UNIVERSITY OF CALIFORNIA, SAN DIEGO

Sea Urchin Sperm Membrane Glycoproteins

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Marine Biology

by

Kathryn J. Mengerink

Committee in charge:

Professor Victor D. Vacquier, Chair
Professor Douglas Bartlett
Professor Bianca Brahamsha
Professor Ron Burton
Professor Nicholas Holland
Professor Douglas Smith

2002

The dissertation of Kathryn J. Mengerink is approved, and it is acceptable in quality and form for publication on microfilm:

University of California, San Diego

2001

DEDICATION

To Mom, my hero.

TABLE OF CONTENTS

ABBREVIATIONS

ABC	ATP-binding cassette
ABCA	ABC transporter subfamily A
ABCe	bacterially expressed polypeptide of sea urchin ABC transporter
ADPKD	autosomal dominant polycystic kidney disease
AR	acrosome reaction
ARIS	acrosome reaction-inducing substance
FSW	filtered sea water
BSA	bovine serum albumin
CRD	carbohydrate recognition domain
EGF	Epidermal Growth Factor
EJ	Egg Jelly
Fab	univalent antigen-binding fragment from IgG
GlcNAc	N-acetyl-D-glucosamine
GPCR	G-protein coupled receptor
GPS	GPCR cleavage site
HDL	high density lipoprotein
PLAT	polycystin/lipoxygenase/alpha toxin
PNGase F	peptide-N-glycosidase F
PVDF	polyvinylidene difluoride

REJ	receptor for egg jelly
S/C	SUEL/CRD
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SMV	sperm membrane vesicle
SUEL	sea urchin egg lectin
TMS	transmembrane segment
TRPC	transient receptor potential channel
VC	vitelline coat
WGA	wheat germ agglutinin
ZP	zona pellucida

LIST OF TABLES AND FIGURES

ACKNOWLEDGEMENTS

Thank you, Vic for being a wonderful advisor and friend. Your enthusiasm for the lab and excitement for science was inspiring. Many thanks to Gary Moy for assisting me with my research, for ensuring that the lab ran smoothly and for always being a great person to work with. Thanks to the Vacquier lab crew for friendship as well as scientific advice, especially Willie Swanson, Joseph Schulz, Silvia Armitano, Brian Hillier, Anna Neill and Jonny Lyon. Many thanks to my dear friends who kept me sane, reminded me to enjoy life, and who have made my experience at Scripps unforgettable. To Dave, for hugs and tackles when I needed them. To Meriah, for always being the optimist. To Laura, for years of laughter, high adventure, and ridiculous fun. To Eric, for the wild ride. To my family who were always with me, even though they were hundreds of miles away. Mom, Dad, Dad, Anne, Matthew, Alaine, the babies, thank you for helping me through this. I would not have made it without you.

The text of Chapter One, in full, is a reprint of the material as it appears in *Glycobiology*. The dissertation author was the primary author, and the second author directed and supervised the research, which forms the basis for this chapter.

The text of Chapter Two, in full, is a reprint of the material as it appears in *Zygote*. The dissertation author was the primary author, the second author assisted with the research and the third author directed and supervised the research, which forms the basis for this chapter.

The text of Chapter Three, in full, is a reprint of the material as it appears in the *Journal of Biological Chemistry*. The dissertation author was the primary author, the second author assisted with the research and the third author directed and supervised the research, which forms the basis for this chapter.

The text of Chapter Four, in full, is a manuscript to be submitted to the *Journal of Biological Chemistry*. The dissertation author was the primary author and the co-authors listed in this publication directed and supervised the research, which forms the basis for this chapter.

VITA

1996	B.S., Texas A & M University, College Station
1996 - 2002	Research Assistant, University of California, San Diego
2002	Ph.D., University of California, San Diego

PUBLICATIONS

Mengerink, K. J. and Vacquier, V. D. (2002) suABCA is an ATP-binding cassette transporter and a major sea urchin sperm membrane glycoprotein. Submitted.

Mengerink, K. J., Moy, G. W., and Vacquier, V. D. (2002) suREJ3: a polycystin-1 protein, is cleaved at the GPS domain and localizes to the acrosomal region of sea urchin sperm. *Journal of Biological Chemistry*, 277(2): 943-948.

Mengerink, K. J. and Vacquier, V. D. (2001) Glycobiology of sperm egg interactions in deuterostomes. *Glycobiology* 11(4): 37R-43R.

Mengerink, K. J., Moy, G. W., and Vacquier, V. D. (2000) suREJ proteins: new signaling molecules in sea urchin spermatozoa. *Zygote* 8(4): S28-30.

