimate success can be crucially dependent on the amino acid residues chosen to define the domain boundaries. The amino acid sequence can be used to predict important structural/functional features of the protein. 107 internal amino acids have been determined by direct sequencing of tryptic peptides of proteins D and E (Xu and Hamilton 1996). In that report, after tryptic digests, proteins D and E were found to differ in the mass of one peptide that corresponds to the amino third of the molecule. Previous attempts to sequence the protein from the *N*-terminus have been unsuccessful because the *N*-terminus of each isoform has been shown to be blocked by an unknown group (Brooks, Means et al. 1986; Xu and Hamilton 1996). However, as shown in Figure 1, the *N*-terminus has been successfully de-blocked by removing a pyrogultamate residue on the *N*-terminus and the first 26 amino acid residues sequenced. This partial amino acid sequence was determined to be 100% identical to the predicted amino acid sequence from the cDNA for protein D (Brooks, Means et al. 1986; Charest, Joseph et al. 1988).

Using an integrated family of amino acid sequence analysis programs (GCG programs), the predicted secondary structural features, regional backbone flexibility, and features relating to the surface accessibility (e.g., possible glycosylation sites) of protein DE are presented in Figure 2A. To a first approximation, the large number of “turn” structures immediately ahead of the carboxyl terminal helices is rather unusual and may be an important structural determinant in the binding of this protein to the sperm surface.

Interestingly, mouse crisp-1, the rat protein DE homologue, was shown to have three intramolecular disulfide bridges located in the *N*-terminal half of the protein, suggesting the existence of two discrete domains in the protein (Eberspaecher, Roosterman et al. 1995). Other features, such as hydrophilic structures around possible glycosylation sites and numerous "bending" motifs, are consistent with the protein exhibiting both α-helical and β-sheet structure. However, none of the CRISP proteins or their homologs' secondary or tertiary structures, including protein DE, has been determined experimentally. Thus, the accuracy of the computer predicted secondary structure couldn't be verified without experimental data (Rost and Sander 1996; Deleage, Blanchet et al. 1997). To assess the accuracy of the predicted structures, the CD spectrum of the protein was determined and is presented in Figure 2B**.** A minimum was observed at 217 nm, indicating the β-sheet motifs were associated with the secondary structure of the protein. In addition, two additional minima were observed, one at 222 nm and the other at 208 nm, indicating that the α-helical motifs were associated with the secondary structure of the protein. Although protein DE contains both α-helical and β-sheet motifs, neither of them is predominant as indicated by the strength of the minima. One would expect, from the predicted secondary structure, a predominance of β-sheet structures, but that is not what has been determined. One of the potential advantages of circular dichroism is that once the spectra are obtained, the percentages of the different secondary structural motifs can be determined. To do this accurately, the protein being examined must match with significance to other proteins in the database of known structures. Protein DE does not, thus the percent of helix or sheets cannot be determined. Circular dichroism suffers from

the limitation of only being able to determine if there is secondary structure, what structure it is and how much is present. It cannot determine the location of those structures.

To determine an exact molecular mass of protein DE, electrospray-ionization mass spectrometry was used Figure 2C. Native protein DE was determined to be a heterogeneous varying in the attached hexose carbohydrate residues. The major species have a mass of 27,084.8, 27,192.8, 27,354.8 and 27,678.8 daltons. This mass distribution of the protein is common with other glycosylated proteins (eg. fetuin). The variances of the hesoxe sugars is due to the less than 100% efficiency of the carbohydrate processing enzymes in the golgi. The predicted molecular mass from the deduced amino acid sequence is 25,711.42 daltons. Thus, six percent of the mass of the native protein is comprised of carbohydrates.

**FIGURE 1** Comparison of the first 26 amino acids predicted from protein DE cDNA and those determined by automated sequencing after deblocking the N-terminal pyroglutamate residue.

**Predicted Sequence:**
1 MALMLVLLFL AAVLPPSLLQ DTTDEWDRDL ENLSTTKLSV QEEIIN
1 _____Q DTTDEWDRDL ENLSTTKLSV QEEIIN
**Determined Sequence** ^==*Signal peptide, cleaved between #19&20*

Pyroglutamate:

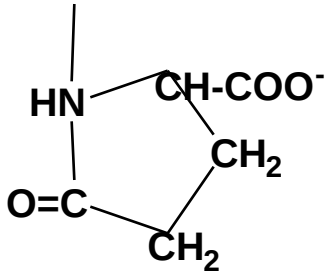
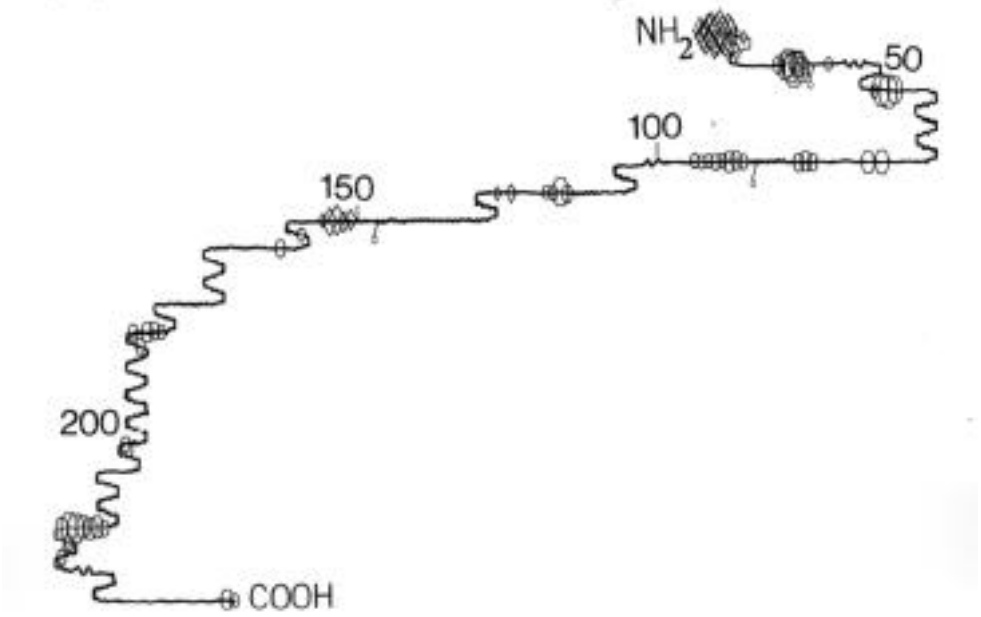


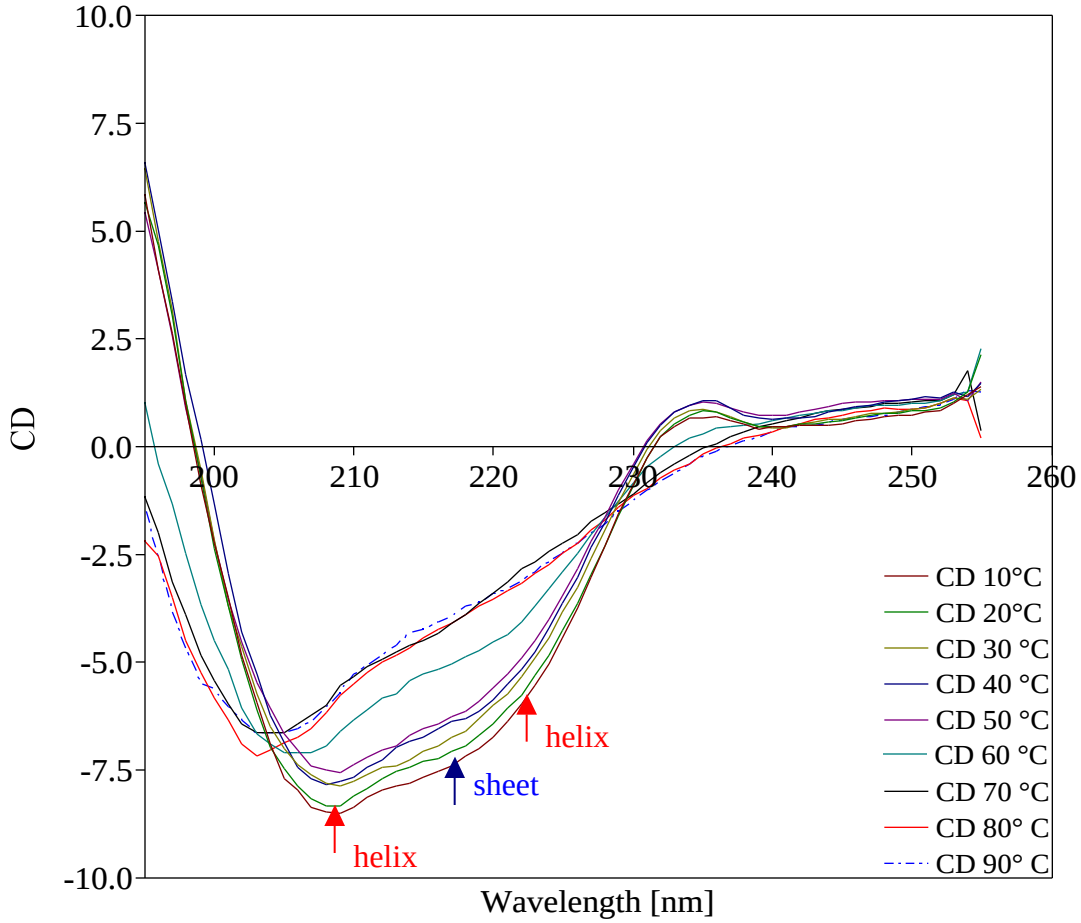
**FIGURE 2** Secondary structure analysis of protein DE **(A)** Secondary structure prediction performed using Genetics Computer Group software package. **(B)** Secondary structural analysis performed by circular dichroism spectroscopy of protein DE before and after heat denaturation. (C) Mass spectral analysis of purified protein D by ESI-MS. The molecular masses are indicated above the peaks on the chromatogram.

**(A)**




**(B)**

Temperature denaturation of protein D/E
analyzed by circular dichroism



**(C)**

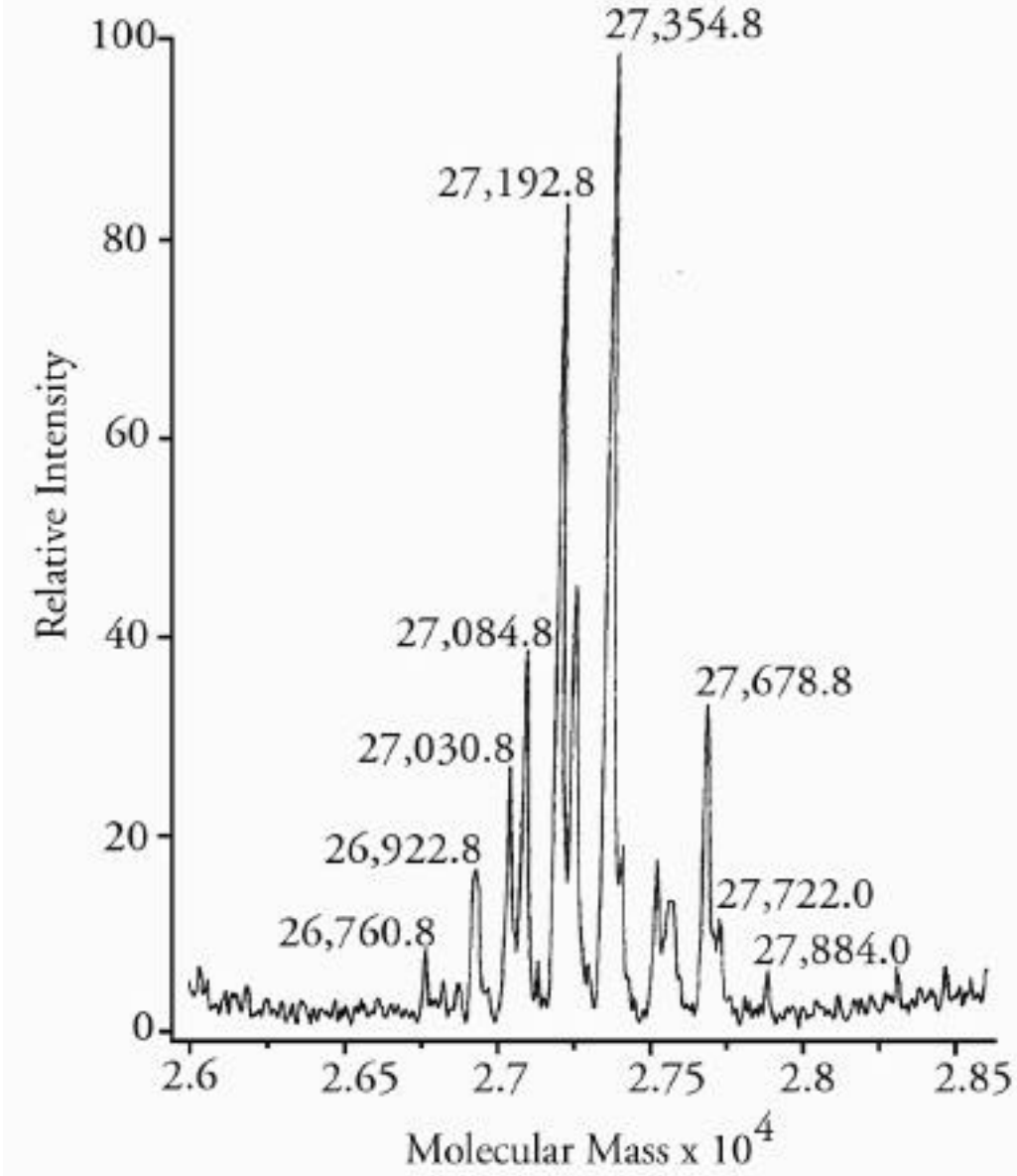
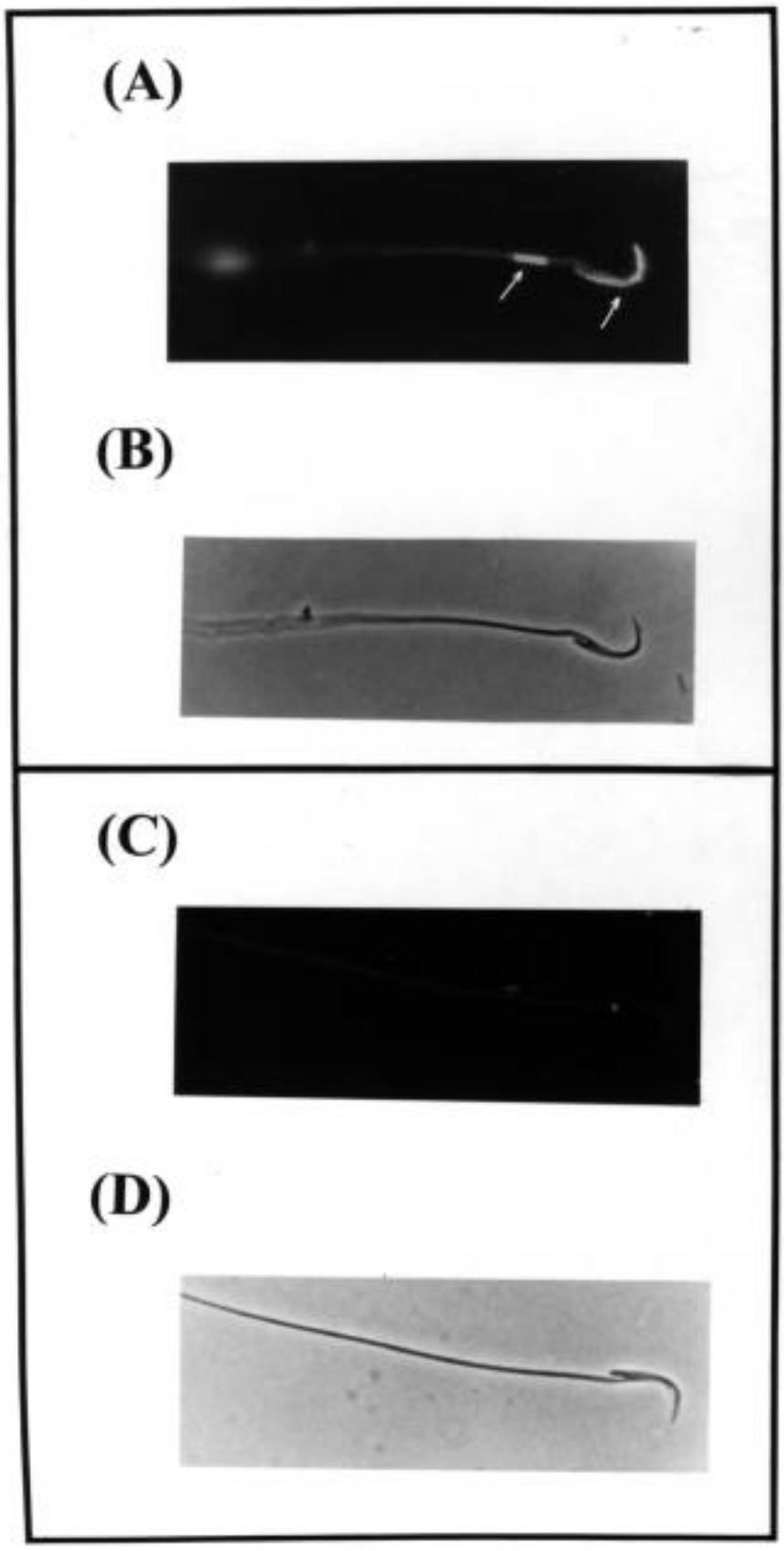