CONFERENCE PARTICIPATION

Invited Speaker, Fertilization and Activation of Development, Gordon Conference, (2001)

Poster Presenter, American Society for Cell Biology Meeting, (2000)

Invited Speaker, Developmental Biology of the Sea Urchin XIII Meeting, (2000)

Invited Speaker, The Molecular and Cellular Biology of the Egg and Embryo Extracellular Matrix Meeting, (2000)

Poster Presenter, Molecular Mechanisms of Cell-Cell Interaction, (2000)

Invited Speaker, Developmental Biology of the Sea Urchin XII Meeting, (1999)

Poster Presenter, American Society for Cell Biology Meeting, (1998)

ABSTRACT OF THE DISSERTATION

Sea Urchin Sperm Membrane Glycoproteins

By

Kathryn J. Mengerink

Doctor of Philosophy in Marine Biology

Unversity of California, San Diego, 2002

Professor Victor D. Vacquier, Chair

Fertilization is a multi-step process that involves contact, binding, and fusion of sperm and eggs. In most deuterostomes, sperm must undergo a process called the acrosome reaction (AR) before fusion can occur. The AR is induced by contact between the egg and sperm, resulting in a complex signaling cascade, which leads to the exocytosis of an acrosomal vesicle found at the tip of the sperm head and the polymerization of actin to form the acrosomal process. This process results in a newly exposed membrane that fuses with the plasma membrane of the egg.

In the sea urchin, *Strongylocentrotus purpuratus*, wheat germ agglutinin (WGA) is capable of blocking the egg jelly induced AR. WGA is a lectin that binds N-acetyl-D-glucosamine and sialic acid. One WGA-binding protein, receptor for egg jelly 1 (suREJ1) has been identified as binding to the AR-inducing substance, fucose

sulfate polymer of egg jelly. Also, monoclonal antibodies to suREJ1 are capable of inducing the AR. The focus of this dissertation is to identify and characterize other WGA-binding proteins in an effort to identify the important cell surface receptors involved in the sea urchin AR.

Chapter I introduces the glycobiology of fertilization in deuterostomes. Chapter II describes the known members of the sea urchin REJ family, suREJ1, suREJ2 and suREJ3. These are large multidomain membrane receptors related to the polycystic kidney disease proteins, polycystin-1 and polycystin-2. suREJ1 is known to be a involved in the AR, and indirect evidence indicates suREJ3's involvement, as is discussed in chapter III. Chapter IV identifies another WGA-binding protein as an ATP-binding cassettte (ABC) transporter belonging to the human subfamily A. Members of this subfamily are involved in lipid and cholesterol transport. It is known that cholesterol must be removed from mammalian sperm before they can acrosome react. suABCA may have a similar function. Chapter VI gives general conclusions about these newly discovered proteins and gives suggestions for future work. Appendix A describes another putative suREJ protein, suREJX, and Appendix B demonstrates the presence of REJ proteins in sea urchin embryos.

CHAPTER I

Glycobiology of Sperm-Egg Interactions in Deuterostomes

Glycobiology vol. 11 no. 4 pp. 37R–43R, 2001

MINI REVIEW

Glycobiology of sperm–egg interactions in deuterostomes

Kathryn J. Mengerink[1] and Victor D. Vacquier

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla CA 92093-0202, USA

Accepted on February 9, 2001

The process of fertilization begins when sperm contact the outermost egg investment and ends with fusion of the two haploid pronuclei in the egg cytoplasm. Many steps in fertilization involve carbohydrate-based molecular recognition between sperm and egg. Although there is conservation of gamete recognition molecules within vertebrates, their homologues have not yet been discovered in echinoderms and ascidians (the invertebrate deuterostomes). In echinoderms, long sulfated polysaccharides act as ligands for sperm receptors. Ascidians employ egg coat glycosides that are recognized by sperm surface glycosidases. Vertebrate egg coats contain zona pellucida (ZP) family glycoproteins, whose carbohydrates bind to sperm receptors. Several candidate sperm receptors for vertebrate ZP proteins have been identified and are discussed here. This brief review focuses on new information concerning fertilization in deuterostomes (the phylogenetic group including echinoderms, ascidians, and vertebrates) and highlights protein–carbohydrate interactions involved in this process.