27,354.8
27,192.8
27,084.8
27,678.8
27,030.8
26,922.8
27,722.0
26,760.8
27,884.0
100
80
60
40
20
0
Relative Intensity
2.6
2.65
2.7
2.75
2.8
2.85
Molecular Mass x 10^4

**FIGURE 3** Protein DE localization on cauda sperm by indirect immunofluorescence. **(A)** Immunodetection of protein DE on cauda sperm (fluorescence image); **(B)** Immunodetection of protein DE on cauda sperm (phase contrast image); **(C)** negative control using secondary antibody only with rat cauda sperm (fluorescence image); **(D)** negative control using secondary antibody only with rat cauda sperm (phase contrast image).


(A)
(B)
(C)
(D)

# CHAPTER IV

## CHARACTERIZATION OF PROTEIN DE SYNTHESIS AND BINDING TO RAT EPIDIDYMAL SPERM BEFORE AND AFTER DEPOSITION IN THE FEMALE REPRODUCTIVE TRACT

The data presented in this chapter will be submitted to *Journal of Andrology.* The co-authors include:

## INTRODUCTION

In mammals, testicular spermatozoa lack the ability for progressive forward movement and to bind and fertilize an ovum (Dacheux and Paquignon, 1980). To acquire these two important biological properties, spermatozoa must leave the testis and pass through the epididymis, a process that is commonly known as *sperm maturation*. Maturation of spermatozoa within the epididymis is dependent on testicular androgens (Fournier-Delpech et al., 1984) and requires epididymal protein synthesis (Orgebin-Crist and Jahad, 1979). During epididymal maturation, both membrane lipids and proteins of spermatozoa have been shown to undergo distinct physical and chemical alterations (Nikolopoulou et al., 1985; Hall et al., 1991; Kohane et al., 1980). Although changes in membrane lipids have been postulated to stabilize the plasma membrane as spermatozoa pass through the epididymis (Parks and Hammerstedt, 1985), alteration of the surface of spermatozoa by secreted epididymal proteins represents a change that may be absolutely required for sperm-egg interactions (Echeverrria et al., 1984; Fouriner-Delpech et al., 1984; More and Hartman 1986; Vreeburg et al., 1992).

Several major secretory proteins of the rat epididymis have been isolated and biochemically characterized (Garberi et al., 1979; Brooks and Higgins, 1980; Jones, et al., 1980; Wong and Tsang, 1982; Brooks, 1982). However, of all the major secretory proteins of the rat epididymis that have been studied to date, only protein DE has been shown to be involved in a stage of the fertilization process (Cuasnicu et al., 1984;

Fournier-Delpech et al. 1985; Rochwerger et al., 1992; Hall et al., 1997; Cohen et al., 1996; Ellerman, Brantua et al., 1998). Previous studies have shown protein DE molecules to be synthesized by the principal cells of the epididymal epithelium and to bind to the surface of spermatozoa during passage through the epididymal duct (Lea et al., 1978; Kohane et al., 1980; Faye et al., 1980; Kohane et al., 1983; Brooks and Tiver, 1983; Turner et al., 1994). Protein DE molecules have been shown to be a member of the Cysteine Rich Secretory Proteins (CRISP), a group of proteins containing 16 conserved cysteine residues (Eberspaecher et al., 1995; Kratzschmar et al., 1996). The current emerging hypothesis is that protein DE molecules function in sperm-egg membrane fusion (Rochwerger et al 1992; Cohen et al., 1996; Ellerman, Brantua et al., 1998).

The aim of the present study was to further our understanding of how protein DE interacts with and bind to the surface of epididymal spermatozoa and how they remain bound after they have been deposited in the female reproductive tract. Western blot analysis, indirect immunofluorescent (IIF) localization, and photochemical cross-linking experiments were used to assess the topology of protein DE molecules on the surface of maturing epididymal spermatozoa and after their deposition in the female reproductive tract.

As illustrated in Table 1, several studies have presented evidence to support the concept that a structural reorganization of the plasma membrane occurs as spermatozoa undergo functional changes, such as maturation within the epididymis, the acrosome reaction, and

capacitation. In total, these useful studies have provided an excellent overview of the dynamic state of the sperm plasma membrane, but they have yet to provide evidence of how protein molecules from the surrounding fluid environment (e.g., epididymal lumen) were bound to the surface of spermatozoa.

## MATERIALS AND METHODS

### *Animals*

Sexually mature male and female Sprague-Dawley rats were purchased from Hilltop Lab Animals, Inc. (Scottsdale, PA), maintained on a 12-hour light:12-hour dark cycle, and given laboratory chow and water *ad libitum*. The rats were terminated by $CO_2$ asphyxiation followed by decapitation. The epididymides and testes of the males were excised from the scrotal sac and the fallopian tubes of the female were excised from the body cavity.

### *Collection of Epididymal Spermatozoa*

Epididymides with the vas deferentia attached were trimmed of extraneous adipose tissue and sectioned into four anatomical segments, as illustrated in Figure 1: proximal caput, distal caput, corpus, and cauda. These regions approximate the anatomical areas of the rat epididymis previously described (Jones et al., 1980). As shown in Table 1, three separate buffers were used to isolate, wash, and resuspend epididymal spermatozoa. These buffers were selected because they approximate the ion composition of the intraluminal contents of the caput, corpus, and cauda region of the epididymis (Setchell and Maddocks, 1994). The intraluminal contents of the caudal segment were flushed out through an incision in the distal caudal region of the epididymis and the attached vas deferens were backflushed with 0.5 mL of caudal buffer. Caput and corpus segments of the epididymis were minced several times with a single-edged razor blade. Spermatozoa released into the caput and corpus buffers were collected with a Pasteur pipette. Epididymal spermatozoa were pelleted and washed in their corresponding buffers by centrifugation (~1,000 x g, for 10 min. at room temperature). Sperm concentration was estimated by hemocytometric count. Spermatozoa recovered from each segment of the epididymis were contaminated by less than 0.1% somatic cells, as estimated by phase-contrast microscopy. Two to four rats were used for each experiment (N = three experimental replicates).

*Collection of Epididymal Sperm After Deposition in the Female Reproductive Tract*

Mating between female and male rats was initiated and observed after sunset by illumination using a red photographic light. A male of proven fertility (i.e., a progeny

producer, as assessed by Hilltop Lab Animal, Inc.) was placed in a rat holding cage with four females. When the male was observed to mount a female, both the male and female were then transferred to a separate holding cage and allowed to continue mating for an additional 15 minutes (e.g., typically 8 to 10 mounts by a male). A female was sacrificed fifteen minutes, one hour and seven hours after the male had deposited spermatozoa into the vagina cavity, and the fallopian tubes were excised. The fallopian tubes were gently squeezed using small metal forceps to recover the spermatozoa. Spermatozoa recovered from the fallopian tubes were suspended in phosphate buffered saline (PBS), pH 7.38 and pelleted by centrifugation (~1,000 x g for five minutes at room temperature). The concentration of spermatozoa and percentage contamination by other cell types were estimated by hemocytometric count. The preparation of sperm was contaminated by less than 0.1% somatic cells, as revealed by phase-contrast microscopy. The mating experiment at each time interval was repeated twice (N = three separate mating experiments).

*Preparation and Analysis of Anti-Protein DE Antibodies*.

Rabbit polyclonal antibodies (Abs) against purified protein DE were raised in two female New Zealand while rabbits weighing ~2.0 kg. After preimmune serum was collected, one milligram of lyophilized protein was dissolved in 500 μL of sterile deionized water and emulsified with 500 μL of Freund's complete adjuvant. Aliquots (100 μL) of the mixture,

which contained the protein at a concentration of 1 μg/μL, were administered intradermally at ten different sites along the rabbit's back.

The presence of protein DE Abs in the serum was assessed by enzyme immunosorbent binding and immunoprecipitation assays (Farrington and Hymer, 1987; Carson et al., 1977) at two, four, and six weeks after the injection of protein DE. A positive Ab titer was measured at the end of the six-week immunization period. The animals were then given a second dose of protein, which was emulsified with 500 mL of Freund's incomplete adjuvant at a protein concentration of 2 mg/mL, by intra-muscular injection. Two weeks after the second immunization, the animals were anesthetized and blood was collected by heart puncture. The blood was allowed to clot at $4^{o}$ C, centrifuged at 2000 x g at $4^{o}$ C to remove cells, and the antiserum stored in 500 mL aliquots at $-20^{o}$ C. The final titer for the rabbit anti-protein DE Abs was 1:40,000.

Chicken polyclonal Abs against purified protein DE were prepared commercially (Strategic Biosolutions, Inc., Ramona, CA) in two hens after pre-immunization egg collection. The purified sample (3.0 mg of total protein) was dissolved in sterile phosphate buffer, pH 7.2, and suspended at a final protein concentration of 0.84 mg/mL. Each hen was given four immunizations, followed by post-immunization egg collection for 15 days. The post-immunized eggs were then pooled into three batches: those collected on days one to five, six to ten, and 11 to 15. IgY Abs were isolated from each of

the three batches plus the preimmune batch and then titered by Strategic Biosolutions using an ELISA. The final titer for the chicken anti-protein DE IgY Abs was 1:8,800 and 1:8,500 for hen one and two, respectively. Both the rabbit and chicken anti-protein DE Abs were determined to be monospecific, as assessed by a Western immunoblot competition assay. Briefly, protein DE samples were subjected to electrophoresis on 10% uniform one-dimensional SDS-PAGE slab gels, transferred to a nitrocellulose membrane, and immunoblotted with anti-protein DE Abs. The immunoblotted nitrocellulose membrane was incubated with either rabbit or chicken anti-protein DE Abs that had been preabsorbed with 100 to 1000 fold of the purified protein. The major 32,000 dalton protein band identified using the anti-protein DE Abs was abolished, indicating that the polyclonal Abs were specific for protein DE.

*SDS-PAGE and Western Blot Analysis*

Protein samples were dissolved in 50 mM Tris/HCL, pH 6.8 containing 10% β-mercaptoethanol (v/v), 10% SDS (w/v), 30% glycerol (v/v), and 0.1% (w/v) bromphenol blue, and then incubated at $40^{o}$ C for 30 minutes to prevent the formation of high molecular weight protein aggregates. SDS-PAGE analysis was performed under reducing and denaturing conditions by use of the buffering system of Laemmli (Laemmli, 1968) and a uniform 8% polyacrylamide vertical slab gel. Each gel track contained an equal amount of total protein for qualitative analysis and was run at a constant current of 15

mA until the tracking dye reached the separating gel. The current was then increased to 30 mA per gel until the tracking dye was approximately one cm from the bottom of the slab gel. To visualize the banding pattern of resolved proteins, gels were stained with either silver using a commercially available staining kit (Bio-Rad) or 0.25% Coomassie Brilliant Blue R-250 dye (CBBD) (w/v) in an aqueous solution containing 50% methanol (v/v) and 7% acetic acid (v/v) for six to eight hours and then destained overnight in the same solution without CBBD. Prestained molecular mass markers (Pierce Chemical Co.) were used to estimate the molecular mass of resolved sperm proteins. The molecular mass protein markers included: myosin (~215,000 daltons), Phosphorylase B (~120,000 daltons), Bovine Serum Albumin (~84,000 daltons), Ovalbumin (~60,000 daltons), Carbonic Anhydrase (~39,200 daltons), Soybean Trypsin Inihbitor (~28,000 daltons), and Lysozyme (~18,000 daltons). As indicated by the manufacturer (i.e., Pierce Chemical Co.), the covalently bound CBBD alters the apparent molecular mass of the proteins relative to unstained proteins and tended to produce broader bands.

Proteins were electrophoretically transferred onto nitrocellulose (Bio-Rad) for eight to 10 hours at 40 volts using a Transphor Electrophoresis (Hoeffer/Pharmacia Scientific Instruments) or for 40 minutes at 24 volts using a Genie Semi-Wet Blotter (Idea-Scientific Co., Mineapolis, MN). To assess the efficiency of protein transfer, the gels were stained with Coomassie Blue dye and/or silver. The nitrocellulose membrane was blocked with commercially purchased 5% nonfat dry milk dissolved in 10 mM Tris/HCL, pH 8.0 containing 150 mM NaCL, and 0.05% Tween 20 for 30 minutes at room

temperature. Polyclonal antiprotein DE Abs were diluted 1:10,000 (rabbit Abs) or 1:5,000 (chicken Abs) and incubated for two hours to overnight. The nitrocellulose sheet was then washed three times (15 minutes each time) using 20 mM Tris/HCL, pH 7.5 containing 150 mM NaCL, 0.05% Tween 20, and 0.02% sodium azide (membrane blot wash buffer). Goat-anti-rabbit HRP (GAR-HRP) was diluted 1:25,000 and incubated for one hour at room temperature and washed three times (15 minutes each time) with membrane blot wash buffer. To detect the biotinylated molecular mass protein markers, NeutraAvidin0HRP was diluted 1:20,000 and incubated with GAR-HRP for one hour. After the one-hour incubation period, the nitrocellulose sheet was washed three times (15 minutes each time) with membrane blot wash buffer. To detect immune positive complexes, the nitrocellulose sheet was incubated for ~30 seconds to one minute with SuperSignal chemiluminescent substrate (Pierce Chemical Co.), covered with plastic wrap, and exposed to Kodak BioMax x-ray film at room temperature for 30 seconds to one minute or longer (i.e., three to five minutes) to visualize the immune positive banding pattern.

*Photoactivatable Cross-linking of Purified Protein DE to the Surface of Epididymal Spermatozoa*

The cross-linking of protein DE molecules to the surface of epididymal spermatozoa was performed in two stages. First, a purified sample of protein DE was dialyzed against phosphate buffered saline (PBS), pH 7.5, resuspended at a concentration of 1.0 µg/µL,

and conjugated to the tri-functional cross-linking reagent, sulfo-succinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido) hexanoamido] ethyl-1,3' dithio-proionate (Sulfo-SBED, Pierce Chemical Co.). The cross-linking, which has a biotin handle that is transferable, was conjugated to protein DE at a molar excess ratio 5:1 (3.6 nmol of Sulfo-SBED to 0.727 nmol of protein). Prior to coupling the cross-linking reagent to the protein, a working stock solution of the cross-linking reagent was prepared at a concentration of 0.1 μg/μL by dissolving one milligram of Sulfo-SBED into 10 mL of dimethyl sulfoxide (DMSO, Sigma Chemical Co.). Soybean trypsin inhibitor protein was subjected to $C_{18}$ reverse phase high-pressure liquid chromatography (HPLC) to assess its purity and then used for competition experiments as a nonspecific competitor protein. The "stock" Sulfo-SBED (3.2 μg) and purified protein DE (0.632 μg) were added to a siliconized 1.7-mL microfuge tube, dissolved in 300 μL of 25 mM sodium phosphate buffer, pH 7.2, and allowed to react at room temperature for one hour. The Sulfo-SBED-conjugated protein was then transferred to a Tube-O-Dialyzer (Geno Technologies, Inc., St. Louis, MO) and dialyzed for 12 hours at $4^{o}$ C to remove uncoupled cross-linking reagent. A schematic diagram illustrating the derivatization reactions involved in conjugating Sulfo-SBED to protein DE is shown in Figure 2. During the second stage, spermatozoa ($1.0 \times 10^{8}$ total cells) were incubated for 30 minutes in 25 mM phosphate buffer, pH. 7.5 containing 400 mM NaCl (Wong and Tsang, 1982) and washed twice in PBS by centrifugation at 1,000 x g for five minutes at room temperature to remove any non-covalently linked protein DE molecules from the sperm surface. The sperm samples were then placed in clear, siliconized glass test-tubes (13 x 75 mm) with buffer and other

reactants. To each test-tube, 0.632 µg (36 pmol) of the Sulfo-SBED-conjugated protein were added in the dark or under a red photographic light using a safelight filter (Eastman Kodak Co., Rochester, NY); the total reaction volume was 750 µL. Each test-tube was mixed by gently hand-vortex action and incubated for one hour at 33°C in the dark. To assess whether the interaction of protein DE molecules with the surface of spermatozoa was specific and competitive, sperm cells were incubated in the presence of a constant amount of Sulfo-SBED-conjugated protein DE, 100 molar excess of Soybean Trypsin Inhibitor protein, and varying concentrations (e.g., 50, 200, 500, 1000 molar excess) of "cold" protein DE (i.e., protein DE without Sulfo-SBED conjugation). After one hour, the test tubes were placed in a Spectronics Ultraviolet (UV) Viewing Cabinet Box (Spectronic Inc., Westbury, NY) and irradiated with short (i.e., 254 nm) UV light for 15 minutes at room temperature. The sperm samples were then brought to a final volume of 1.5 mL with PBS, centrifuged at 1,000 x g for 10 minutes at room temperature to pellet the spermatozoa, and the pellet was resuspended in 100 µL of 50 mM Tris/HCL, pH 7.5 containing 50 mM NaCl, 1 mM EDTA, 0.1% Nidonet P-40 (NP-40) detergent (solubilization buffer). The pelleted sperm samples were solubilized overnight by vortex action at room temperature and centrifuged at 12,000 x g for 10 minutes at room temperature to remove the insoluble material and nucleic acids. The pelleted material (i.e., large pieces of nucleic acids, undissolved mitochondria and sperm tails, etc.) was discarded. The proteins in the membrane-supernatant were then resolved by one-dimensional SDS-PAGE using an 8% uniform acrylamide slab gel under reducing and denaturing conditions as previously described in the *Materials and Methods.* The

photoaffinity labeled proteins were visualized with chemiluminescence as described elsewhere.

*Treatment of Spermatozoa with Phospholipase C (Phosphatidylinositol-specific)*

Caudal spermatozoa (1.0 x $10^8$ total cells) were suspended in 25 mM phosphate buffer, pH 7.5 containing 400 mM NaCl and incubated in a 1.7-mL plastic, siliconized microcentrifuge test-tube for 30 minutes at room temperature. The sperm cells were resuspended in PBS, washed twice in PBS by centrifugation (i.e., 1,000 x g for five minutes at room temperature), and reacted with 5 units of Phospholipase C (PLC) for 30 minutes, one hour, and two hours at 37$^o$ C, with gentle agitation in a rotating water bath. A control experiment consisted of incubating a sperm sample for 2 hours at 37$^\circ$ C without enzyme (e.g., the PLC solution was replaced with an equal volume of PBS) was used to assess nonenzymatic dissociation of the protein under the reaction conditions. After the various incubation periods, the sperm samples were centrifuged at 1,000 x g for five minutes, and the supernatant solutions were removed and stored at –20$^o$ C until further analysis. The sperm pellets were then resuspended in PBS and washed three time in PBS by centrifugation at 1,000 x g for five minutes at room temperature. The sperm pellets were then resuspended in 100 μL solubilization buffer, vortexed overnight at room temperature, and centrifuged at 12,000 x g for 10 minutes at room temperature. The proteins in the supernatant solutions (i.e., the 1,000 x g and 12,000 x g centrifugations)

were resolved by one-dimensional SDS-PAGE under reducing and denaturing conditions on 8% uniform acrylamide slab gel as previously described in the Materials and Methods.

*Immunofluorescent Localization of Protein DE Molecules on the Sperm Surface*

All immunofluorescent microscopic procedures were performed at room temperature. Spermatozoa recovered from the male epididymis and female reproductive tract were suspended in PBS, washed three times in PBS by centrifugation at ~700 x g for five minutes, and prepared for immunofluorescence at ~3.0 x $10^7$ total cells. The sperm samples were incubated for 30 minutes in Bouin's fixative, which was prepared fresh for each sperm isolation. The fixed spermatozoa were washed four to five times in PBS to remove excess fixative and pelleted by centrifugation at

700 x g. The pelleted spermatozoa were resuspended in PBS containing 1% bovine serum albumin (i.e., used as a blocking reagent) for one to two hours at $4^o$ C. Spermatozoa were centrifuged at 700 x g for five minutes and resuspended in normal donkey serum for 30 minutes. After the incubation period, spermatozoa were centrifuged at ~700 x g for five minutes and washed three time in PBS. The pellet was resuspended in 198 μL of 5% nonfat milk/PBS (w/v) and 2 μL of chicken anti-protein DE IgY Abs for one hour; this yielded a 1:100 dilution of the primary Abs. After incubation with the primary Abs, the spermatozoa were resuspended in PBS, washed three times by centrifugation at ~700 x g at room temperature, and the supernatant discarded. A drop of gel mount solution was placed on a glass microscope slide, 25 μL of the sperm pellet were injected directly into

the gel mount, and a cover slip placed on the microscope slide. Control experiments to assess nonspecific Ab staining were performed through replacement of the immune Abs with preimmune chicken IgY fraction, preabsorption of the immune Abs with purified antigen, dilution or elimination of immune Abs, and dilution or elimination of the secondary Abs. Sperm were examined using a Nikon LabPhot microscope equipped with an FITC epifluorescence apparatus, and photomicrographs were taken using Kodak T-Max 400 black/white film (400 ASA).

*Protein Assay*

Soluble protein was measured using the Lowry Method (Lowry et al., 1951), with bovine serum albumin (BSA) as the standard protein. Samples were assayed in duplicate with the concentration of BSA ranging from 10 to 80 μg/mL in 10-μg increments.

## RESULTS

A chicken-anti-protein DE was generated to perform the immunolocalization and western blot analyses. Prior to using the antibody, it was tested for its specificity to protein DE. To assess the specificity of chicken anti-protein IgY Abs, a competition assay was performed, and the results of the assay are presented in Fig 1. No protein DE immune positive staining was detected when the Abs were preabsorbed with 100-fold excess

purified protein DE (fig 1a, lane 2). As shown in fig 4b, protein DE was detected in the protein extracts of tissue and spermatozoa obtained from the distal caput, corpus, and caudal regions, but not in the proximal caput region of the epididymis. Densitometric scanning of the western blots revealed that the amount of protein DE detected increased from the distal caput to the caudal region of the epididymis (Fig 2).

The localization of protein DE on ejaculated sperm was assessed by IIF. Figure 3 shows the IIF localization of protein DE molecules on the surface of ejaculated spermatozoa deposited immediately (i.e., minutes) in the female vaginal cavity and after being deposited in the female vaginal cavity for one and seven hours. Spermatozoa recovered immediately from the vaginal cavity exhibited intense fluorescent staining that was localized over the entire surface of the spermatozoa (fig 3B). In contrast, an intense fluorescent staining pattern was primarily localized to the head and mid-piece regions of spermatozoa recovered from the vaginal cavity after one hour (fig 3C). After seven hours of being deposited in the female vaginal cavity (fig 3D), the fluorescent staining intensity on the surface of the spermatozoa was comparable to that of the control samples (secondary antibody-FITC after seven hours).

An experiment to assess the interaction of protein DE molecules with the plasma membrane of epididymal spermatozoa is presented in figure 5. As shown in figure 6, in the presence of soybean trypsin inhibitor protein and conjugated-protein DE, five

prominent protein bands exhibiting a molecular mass of ~120,000, ~48,000, ~82,000, ~76,000, and ~75,000 daltons were labeled (figure 6, see arrows). The competition experiment using "cold" protein DE (i.e., unconjugated) at 50, 200, 500 and 1000 fold molar excess in the presence of a constant amount of Sulfo-SBED-conjugated protein DE eliminated the banding intensity a protein exhibiting a molecular mass of approximately 120,000 daltons (figure 6).

The effect of phospholipase C treatment for various periods of time on the release of protein DE molecules from the surface of spermatozoa is presented in figure 7. In comparison to the control, spermatozoa treated for 30 minutes, one hour, and two hours with phospholipase C exhibit a marked decrease in the fluorescence intensity over the head region of the spermatozoa, but not over the regions covering the mid-piece and tail (fig 7A). The release of protein DE molecules from the surface of spermatozoa into the supernatant by phospholipase C treatment, as assessed by western blot analysis, is presented in Figure 7B. After two hours of enzyme treatment, a marked increase was observed in the amount of protein DE molecules released into the supernatant, compared to the control supernatant (fig 7B, lanes 3,5 and 7).

## DISCUSSION

The results of the present study confirm earlier findings (Lea, Petrusz et al. 1978; Faye, Duguet et al. 1980; Kohane, Cameo et al. 1980; Brown, von Glos et al. 1983; Kohane,

Pineiro et al. 1983; Brooks 1987) (table 1**)** that protein DE molecules secreted by the epididymal epithelium initially bind to spermatozoa in the caput region and demonstrate for the first time that protein DE interacts with a specific plasma membrane protein, and is subsequently bound to the sperm surface through a glycosylphosphatidyl inositol linkage

These results demonstrating regional differences in the immunofluorescent staining pattern of protein DE molecules on the surface of epididymal spermatozoa suggest that rearrangement of "preexisting" membrane-bound protein DE molecules occurs as spermatozoa pass through the epididymis. The rearrangement of preexisting membrane components (e.g., proteins and/or lipids) within the plane of the plasma membrane using biophysical techniques has been demonstrated previously using ram (Wong and Tsang 1982), guinea pig (Myles and Primakoff 1984; Myles, Primakoff et al. 1984; Cowan, Primakoff et al. 1986), and rat (Gaunt 1983; Gaunt, Brown et al. 1983). The rearrangement of protein DE molecules as spermatozoa mature within the epididymis could possibly account for the regional differences in the immunofluorescent staining patterns of spermatozoa obtained from the caput, corpus, and caudal regions of the epididymis. It is also possible that the observed regional differences in the IIF staining patterns on the surface of epididymal spermatozoa may be due to the loss of "preexisting" membrane proteins (e.g., coating proteins) during passage through the epididymis. This may result in the exposure of protein DE molecules on the head region to antibody binding, but not on the tail region of spermatozoa as they pass through the epididymis.