Key words: acrosome reaction/fertilization/protein–carbohydrate recognition/sperm–egg interaction/zona pellucida

Introduction

Fertilization is a multistep process and a unique event that involves fusion of two haploid gametes to form a diploid zygote. Sperm must locate, adhere to, and fuse with the egg. The egg must prevent further sperm fusion to avoid pathological polyspermy, a lethal condition. Eggs are surrounded by an extracellular matrix, which varies in composition among animal groups (Figure 1). When sperm contact this matrix, there are primary binding events that, in most deuterostomes, lead to the sperm acrosome reaction (AR). The AR is triggered by increases in intracellular Ca^{2+} and pH (Darszon *et al.*, 1999) and results in exocytosis of the acrosomal vesicle (an organelle in the sperm head). Following the AR, secondary binding events occur, and the membrane exposed by the AR fuses with the egg plasma membrane. Protein–carbohydrate interactions play a critical role in this complex process. Because there is such a vast literature, this brief review will focus only on deuterostome sperm–egg recognition events that involve carbohydrate–protein interactions.

Nonvertebrate deuterostomes

Echinoderms

Echinoderms are marine invertebrates found at the base of the deuterostome lineage. Because they spawn large quantities of gametes into sea water, they make excellent model organisms for studying molecular events involved in sperm–egg interaction.

Sea urchins In sea urchins, protein–carbohydrate interactions take place both at the egg jelly and the vitelline layers. Sperm first contact the egg jelly layer. A 210-kDa multidomain

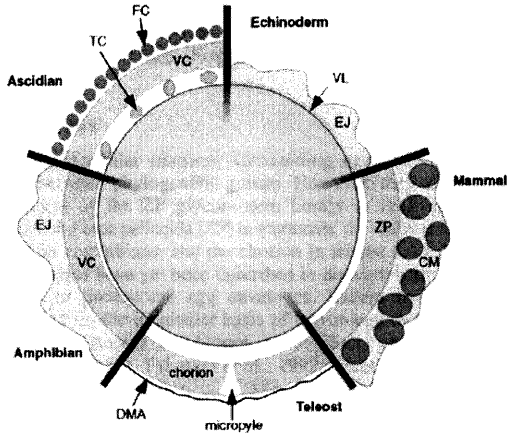


Fig. 1. A diagram showing the various types of extracellular matrices surrounding deuterostome eggs. The ascidian extracellular matrix is composed of follicle cells (FC), a vitelline coat (VC), and a perivitelline space containing test cells (TC). The echinoderm has egg jelly (EJ) and a vitelline layer (VL). The mammalian extracellular matrix contains a cumulus matrix (CM) including cumulus cells and a zona pellucida (ZP). The teleost extracellular matrix has a dilute mucous area (DMA) and a thick chorion that has a hole through it called the micropyle. Amphibians have an EJ and VC.

[1]To whom correspondence should be addressed

receptor for egg jelly (REJ) on sperm (Moy *et al.*, 1996) binds to the fucose sulfate polymer (FSP) of egg jelly triggering the AR (Vacquier and Moy, 1997). Females of *Strongylocentrotus purpuratus* have one of two forms of FSP that are equally potent at AR induction. These FSPs are linear polysaccharides of 1→3-linked α-L-fucopyranosyl units that differ in their sulfation pattern at the C-2 and C-4 positions (Alves *et al.*, 1998). REJ contains two C-type lectin domains (CRDs, reviewed by Drickamer, 1988; Moy *et al.*, 1996), and it may be that each of the two CRDs of REJ binds to each of the forms of FSP. The structures of sulfated polysaccharides from the egg jelly of other sea urchin species have been determined and are species-specific inducers of the AR. Like *S. purpuratus*, the species *Strongylocentrotus franciscanus*, *Arbacia lixula*, and *Lytechinus variegatus* contain sulfated α-L-fucans, and the sulfated polysaccharide of *Echinometra lucunter* is a homopolymer of 2-sulfated, 3-linked α-L-galactan (Alves *et al.*, 1997; Vilela-Silva *et al.*, 1999). These polysaccharides are AR inducers in the complete absence of a polypeptide.

Following the initial egg jelly–sperm interaction, the AR expels the protein bindin from the acrosomal vesicle. Bindin coats the sperm acrosomal process, mediates sperm adhesion to eggs, and may mediate sperm–egg fusion (Vacquier *et al.*, 1995). A 350-kDa glycoprotein receptor for bindin was identified from the vitelline layer of sea urchin eggs (reviewed by Ohlendieck and Lennarz, 1995). The N-terminus of the protein has a relatively high content of Cys and Pro residues and contains 17 potential O-linked and 5 potential N-linked glycosylation sites (Just and Lennarz, 1997). The receptor is 70% carbohydrate (Kitazume-Kawaguchi *et al.*, 1997), and both carbohydrate moieties and the protein backbone are needed for sperm–egg binding. O-linked, sulfated oligosialylated chains, isolated from the 350-kDa receptor, inhibit fertilization by binding to acrosome-reacted sperm (Dhume and Lennarz, 1995; Kitazume-Kawaguchi *et al.*, 1997). Furthermore, bindin binds to sulfated fucose polymers from the egg surface (DeAngelis and Glabe, 1990). However, the function of the polypeptide chain has been questioned because of its similarity to heat shock protein 110 (Mauk *et al.*, 1997).