The observation that protein DE molecules remain on the surface of ejaculated spermatozoa recovered from the female vaginal cavity (Fig. 3) suggests that protein DE molecules may be covalently linked to the surface of spermatozoa. Although protein DE molecules were observed to redistribute to the equatorial region of the sperm head (Fig. 3), as observed in a previous study (Rochwerger and Cuasnicu 1992), demonstrating redistribution of protein DE under *in vivo* conditions, the immunofluorescent staining pattern was different. The differences may be due to the fact that ejaculated spermatozoa, spermatozoa which were subjected to interaction with seminal fluid, were used in the present study while in the earlier study *in vitro* capacitated spermatozoa were injected into the uterine horns of the female rats and recovered after six to seven hours of incubation. Alternatively, the polyclonal antibodies used in the present study may recognize additional protein DE molecule epitopes. Nonetheless, the observation that protein DE molecules were found on the head regions of both ejaculated spermatozoa recovered from the female vaginal cavity (Fig. 3) and spermatozoa that had been subjected to *in vitro* capacitation (Rochwerger and Cuasnicu 1992) suggests that the protein DE molecules located over the head region were covalently bound. Although earlier studies (Sattayasai, Sattayasai et al. 1984) have produced data implicating the participation of membrane-protein sulfhydryl groups in the epididymal maturation of spermatozoa, a more recent study (Rooney, Atkinson et al. 1993) suggests a possible role of GPI anchors in the binding of proteins to the surface of spermatozoa. The release of protein DE molecules by phospholipase C treatment from the head region, but not the tail

region of spermatozoa (Fig. 7), has provided direct evidence that some protein DE molecules were covalently bound via a GPI anchor.

Previous reports that protein DE molecules were secreted by the epithelium of the rat epididymis and bind to the surface of rat spermatozoa (Lea, Petrusz et al. 1978; Faye, Duguet et al. 1980; Wong and Tsang 1982; Brown, von Glos et al. 1983; Kohane, Pineiro et al. 1983; Brooks 1987; Hall and Hadley 1990; Hall and Tubbs 1997) suggest that protein DE molecules interact with the plasma membrane of spermatozoa through a binding or receptor type membrane protein. Experiments designed to assess the mechanism(s) underlying the interaction of protein DE molecules with the plasma membrane of spermatozoa indicated that protein DE molecules interact with at least two membrane proteins with a subunit molecular mass of ~130,000 and ~120,000 daltons, respectively (Fig. 6). Since these two protein bands were eliminated by using a molar concentration of unconjugated ("cold") protein DE molecules at 100-fold excess, the interaction was deemed specific. However, our findings (Fig. 6) as well as previous reports by another laboratory (Moore, White et al. 1989) that a "maturation-dependent glycoprotein" (i.e., protein DE) may be bound to the surface of spermatozoa through a GPI anchor has led us to speculate about the mechanism(s) of how protein DE molecules interact and bind to the surface of epididymal spermatozoa. The interaction of protein DE molecules with the plasma membrane of spermatozoa appears to be more complex than a simple receptor-ligand type of interaction (Wong and Tsang 1982; Hall and Hadley 1990; Hall, Tubbs et al. 1996). Since protein DE contains 14 cysteine residues, which are

clustered in the C-terminal region of the protein (Eberspaecher, Roosterman et al. 1995) and various enzymatic activities have been found in association with the plasma membrane of rat spermatozoa (Chulavatnatol and Ruenwongsa 1976; Chulavatnatol and Yindepit 1976; Curry and Atherton 1992), it is possible that the interaction of protein DE molecules with the surface of spermatozoa may be a two-step process. The initial step may involve the clustered 14 cysteine residues in the C-terminal region. This region of the protein may serve to bind and/or orient protein D molecules toward the plasma membrane overlying the head region while protein E molecules may be directed to the tail region. The two protein molecules have been shown to have differences in their structural features (Xu and Hamilton 1996), suggesting that protein D and E molecules may exhibit different "functional" domains. The second step may involve an enzyme-mediated covalent linkage of protein D molecules to a GPI anchor located on the plasma membrane covering the head region. This explanation could account for our finding that glycosylphosphatidylinositol-specific phospholipase C treatment of spermatozoa removed protein DE molecules from the plasma membrane over the head region, but not from the tail region of epididymal spermatozoa (Fig. 7).

Alternatively, it is also possible that the membrane anchor or the lack of specific type of membrane anchor in different regions of the plasma membrane (e.g., head region vs. tail region) may influence both the kinetics and binding affinity for a specific receptor. A previous study (Chesla, Li et al. 2000) using Chinese Hamster Ovary cells has demonstrated that the membrane anchor influences both the binding kinetic rate and

affinity of a cell surface receptor for monomeric IgG (CD16). The receptor is one of only four eukaryotic receptors known to exist in both the transmembrane (CD16-TM) and glycosylphosphatidylinositol (CD16-GPI) isoforms (Chesla, Li et al. 2000). Although the biological significance of the two receptor isoforms is not known, this study showed that, compared with the CD16-TM, the CD16-GPI bound faster and with higher affinity to human and rabbit IgGs but slower and with lower affinity to mouse IgG (Chesla, Li et al., 2000). This may account for earlier reports (Wong and Tsang 1982) of Scatchard binding data that revealed two binding kinetics patterns, one in which protein DE molecules bound with high affinity (e.g., $K_d$ = 2.6 x $10^{-10}$ M) but low capacity to the surface of caudal spermatozoa, and the other in which protein DE molecules bound to the surface of caudal spermatozoa with lower affinity (e.g., $K_d$ = 2.2 x $10^{-9}$ M) but high capacity.

Although protein DE molecules, which are members of the CRISP family, have been identified in different organs of a variety of mammalian species (Foster and Gerton 1996; Kratzschmar, Haendler et al. 1996; Haendler, Toda et al. 1999; Klemme, Roberts et al. 1999) and have been postulated to participate in a more specific stage of fertilization, such as the sperm-egg fusion event (Rochwerger, Cohen et al. 1992; Rochwerger and Cuasnicu 1992; Cohen, Munuce et al. 1996) because protein DE molecules have been localized to an area of the plasma membrane overlying the head region (Faye, Duguet et al. 1980; Brooks and Tiver 1983; Cameo, Echeverra et al. 1986; Rochwerger, Cohen et al. 1992; Rochwerger and Cuasnicu 1992; Cohen, Munuce et al. 1996), several important functional questions regarding this protein remain unanswered.

**FIGURE 1** Specificity of Chicken–anti–protein DE.

Purified protein DE was resolved via SDS-PAGE and blotted to nitrocellulose. (1) Protein DE was visualized with Chicken–anti–protein DE; (2) Chicken–anti–protein DE was preabsorbed for 1 hour with 100 fold excess purified protein DE then used to visualize transblotted protein DE. No protein DE is detected demonstrating the specificity of competition when using purified protein DE.

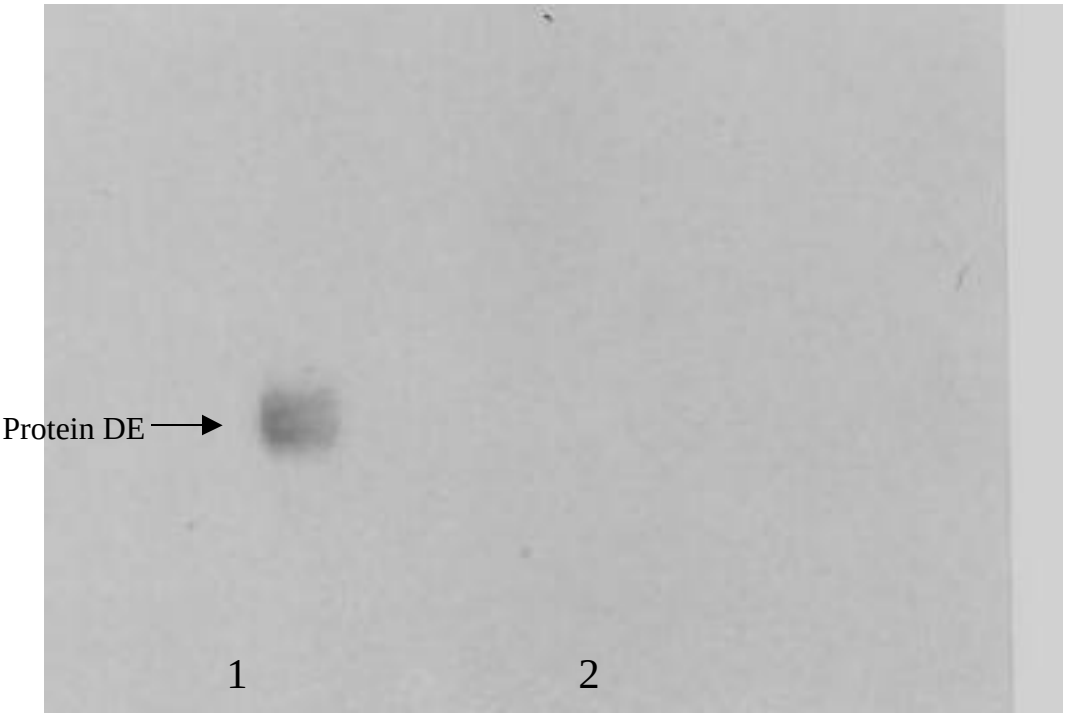
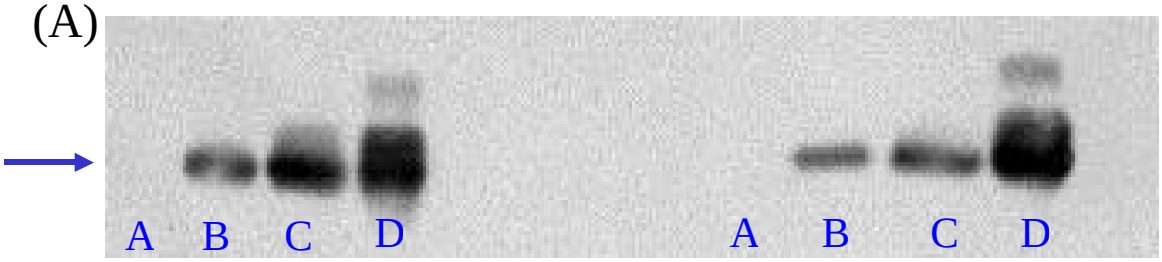

Protein DE
1
2

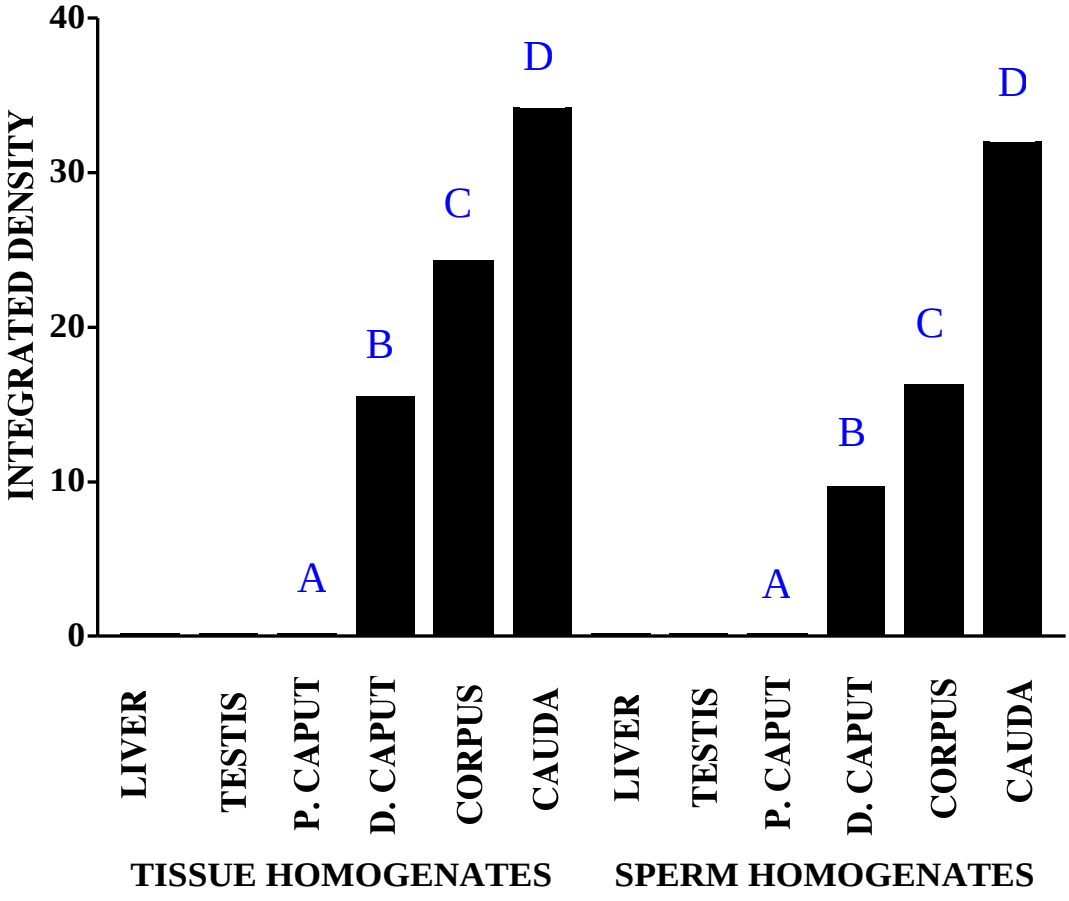
**FIGURE 2** **(A)** Western blot analysis using rabbit–anti–DE, epididymal tissue and tissue from liver; testis; (a) proximal caput; (b) distal caput; (c) corpus; (d) cauda epididymis

**(B)** Indirect immunofluorescence of protein D on sperm recovered from various epididymal regions.

(A)




**ANTI-PROTEIN D IMMUNOREACTIVITY**



(B)

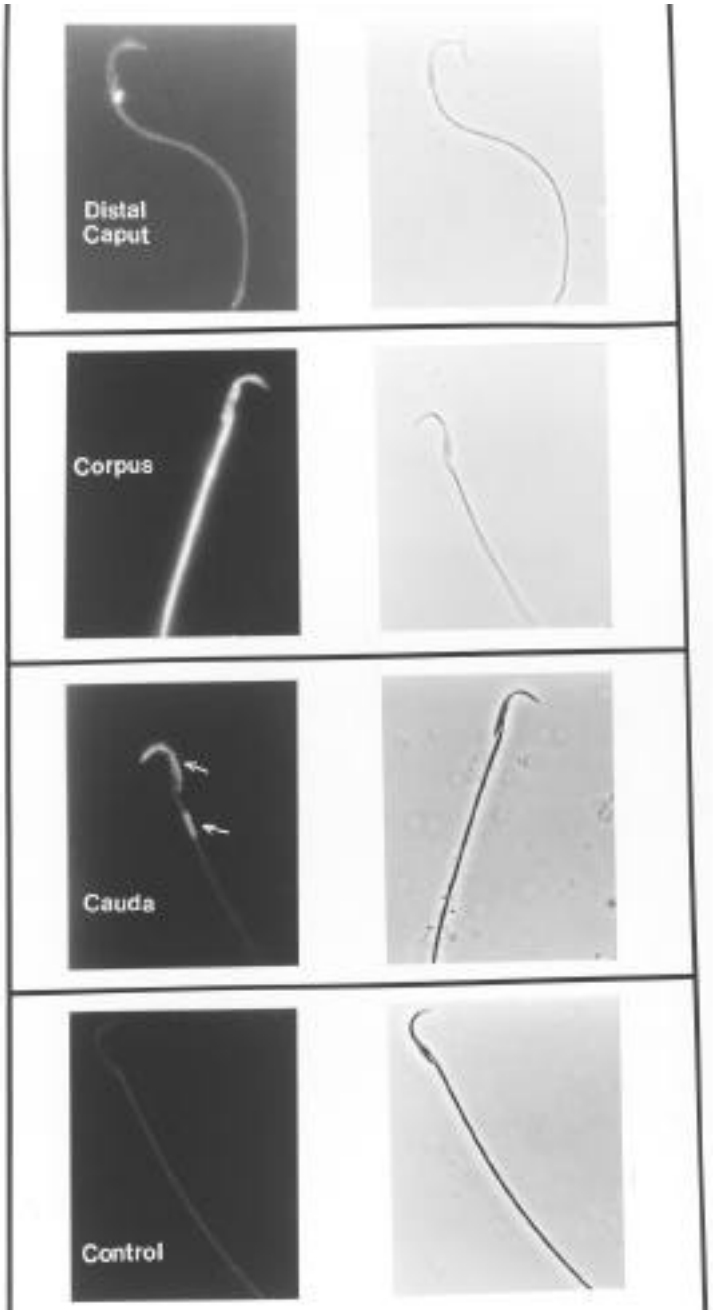


**FIGURE 3** **(A)** Indirect immunofluorescene of protein DE on ejaculated sperm recovered from the female reproductive tract after 10 minutes (ejaculate) ; one hour; and seven hours.

**(B)** Western blot analysis of samples in **(A)** using chicken–anti–DE and visualized with enhanced chemiluminescence. Sperm recovered from the female reproductive tract (3) 10 minutes; (2) one hour; and (1) seven hours after ejaculation. In figure B, Lanes 4 and 5 are positive controls: solubilized caudal sperm and purified protein DE, respectively.

Protein DE is found to initially relocalize over the entire surface of the sperm head immediately after ejaculation, then to the postacrosomal region after 1 hour and finally cannot be detected after seven hours by either IIF (**A) ,** or western blot analysis. (**B**,1).

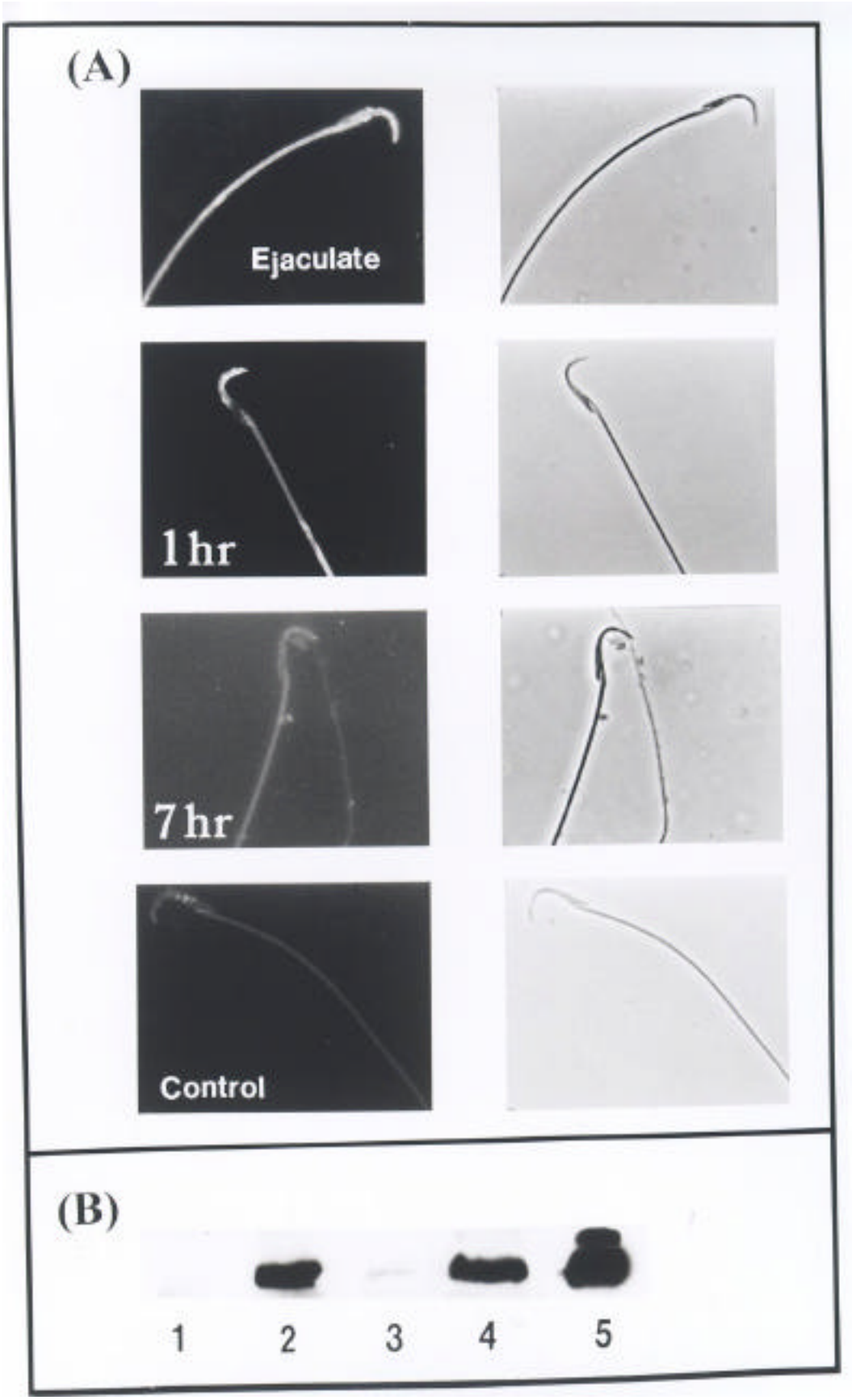
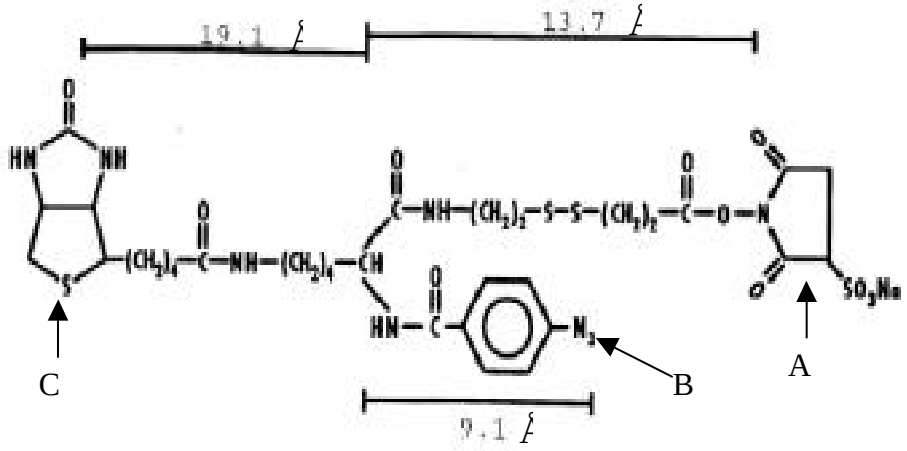

(A)
Ejaculate
1 hr
7 hr
Control
(B)
1
2
3
4
5

**FIGURE 4** Photoactivatible cross-linker molecule Sulfo-SBED

The cross-linking molecule is heterotrifunctional: (a) NHS-ester functional group for covalent linkage to free amines; (b) Azido group for non-specifically cross linking into peptide bonds; and (c) biotin moiety that is used for sensitive detection with Avidin-enhanced chemiluminescence detection.




**Sulfo-SBED**

**M.W. 879.97**

**FIGURE 5** Schematic Illustration of the photoactivatible cross linking proceedure.

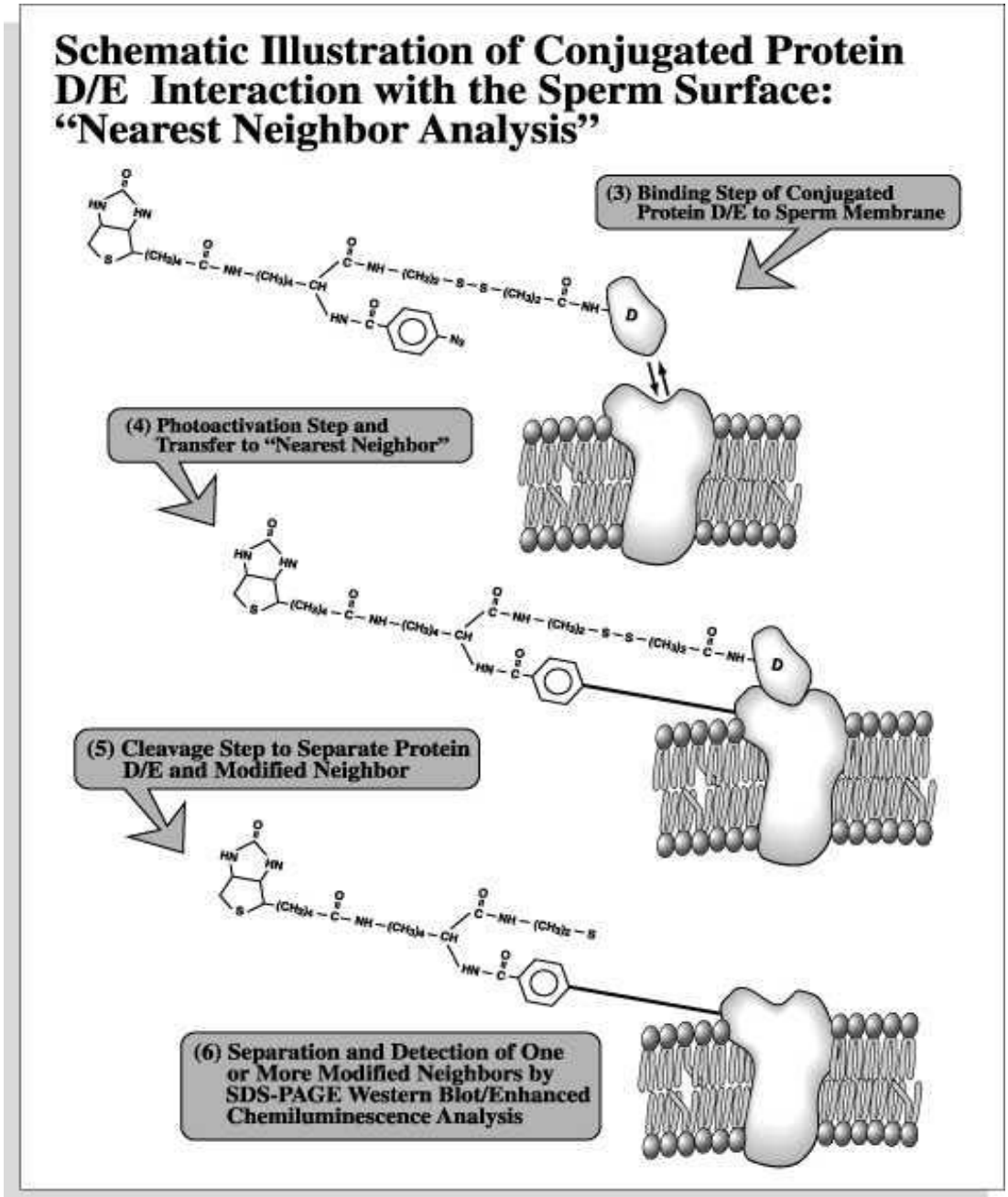
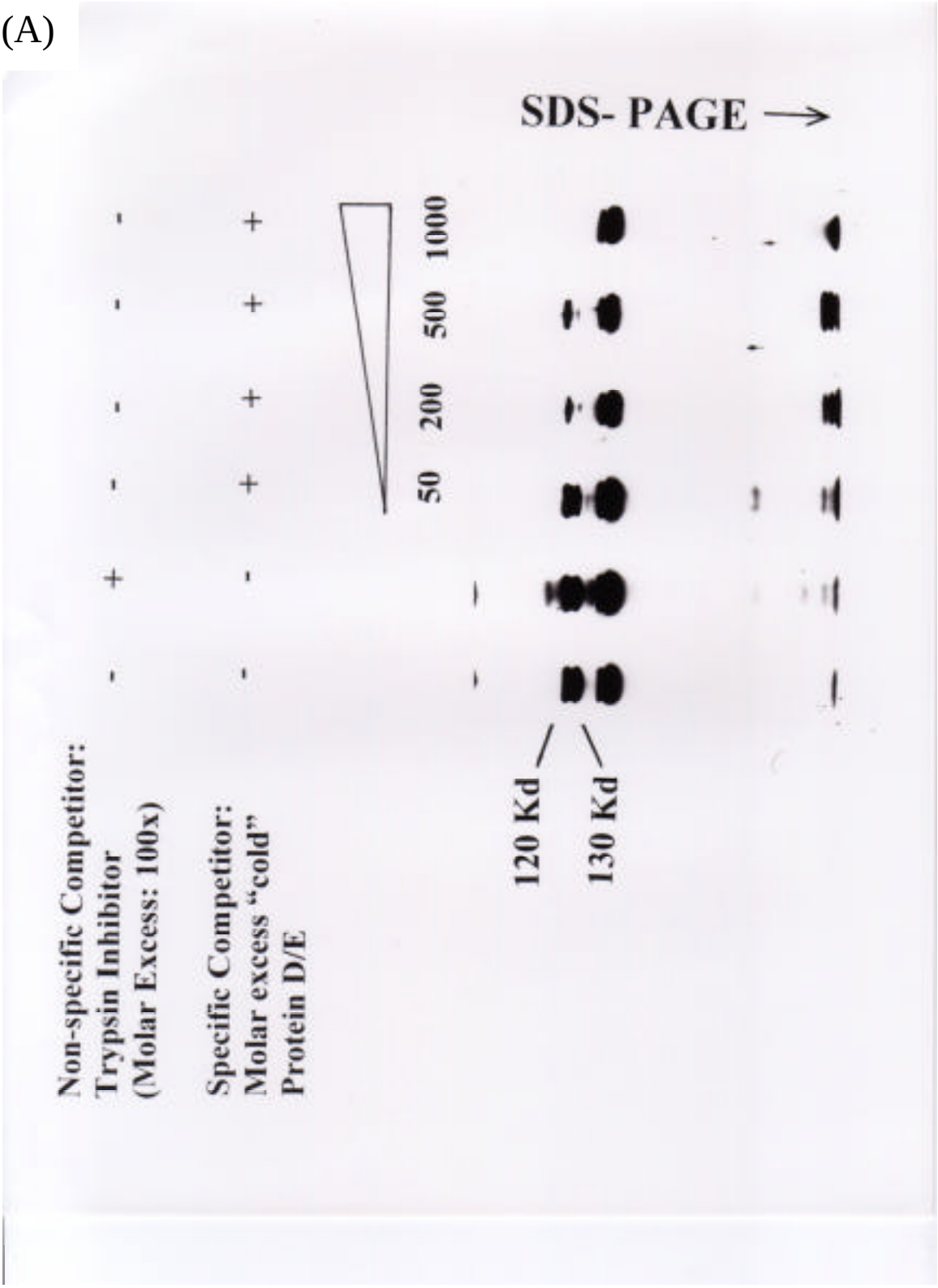

Schematic Illustration of Conjugated Protein D/E Interaction with the Sperm Surface: "Nearest Neighbor Analysis"
(3) Binding Step of Conjugated Protein D/E to Sperm Membrane
(4) Photoactivation Step and Transfer to "Nearest Neighbor"
(5) Cleavage Step to Separate Protein D/E and Modified Neighbor
(6) Separation and Detection of One or More Modified Neighbors by SDS-PAGE Western Blot/Enhanced Chemiluminescence Analysis

**FIGURE 6** Identification of protein DE binding protein on the surface of rat sperm using photoactivatible cross-linking.

Caudal sperm that were washed of loosely bound protein DE using 0.4M NaCl were incubated with SBED-protein DE and (A) increasing amounts of purified protein DE or (B) 100-fold excess purified protein DE. Specificity of competition was demonstrated with 100-fold excess trypsin inhibitor instead of purified protein DE. The 120 KDa band is specifically competed away in the presence of 200 fold excess pure protein DE, respectively.

(A)


SDS- PAGE →
Non-specific Competitor:
Trypsin Inhibitor
(Molar Excess: 100x)
Specific Competitor:
Molar excess "cold"
Protein D/E
- + - -
- - + +
- - + +
- - + +
50 200 500 1000
120 Kd
130 Kd

(B)

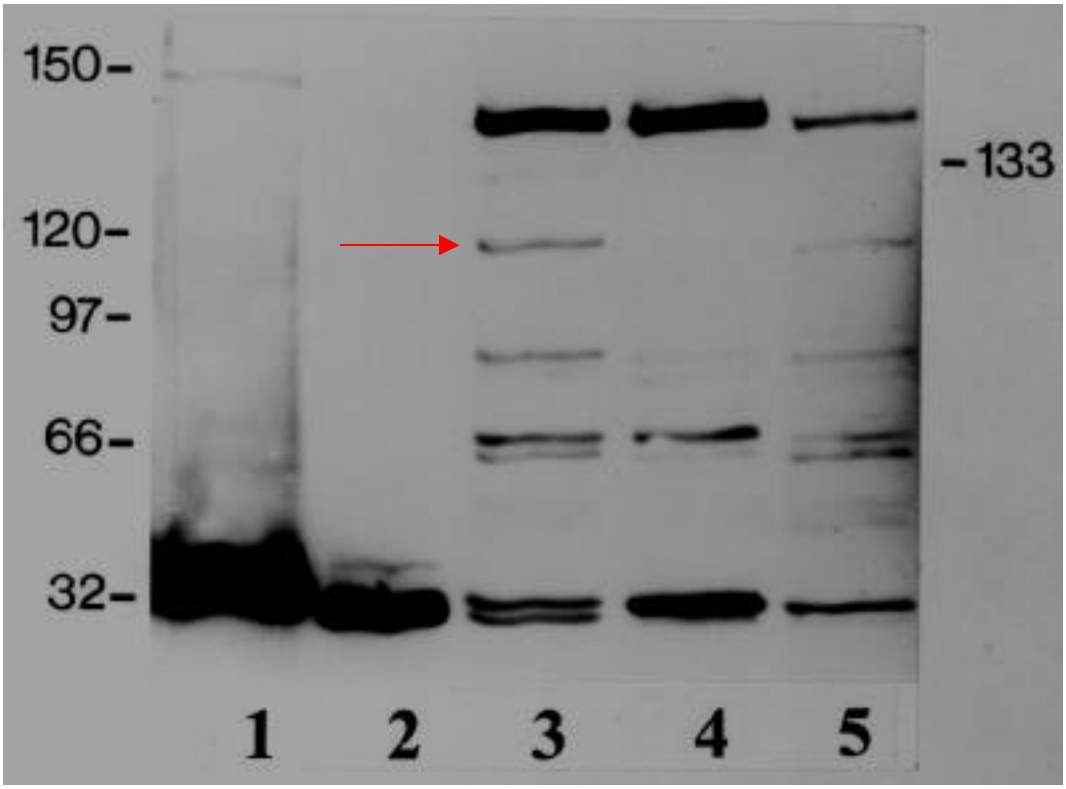


**FIGURE 7** Incubation of caudal sperm with phosphatidyl inositol specific phospholipase C. (PI-PLC) and protein DE is visualized by indirect immunofluorescene after (A) 30 minutes, (B) one hour; and (C) two hours after incubation with PI-PLC and (D) in the absence of PI-PLC.

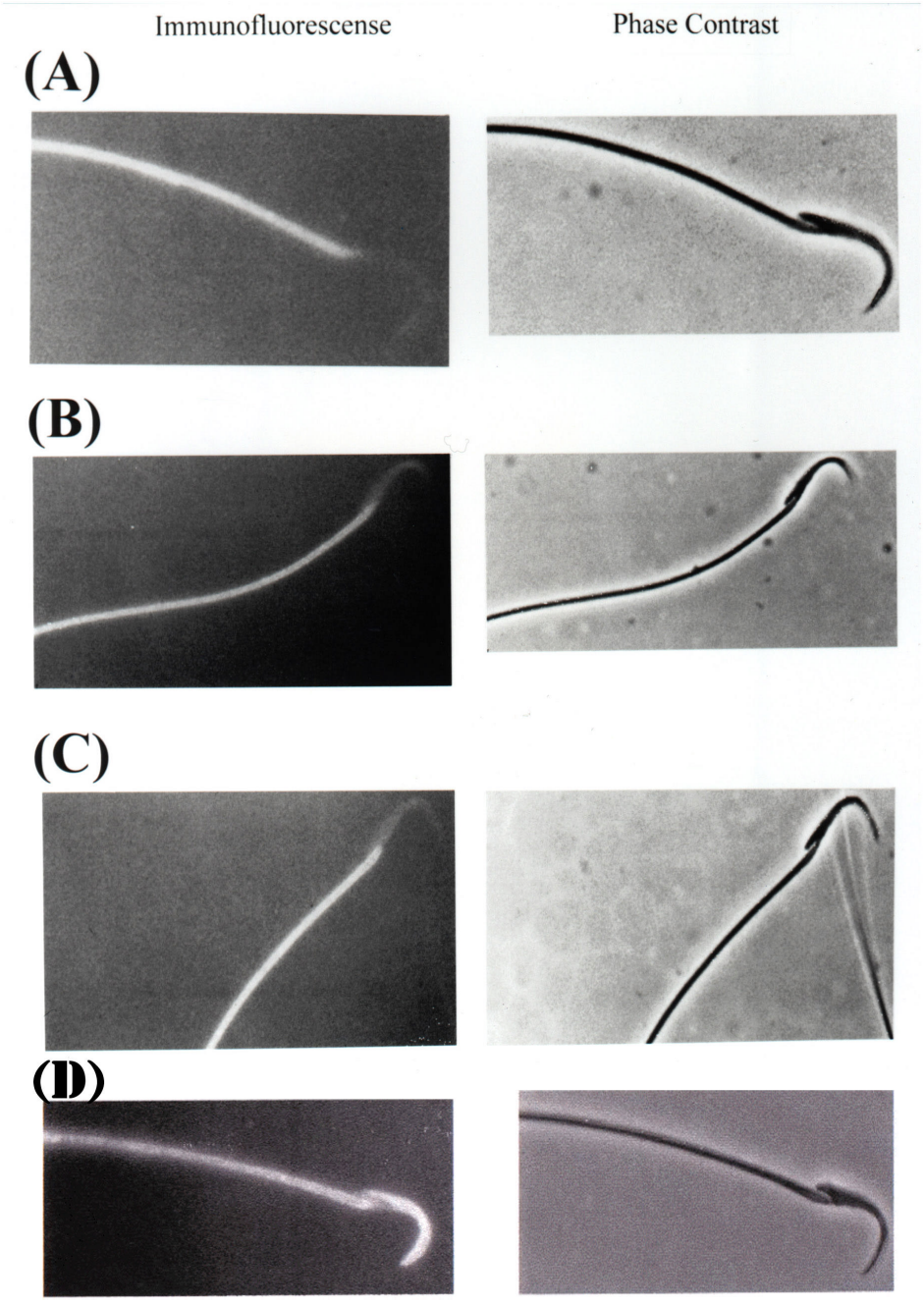

Immunofluorescense
Phase Contrast
(A)
(B)
(C)
(D)

**TABLE 1** Table of various sperm membrane proteins and their localization. All of the proteins have been determined to relocalize from one domain to a different domain on the sperm surface after significant maturational events.

| Protein | When Changed | Domain Change | Reference: |
|---|---|---|---|
| PH-20 | Epididymal passage | Whole cell to posterior | [5] |
| Fertilin (PH-30) | Epididymal passage | Whole head to posterior head | [6] |
| AH-50 | Epididymal passage | Whole head to anterior head | [7] |
| CE9 | Epididymal passage | Posterior tail to anterior tail | [8] |
| Surface galactosyl transferase | Epididymal passage | Anterior head over acrosome to more restricted acrosomal "cap" | |
| Protein DE | Epididymal passage | Concave surface of the anterior region of the head to the lateral head surfaces in the post-acrosomal region | [1] |
| PH-20 | Acrosome reaction | Posterior head to inner acrosomal membrane | [4] |
| Protein DE | Acrosome reaction | | [2] |
| Protein DE | Capacitation | Dorsal region of head to equatorial region | [3] |

1. Brooks, D.E. and K. Tiver, *Localization of epididymal secretory proteins on rat spermatozoa.* J. Reprod Fertil, 1983. **69**(2):p. 651-7

2. Cameo, M.S., et al., *Immunochemical Localization of Epididymal Protein DE on Rat Spermatozoa: Its Fate After Induced Acrosome Reaction.* Gamete Research, 1986. **15**:p.247-257.

3. Rochwerger, L. and P.S. Cuasnicu, *Redistribution of a rat sperm epididymal glycoprotein after in vitro and in vivo capacitation.* Mol Reprod Dev, 1992. **31**(1):p. 34-41.

4. Cowan, A.E., D.G. Myles, et al. *Lateral Diffusion of the PH-20 Protein on Guinea Pig Sperm: evidence that barriers to diffusion maintain plasma membrane domains in mammalian sperm.* 1987. J Cell Bio **104**(4):917-23

5. Deng, X., K. Czymmek, et al. *Biochemical maturation of PAM1(PH-20) during epididymal transit of mouse sperm involves modifications of N-linked oligosaccharides.* Mol Reprod Dev **52**(2): 196-206

6. Hunnicutt, G.R., D.E. Koppel, et al. *Analysis of the process of localization of fertilin to the sperm posterior head plasma membrane domain during sperm maturation in the epididymis.* Dev Biol **191**(1): 146-59

7. Phelps, B.M., D.E. Koppel, et al. *Evidence that proteolysis of the surface is an initial step in the mechanism of formation of sperm cell surface domains*. J Cell Biol **111** (5 Pt.. 1): 1839-47

8. Petruszak, J.A., C.L. Nehme, et al. *Endoprtoeolytic cleavage in the extracellulardomain of the integral plasma membrane protein CE9 precedes its redistribution from the posterior to the anterior tail of the rat spermatozoon during epididymal maturation.* J Cell Biol **114**(5): 917-27

# CHAPTER V

## SUMMARY AND CONCLUSIONS

In this study, a rapid procedure for the purification of rat protein DE (CRISP-1) was developed using classical column chromatography. The purified protein was then biochemically characterized by two-dimensional electrophoresis, electrospray-ionization mass spectrometry, and circular dichroism. For the first time, the amino-terminus of protein D was deblocked and sequenced by automated Edman degradation. Then, using the purified protein, the synthesis, secretion, and binding of protein DE to the membrane of epididymal sperm was correlated using western blot analysis and indirect immunofluorescence. Ejaculated sperm were recovered from the female reproductive tract at various times, and the binding of epididymal protein DE was characterized by indirect immunofluorescence and western blot analysis. UV cross-linking of protein DE was used to identify a 120 kDa binding protein for protein DE on the surface of epididymal sperm. Finally, phosphatidylinositol-specific phospholipase C was used to demonstrate that the interaction of protein DE with sperm is phosphatidylinositol mediated.

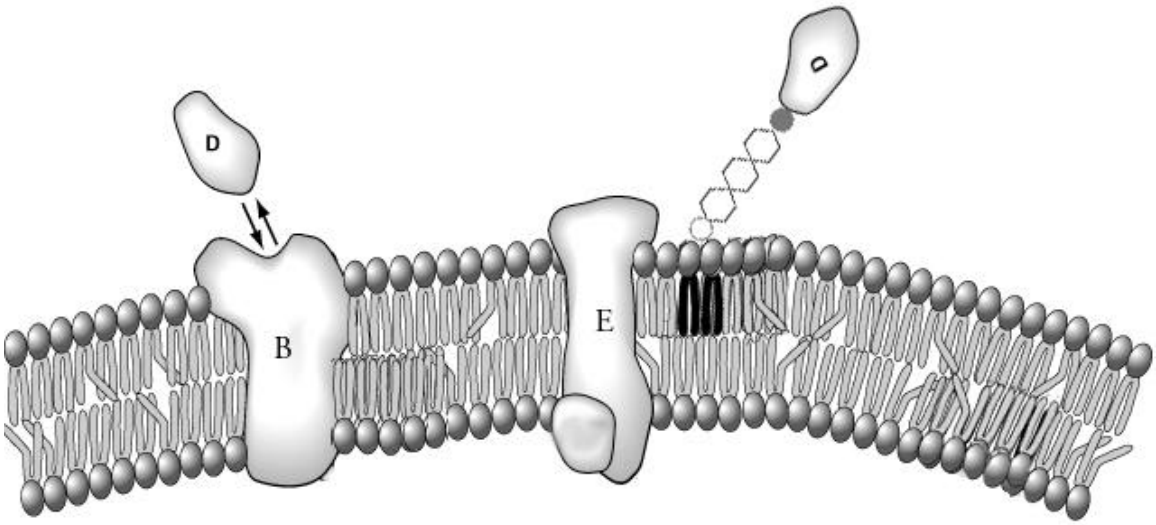
Since protein DE has been shown to bind to sperm with high affinity and a population of it bids irreversibly, I attempted to identify what protein DE binds to on the surface of epididymal sperm. Using photoactivatible cross-linking, a 120 KDa sperm surface protein, was identified as specifically binding to protein DE. The next question that was asked is how the irreversible protein DE remained associated with sperm as it was not ionic or due to disulfide bonds. GPI anchoring of proteins to the extracellular

surface of cells is a well characterized post-translational modification. When Epididymal sperm, that had been washed of reversibly bound protein DE were incubated in the presence of phosphatidylinositol-specific phospholipase C, protein D and not protein E was liberated as determined by western blot analysis and indirect immunofluorescence. These data suggest that protein DE is binding to the sperm membrane either by (a) Initially, binding to a 120 KDa binding protein, and then is enzymatically anchored to the plasma membrane via a GPI-anchor; or (b) binding to a 120 Kda protein and then covalently binds to a protein that is anchored to the plasma membrane via a GPI linkage (Figure 1). In somatic cells, GPI anchoring of proteins occurs during the posttranslational modification of the newly synthesized molecule. As the GPI-targeted protein passes through the endoplasmic reticulum, the carboxy terminus is cleaved and the GPI anchor is transferred en bloc through a peptide bond to the C-terminal residue (Ferguson and Williams 1988; Doering, Masterson et al. 1990; Udenfriend and Kodukula 1995). How then, would a secreted, soluble protein become attached to the sperm membrane via a GPI-anchor? One possibility is the enzyme that is responsible for the C-terminal attachment of the GPI anchor is present on the plasma membrane of epididymal sperm. In somatic cells, the GPI-anchor transferase is present in the endoplastic reticulum (Doering, Masterson et al. 1990). Sperm, as they develop, extrude many of their cytoplasmic organelles (ie golgi, endoplasmic reticulum and lysosomes) and cytoplasm in a cytoplasmic droplet (Roberts, Scouten et al. 1976). As the spermatozoa traverse the epididymis, the cytoplasmic droplet is associated with the plasma mebrane and migrates from the

head to the tail region and is eventually lost in the cauda region. The Enzyme markers for the golgi apparatus alpha 2,6 sialyltransferase, and anti-human beta 1,4 galactosyltransferase have been immunolocalized in the cytoplasmic droplet (Oko, Hermo et al. 1993). Thus is it possible that as the cytoplasmic droplets migrate along the length of the sperm, various membrane components (ie lipids and proteins) are exchanged, including those that are normally associated with cytoplasmic organelles. This in turn provides several interesting follow up questions. For example: 1) What structural features of protein D and/or protein E molecules are required for their association, binding, and subsequent covalent linkage to GPI anchors in the plasma membrane of spermatozoa? 2) Are there two types of GPI anchors found in association with the plasma membrane of mammalian spermatozoa, one in the head region and another in the tail region? 3) If protein D molecules have been postulated to participate in sperm-egg fusion, what is the function of protein E? and 4) Is the structure of protein D or E molecules on the surface of spermatozoa altered (e.g., carbohydrate residues) during the process of ejaculation, capacitation, or acrosome reaction *in vivo* ? The present findings should stimulate further future investigation, which could answer these questions

Figure 1. Proposed model of the initial binding of protein DE to sperm and the subsequent covalent anchoring via a GPI moiety.

An alternative possibility is that after protein DE binds it is covalently linked to another surface protein that is GPI anchored (not shown).


D
D
B
E

# BIBLIOGRAPHY

Acott, T. S. and D. D. Hoskins (1978). “Bovine sperm forward motility protein. Partial purification and characterization.” J Biol Chem **253**(19): 6744-50.

Almeida, E. A., A. P. Huovila, et al. (1995). “Mouse egg integrin alpha 6 beta 1 functions as a sperm receptor.” Cell **81**(7): 1095-104.

Amann, R. P. (1981). “A Critical Review of Methods for Evaluation of Spermatogenesis from Seminal Characteristics.” Jouranl of Andrology **2**: 37-58.

Austin, C. R. (1985). “Sperm maturation in the male and female genital tracts.” Biology of Fertilization **2**: 121-155.

Aveldano, M. I., N. P. Rotstein, et al. (1992). “Lipid remodelling during epididymal maturation of rat spermatozoa. Enrichment in plasmenylcholines containing long-chain polyenoic fatty acids of the n-9 series.” Biochem J **283**(Pt 1): 235-41.

Ball, G. D., M. L. Leibfried, et al. (1983). “Factors affecting successful in vitro fertilization of bovine follicular oocytes.” Biol Reprod **28**(3): 717-25.

Barros, C., J. M. Bedford, et al. (1967). “Membrane vesiculation as a feature of the mammalian acrosome reaction.” J Cell Biol **34**(3): C1-5.

Bedford, J. M., and and G. W. Cooper (1978). Membrane fusion events in fertilization of vertebrate eggs. Amsterdam: North-Holland.

Blobel, C. P., D. G. Myles, et al. (1990). “Proteolytic processing of a protein involved in sperm-egg fusion correlates with acquisition of fertilization competence.” J Cell Biol **111**(1): 69-78.

Blobel, C. P., T. G. Wolfsberg, et al. (1992). “A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion [see comments].” Nature **356**(6366): 248-52.

Brandt, H., T. S. Acott, et al. (1978). “Evidence for an epididymal origin of bovine sperm forward motility protein.” Biol Reprod **19**(4): 830-5.

Brooks, D. E. (1973). “Epididymal and testicular temperature in the unrestrained conscious rat.” Journal of Reproductive Fertility **35**: 157-160.

Brooks, D. E. (1981). “Metabolic Activity in Epididymis and its Regulation by Androgens.” Physiological Reviews **61**: 515-555.

Brooks, D. E. (1981). “Secretion of proteins and glycoproteins by the rat epididymis: regional differences, androgen-dependence, and effects of protease inhibitors, procaine, and tunicamycin.” Biol Reprod **25**(5): 1099-117.

Brooks, D. E. (1982). “Purification of rat epididymal proteins "D' and "E', demonstration of shared immunological determinants, and identification of regional synthesis and secretion.” Int J Androl **5**(5): 513-24.

Brooks, D. E. (1983). “Effect of androgens on protein synthesis and secretion in various regions of the rat epididymis, as analysed by two-dimensional gel electrophoresis.” Mol Cell Endocrinol **29**(3): 255-70.

Brooks, D. E. (1987). “Androgen-regulated epididymal secretory proteins associated with post- testicular sperm development.” Ann N Y Acad Sci **513**: 179-94.

Brooks, D. E. and S. J. Higgins (1980). “Characterization and androgen-dependence of proteins associated with luminal fluid and spermatozoa in the rat epididymis.” J Reprod Fertil **59**(2): 363-75.

Brooks, D. E., A. R. Means, et al. (1986). “Molecular cloning of the cDNA for androgen-dependent sperm-coating glycoproteins secreted by the rat epididymis.” Eur J Biochem **161**(1): 13-8.

Brooks, D. E. and K. Tiver (1983). “Localization of epididymal secretory proteins on rat spermatozoa.” J Reprod Fertil **69**(2): 651-7.

Brown, C. R., K. I. von Glos, et al. (1983). “Changes in plasma membrane glycoproteins of rat spermatozoa during maturation in the epididymis.” J Cell Biol **96**(1): 256-64.

Cameo, M. S. and J. A. Blaquier (1976). “Androgen-controlled specific proteins in rat epididymis.” J Endocrinol **69**(1): 47-55.

Cameo, M. S., F. G. Echeverra, et al. (1986). “Immunochemical Localization of Epididymal Protein DE on Rat Spermatozoa: Its Fate After Induced Acrosome Reaction.” Gamete Research **15**: 247-257.

Cameo, M. S., F. G. Echeverria, et al. (1986). “Immunochemical localization of epididymal protein DE on rat spermatozoa: its fate after induced acrosome reaction.” Gamete Research **15**: 247-257.

Charest, N. J., D. R. Joseph, et al. (1988). “Molecular cloning of complementary deoxyribonucleic acid for an androgen-regulated epididymal protein: sequence homology with metalloproteins.” Mol Endocrinol **2**(10): 999-1004.

Chesla, S. E., P. Li, et al. (2000). “The membrane anchor influences ligand binding two-dimensional kinetic rates and three-dimensional affinity of FcgammaRIII (CD16).” J Biol Chem **275**(14): 10235-46.

Chulavatnatol, M. and P. Ruenwongsa (1976). “Activation of proenzyme of acidic protease from human seminal plasma.” Biochim Biophys Acta **452**(2): 525-32.

Chulavatnatol, M. and S. Yindepit (1976). “Changes in surface ATPase of rat spermatozoa in transit from the caput to the cauda epididymidis.” J Reprod Fertil **48**(1): 91-7.

Cohen, D. J., M. J. Munuce, et al. (1996). “Mammalian sperm-egg fusion: the development of rat oolemma fusibility during oogenesis involves the appearance of binding sites for sperm protein "DE".” Biol Reprod **55**(1): 200-6.

Cohen, D. J., L. Rochwerger, et al. (2000). “Relationship between the association of rat epididymal protein "DE" with spermatozoa and the behavior and function of the protein.” Mol Reprod Dev **56**(2): 180-8.

Courtens, J. L., J. Rozinek, et al. (1982). “Binding of epididymal proteins to the spermatozoa of ram.” Andrologia **14**(6): 509-14.

Cowan, A. E., P. Primakoff, et al. (1986). “Sperm exocytosis increases the amount of PH-20 antigen on the surface of guinea pig sperm.” J Cell Biol **103**(4): 1289-97.

Cuasnicu, P., D. a. Conesa, et al. (1990). Potential contraceptive use of an epididymal protein that participates in fertilization: Gamete Interaction: Prospects for Immunocontraception. New York, Wiley-Liss.

Cuasnicu, P. S., F. Gonzalez Echeverria, et al. (1984). “Epididymal proteins mimic the androgenic effect on zona pellucida recognition by immature hamster spermatozoa.” J Reprod Fertil **71**(2): 427-31.

Cuasnicu, P. S., F. Gonzalez Echeverria, et al. (1984). “Antibodies against epididymal glycoproteins block fertilizing ability in rat.” J Reprod Fertil **72**(2): 467-71.

Curry, P. T. and R. W. Atherton (1992). “Cauda epididymal sperm interactions with seminal vesicle fluid.” Mol Reprod Dev **33**(1): 67-73.

Dacheux, J. L., C. Chevrier, et al. (1990). Sperm Biochemical Changes During Epididymal Maturation. Gamete Interaction: Prospects for Immunocontraception, Wiley-Liss, Inc.**:** 111-128.

Deleage, G., C. Blanchet, et al. (1997). "Protein structure prediction. Implications for the biologist." Biochimie **79**(11): 681-6.

Doering, T. L., W. J. Masterson, et al. (1990). "Biosynthesis of glycosyl phosphatidylinositol membrane anchors." J Biol Chem **265**(2): 611-4.

Eberspaecher, U., D. Roosterman, et al. (1995). "Mouse androgen-dependent epididymal glycoprotein CRISP-1 (DE/AEG): isolation, biochemical characterization, and expression in recombinant form." Mol Reprod Dev **42**(2): 157-72.

Echeverria F.M.G., a. Cuasnicu P.S., et al. (1982). "Identification of androgen dependent glycoproteins in the hamster epididymis and thier association with spermatozoa." Journal of Reproductive Fertility **64**: 1-7.

Eddy, E. M., R. B. Vernon, et al. (1985). "Immunodissection of sperm surface modifications during epididymal maturation." Am J Anat **174**(3): 225-37.

Ellerman, D. A., V. S. Brantua, et al. (1998). “Potential contraceptive use of epididymal proteins: immunization of male rats with epididymal protein DE inhibits sperm fusion ability.” Biol Reprod **59**(5): 1029-36.

Ensrud, K. M. and D. W. Hamilton (1991). “Use of neonatal tolerization and chemical immunosuppression for the production of monoclonal antibodies to maturation-specific sperm surface molecules.” J Androl **12**(5): 305-14.

Ensrud, K. M., J. E. Siiteri, and , et al. (1995). “Monoclonal antibodies against rat epididymal protein D localize to the sperm head and do not recognize the membrane form of protein E.” Molecular Biology of the Cell **6**(Supplement #2479).

Faye, J. C., L. Duguet, et al. (1980). “Purification, radioimmunoassay, and immunohistochemical localization of a glycoprotein produced by the rat epididymis.” Biol Reprod **23**(2): 423-32.

Ferguson, M. A. and A. F. Williams (1988). “Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures.” Annu Rev Biochem **57**: 285-320.

Foster, J. A. and G. L. Gerton (1996). “Autoantigen 1 of the guinea pig sperm acrosome is the homologue of mouse Tpx-1 and human TPX1 and is a member of the cysteine-rich secretory protein (CRISP) family.” Mol Reprod Dev **44**(2): 221-9.

Fournier-Delpech, S., M. Courot, et al. (1985). “Decreased fertility and motility of spermatozoa from rats immunized with a prealbumin epididymal-specific glycoprotein.” J Androl **6**(4): 246-50.

Gaddum-Rose, P. (1985). “Mammalian gamete interactions: what can be gained from observation on living eggs.” American Journal of Anatomy **173**: 347-356.

Garberi, J. C., A. C. Kohane, et al. (1979). “Isolation and characterization of specific rat epididymal proteins.” Mol Cell Endocrinol **13**(1): 73-82.

Gaunt, S. J. (1983). “Spreading of a sperm surface antigen within the plasma membrane of the egg after fertilization in the rat.” J Embryol Exp Morphol **75**: 259-70.

Gaunt, S. J., C. R. Brown, et al. (1983). “Identification of mobile and fixed antigens on the plasma membrane of rat spermatozoa using monoclonal antibodies.” Exp Cell Res **144**(2): 275-84.

Gonzalez Echeverria, F. M., P. S. Cuasnicu, et al. (1982). “Identification of androgen-dependent glycoproteins in the hamster epididymis and their association with spermatozoa.” J Reprod Fertil **64**(1): 1-7.

Haendler, B., I. Toda, et al. (1999). “Expression of transcripts for cysteine-rich secretory proteins (CRISPs) in the murine lacrimal gland.” J Cell Physiol **178**(3): 371-8.

Hall, J. C., J. Hadley, et al. (1991). “Correlation between changes in rat sperm membrane lipids, protein, and the membrane physical state during epididymal maturation.” J Androl **12**(1): 76-87.

Hall, J. C. and J. A. Hadley (1990). “Kinetics of receptor-mediated binding of a 32K epididymal glycoprotein to surface of testicular/epididymal sperm in rats.” Mol. Androl I **1**: 232-240.

Hall, J. C. and G. J. Killian (1989). “Two-dimensional gel electrophoretic analysis of rat sperm membrane interaction with cauda epididymal fluid.” J Androl **10**(1): 64-76.

Hall, J. C. and C. E. Tubbs (1997). “Purification and characterization of protein D/E, a putative sperm- binding protein involved in fertilization.” Prep Biochem Biotechnol **27**(4): 239-51.

Hall, J. C., C. E. Tubbs, et al. (1996). “Characterization of high-affinity protein D binding sites on the surface of rat epididymal spermatozoa.” Biochem Mol Biol Int **40**(5): 1003-10.

Hall, J. C., C. E. Tubbs, et al. (1997). “Affinity Purification of Characterization of a 32Kd Glycoprotein from the Rat Sperm Plasma Membrane that is Required for Egg Zona Pellucida Binding.” International Journal of BioChromatography **3**: 155-176.

Hamilton, D. (1975). Structure and Function of the Epithelium Lining the Ductuli Efferents, Ductus Epididymidis, nd Ductus Deferens in the Rat. Handbook of Physiology, Section 7: Endocrinology. G. R. O. a. E. B. A. D.W. Hamilton. Bethesda, The American Physiological Society**:** 259-301.

Hamilton, D. W. (1980). “UDP-galactose:N-acetylglucosamine galactosyltransferase in fluids from rat rete testis and epididymis.” Biol Reprod **23**(2): 377-85.

Hinton, B. T. (1985). Physiological Aspects of the Blood-Dpididymis Barrier. Male Fertility and its Regulation. T. J. L. a. E. S. E. Hafez. New York, MTP Press**:** 371-382.

Hinton, B. T. and S. S. Howards (1982). “Micropuncture and microperfusion techniques for the study of testicular physiology.” Ann N Y Acad Sci **383**: 29-43.

Hohlbrugger, G., H. Schweisfurth, et al. (1982). “Angiotensin I converting enzyme in rat testis, epididymis and vas deferens under different conditions.” J Reprod Fertil **65**(1): 97-103.

Hoskins, D. D., H. Brandt, et al. (1978). “Initiation of sperm motility in the mammalian epididymis.” Fed Proc **37**(11): 2534-42.

Jenkins, A. D., C. P. Lechene, et al. (1980). “Concentrations of seven elements in the intraluminal fluids of the rat seminiferous tubules, rate testis, and epididymis.” Biol Reprod **23**(5): 981-7.

Jones, R. and C. R. Brown (1982). “Association of epididymal secretory proteins showing alpha-lactalbumin- like activity with the plasma membrane of rat spermatozoa.” Biochem J **206**(1): 161-4.

Jones, R., S. Fournier-Delpech, et al. (1982). “Identification of androgen-dependent proteins synthesized in vitro by the ram epididymis.” Reprod Nutr Dev **22**(3): 495-504.

Junqueira, L. C., J. Carneiro, and , et al. (1989). Basic Histology. East Norwalk, Conneticut, Appleton & Lange.

Klemme, L. M., K. P. Roberts, et al. (1999). “Cloning and characterization of the rat Crisp-1 gene.” Gene **240**(2): 279-88.

Kohane, A. C., M. S. Cameo, et al. (1980). “Distribution and site of production of specific proteins in the rat epididymis.” Biol Reprod **23**(1): 181-7.

Kohane, A. C., J. C. Garberi, et al. (1979). “Quantitative determination of specific proteins in rat epididymis.” J Steroid Biochem **11**(1B): 671-4.

Kohane, A. C., F. M. Gonzalez Echeverria, et al. (1980). “Interaction of proteins of epididymal origin with spermatozoa.” Biol Reprod **23**(4): 737-42.

Kohane, A. C., L. Pineiro, et al. (1983). “Androgen-controlled synthesis of specific proteins in the rat epididymis.” Endocrinology **112**(5): 1590-6.

Kratzschmar, J., B. Haendler, et al. (1996). “The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3.” Eur J Biochem **236**(3): 827-36.

Lea, O. A. and F. S. French (1981). “Characterization of an acidic glycoprotein secreted by principal cells of the rat epididymis.” Biochim Biophys Acta **668**(3): 370-6.

Lea, O. A., P. Petrusz, et al. (1978). “Purification and localization of acidic epididymal glycoprotein (AEG): a serm coating protein secreted by the rat epididymis.” In: Endocrine approach to male contraception. Copenhagen, Scriptor,: 592-607.

Legault, Y., G. Bleau, et al. (1979). “The binding of sterol sulfates to hamster spermatozoa.” Steroids **34**(1): 89-99.

Legault, Y., M. Bouthillier, et al. (1979). “The sterol and sterol sulfate content of the male hamster reproductive tract.” Biol Reprod **20**(5): 1213-9.

Levine, N. and D. J. Marsh (1971). “Micropuncture studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats.” J Physiol (Lond) **213**(3): 557-70.

Lowry, O. H., N. J. Rosebrough, et al. (1951). J. Biol. Chem. **193**: 265-275.

Martinez, S. P., D. Conesa, and , et al. (1995). “Potential contraceptive use of epididymal proteins: Evidence for the participation of specific antibodies against rat epididymal protein DE in male and female fertility inhibition.” Journal of Reproductive Immunology **29**: 31-45.

Moore, A., K. M. Ensrud, et al. (1994). “Rat epididymis-specific sperm maturation antigens. I. Evidence that the 26 kD 4E9 antigen found on rat caudal epididymal sperm tail is derived from a protein secreted by the epididymis.” Mol Reprod Dev **37**(2): 181-94.

Moore, A., T. W. White, et al. (1989). “The major maturation glycoprotein found on rat cauda epididymal sperm surface is linked to the membrane via phosphatidylinositol.” Biochem Biophys Res Commun **160**(2): 460-8.

Moore, H. D. (1981). “Glycoprotein secretions of the epididymis in the rabbit and hamster: localization on epididymal spermatozoa and the effect of specific antibodies on fertilization in vivo.” J Exp Zool **215**(1): 77-85.

Moore, H. D. and J. M. Bedford (1979). “Short-term effects of androgen withdrawal on the structure of different epithelial cells in the rat epididymis.” Anat Rec **193**(2): 293-311.

Morrissette, J., J. Kratzschmar, et al. (1995). “Primary structure and properties of helothermine, a peptide toxin that blocks ryanodine receptors.” Biophys J **68**(6): 2280-8.

Murakami, M., Sugita A., et al. (1982). “Scanning Electron Microscopic Observations of the Vas Deferens in Man and Monkey with Special Reference to Spermiophagy in its Ampullary region.” Scanning Electron Microscopy **3**: 1333-1339.

Myles, D. G., L. H. Kimmel, et al. (1994). “Identification of a binding site in the disintegrin domain of fertilin required for sperm-egg fusion.” Proc Natl Acad Sci U S A **91**(10): 4195-8.

Myles, D. G. and P. Primakoff (1984). “Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization.” J Cell Biol **99**(5): 1634-41.

Myles, D. G., P. Primakoff, et al. (1984). “A localized surface protein of guinea pig sperm exhibits free diffusion in its domain.” J Cell Biol **98**(5): 1905-9.

Nikolopoulou, M., D. A. Soucek, et al. (1985). “Changes in the lipid content of boar sperm plasma membranes during epididymal maturation.” Biochim Biophys Acta **815**(3): 486-98.

Oko, R., L. Hermo, et al. (1993). “The cytoplasmic droplet of rat epididymal spermatozoa contains saccular elements with Golgi characteristics.” J Cell Biol **123**(4): 809-21.

Oliphant, G., A. B. Reynolds, et al. (1985). “Sperm surface components involved in the control of the acrosome reaction.” Am J Anat **174**(3): 269-83.

Olson, G. E. and D. W. Hamilton (1978). “Characterization of the surface glycoproteins of rat spermatozoa.” Biol Reprod **19**(1): 26-35.

Olson, G. E. and B. T. Hinton (1985). “Regional differences in luminal fluid polypeptides of the rat testis and epididymis revealed by two-dimensional gel electrophoresis.” J Androl **6**(1): 20-34.

Orgebin-Crist, M. C. and G. E. and Olson (1984). Epididymal sperm maturation. The Male in Farm Animal Reproduction. M. Courot, Martinus Nijhoff**:** 80.

Phelps, B. M., D. E. Koppel, et al. (1990). “Evidence that proteolysis of the surface is an initial step in the mechanism of formation of sperm cell surface domains.” J Cell Biol **111**(5 Pt 1): 1839-47.

Phelps, B. M. and D. G. Myles (1987). “The guinea pig sperm plasma membrane protein, PH-20, reaches the surface via two transport pathways and becomes localized to a domain after an initial uniform distribution.” Dev Biol **123**(1): 63-72.

Phelps, B. M., P. Primakoff, et al. (1988). “Restricted lateral diffusion of PH-20, a PI-anchored sperm membrane protein.” Science **240**(4860): 1780-2.

Pholpramool, C., O. A. Lea, et al. (1983). “The effects of acidic epididymal glycoprotein (AEG) and some other proteins on the motility of rat epididymal spermatozoa.” Int J Androl **6**(3): 240-8.

Plaquin, R., P. chapdelaine, et al. (1984). “Similar Biochemical Properties of Human Seminal Plasma and Epididymal α−1,4-glucosidase.” Journal of Andrology **5**: 277-282.

Podell, D. N. and G. N. Abraham (1978). “A technique for the removal of pyroglutamic acid from the amino terminus of proteins using calf liver pyroglutamate amino peptidase.” Biochem Biophys Res Commun **81**(1): 176-85.

Primakoff, P., H. Hyatt, et al. (1985). “A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg zona pellucida.” J Cell Biol **101**(6): 2239-44.

Primakoff, P., H. Hyatt, et al. (1987). “Identification and purification of a sperm surface protein with a potential role in sperm-egg membrane fusion.” J Cell Biol **104**(1): 141-9.

Reid, B. L. and K. W. Cleland (1957). “The Structure and Function of the Epididymis.” Aust. J. Zool. **5**(3): 223-246.

Rifkin J.M, a. and O. G.E. (1984). “Characterization of maturation-dependent extrinsic proteins of the rat sperm surface.” Journal of Cell Biology **100**: 1582-1591.

Robaire, B. and L. Hermo (1988). Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. The Physiology of Reproduction. E. Knobil, J.D. Neill, G.S. Greenwald, C. L. M. and and D. W. Pfaff. New York, Raven Press**:** 999-1080.

Robb, G. W., R. P. Amann, et al. (1978). "Daily sperm production and epididymal sperm reserves of pubertal and adult rats." J Reprod Fertil **54**(1): 103-7.

Roberts, M. L., W. H. Scouten, et al. (1976). "Isolation and characterization of the cytoplasmic droplet in the rat." Biol Reprod **14**(4): 421-4.

Rochwerger, L., D. J. Cohen, et al. (1992). "Mammalian sperm-egg fusion: the rat egg has complementary sites for a sperm protein that mediates gamete fusion." Dev Biol **153**(1): 83-90.

Rochwerger, L. and P. S. Cuasnicu (1992). "Redistribution of a rat sperm epididymal glycoprotein after in vitro and in vivo capacitation." Mol Reprod Dev **31**(1): 34-41.

Ronkko, S. (1992). "Separation of phospholipase A2 in the testis and cauda epididymis of the adult rat by chromatofocusing." Int J Androl **15**(1): 62-72.

Rooney, I. A., J. P. Atkinson, et al. (1993). "Physiologic relevance of the membrane attack complex inhibitory protein CD59 in human seminal plasma: CD59 is present on

extracellular organelles (prostasomes), binds cell membranes, and inhibits complement-mediated lysis." J Exp Med **177**(5): 1409-20.

Rost, B. and C. Sander (1996). "Bridging the protein sequence-structure gap by structure predictions." Annu Rev Biophys Biomol Struct **25**: 113-36.

Sattayasai, N., J. Sattayasai, et al. (1984). "High-performance liquid chromatographic determination of gossypol in plasma." J Chromatogr **307**(1): 235-9.

Schwidetzky, U., B. Haendler, et al. (1995). "Isolation and characterization of the androgen-dependent mouse cysteine- rich secretory protein-3 (CRISP-3) gene." Biochem J **309**(Pt 3): 831-6.

Sivashanmugam, P., R. T. Richardson, et al. (1999). "Cloning and characterization of an androgen-dependent acidic epididymal glycoprotein/CRISP1-like protein from the monkey [In Process Citation]." J Androl **20**(3): 384-93.

Tezon, J. G., E. Ramella, et al. (1985). "Immunochemical localization of secretory antigens in the human epididymis and their association with spermatozoa." Biol Reprod **32**(3): 591-7.

Turner, T. T. and R. D. Giles (1982). “Sperm motility-inhibiting factor in rat epididymis.” Am J Physiol **242**(3): R199-203.

Turner, T. T., C. E. Jones, et al. (1984). “On the androgen microenvironment of maturing spermatozoa.” Endocrinology **115**(5): 1925-32.

Udenfriend, S. and K. Kodukula (1995). “How glycosylphosphatidylinositol-anchored membrane proteins are made.” Annu Rev Biochem **64**: 563-91.

Vreeburg, J. T., M. K. Holland, et al. (1992). “Binding of epididymal proteins to rat spermatozoa in vivo.” Biol Reprod **47**(4): 588-97.

Wolfsberg, T. G., J. F. Bazan, et al. (1993). “The precursor region of a protein active in sperm-egg fusion contains a metalloprotease and a disintegrin domain: structural, functional, and evolutionary implications.” Proc Natl Acad Sci U S A **90**(22): 10783-7.

Wong, P. Y. and C. N. Uchendu (1990). “The role of angiotensin-converting enzyme in the rat epididymis.” J Endocrinol **125**(3): 457-65.

Wong, P. Y. D. and A. Y. F. Tsang (1982). “Studies on the Binding of a 32K Rat Epididymal Protein to Rat Epididymal Spermatozoa.” Biology of Reproduction **27**: 1239-1246.

Xu, W. and D. W. Hamilton (1996). “Identification of the rat epididymis-secreted 4E9 antigen as protein E: further biochemical characterization of the highly homologous epididymal secretory proteins D and E.” Mol Reprod Dev **43**(3): 347-57.

Yanagimachi, R. (1981). Mechanisms of fertilization in mammals. New York, NY, Plenum Press.

Yanagimachi, R. (1988). Mammalian Fertilization. The Physiology of Reproduction. a. K. J. Knobil E., et al. New York, NY, Plenum Press**:** 135-185.