Starfish There are three major components of starfish (*Asteria amurensis*) egg jelly—glycoproteins, sulfated steroid saponins, and oligopeptides. The AR-inducing substance (ARIS), is an enormous molecule composed of approximately 33% protein, 47% carbohydrate, and 10% sulfate. ARIS has a molecular mass of $>10^4$ kDa, but the minimum functional size is ~14 kDa (Ushiyama *et al.*, 1995). Isolated ARIS is capable of inducing the AR in high Ca^{2+} or high pH sea water. However, in normal sea water, ARIS requires the sulfated steroid saponins (Co-ARIS) to induce the AR (Hoshi *et al.*, 1991). The structure of the main saccharide chain has been determined to be a repeat of the pentasaccharide, →4-β-D-Xyl-1→3-α-D-Gal-1→3-α-L-Fuc-4(SO_3^-)-1→3-α-L-Fuc4(SO_3^-)-1→4-α-L-Fuc-1→. Like ARIS alone, isolated polymers composed of 10–11 pentasaccharide repeating units induce the acrosome reaction at high Ca^{2+} concentrations (Koyota *et al.*, 1997). Thus, sperm–egg recognition and induction of the AR in both sea urchins and starfish involves large sulfated polysaccharides. It will be interesting to learn if the starfish sperm receptor for the pentasaccharide repeat is similar to the sea urchin sperm receptor REJ.

Ascidians

Glycosidases are enzymes that are typically secreted or stored in lysosomes. In ascidians (sessile marine invertebrates), glycosidases are found on the surface of sperm, and they play a critical role in sperm–egg recognition. Based on studies of *Ciona intestinalis* and *Phallusia mammillata* (Hoshi *et al.*, 1983, 1985), a glycosidase on the surface of sperm was proposed to recognize and bind to glycosides within the vitelline coat (VC) of the egg. In the case of *C. intestinalis*, the sperm glycosidase, α-L-fucosidase, binds to terminal L-fucose residues of the VC. For *P. mammillata*, N-acetylglucosaminidase recognizes terminal GlcNAc. The enzymes are thought to act as lectins because their pH optima (~3.9) is well below that of sea water (pH 8) and the rate of hydrolysis is drastically reduced. Examination of glycans present in the vitelline coat of *Halocynthia roretzi* eggs, where sperm–egg binding is mediated through α-L-fucosidase on the sperm surface, indicates that the crucial glycans are O-linked and sulfated (Baginski *et al.*, 1999). Not only do glycosidases occur on the surfaces of sperm, they are also released from the eggs at fertilization as a block to polyspermy (Lambert, 1986, 1989; Matsuura *et al.*, 1993, 1995). The evidence suggests that a specific glycosidase on the sperm surface binds to its respective glycoside on the egg VC. This binding triggers eggs to release large quantities of a similar glycosidase, which prevents the binding of supernumerary sperm.

Carbohydrates also play a role in ascidian sperm–egg interactions at the egg plasma membrane. When vitelline envelopes are removed from the eggs of *Ascidia ceratodes*, application of wheat germ agglutinin (WGA; >10 μg/ml) will biochemically activate the eggs, as if they had been fertilized. Lower concentrations of WGA do not activate eggs but reduce the ability of sperm to fertilize eggs (Flannery and Epel, 1998).

Vertebrates

The extracellular matrices surrounding eggs of vertebrates vary between phylogenetic groups. However, they all contain members of the ZP glycoprotein family in their egg coats (called the zona pellucida [ZP] in mammals, the vitelline envelope [VE] in amphibians, and the chorion in teleost fish). No such ZP proteins have yet been described in nonvertebrate deuterostome or protostome egg envelopes. Recent reviews have appeared on the molecular basis of sperm–egg interactions in mammals (Brewis and Moore, 1997; Shalgi and Raz, 1997; Dell *et al.*, 1999; Takasaki *et al.*, 1999; Wassarman, 1999a,b; Evans, 2000; Prasad *et al.*, 2000). Therefore, only the most recent findings regarding the role of carbohydrates in sperm–egg interaction will be mentioned. Because most of the work on ZP proteins concerns the mouse, this portion of the review will focus on the mouse model and include additional information from other vertebrates.

Glycoproteins of the ZP

ZP3 The mouse ZP is composed of three glycoproteins, mZP1, mZP2, and mZP3 (also called ZPB, ZPA, and ZPC), that are crucial for its structural integrity. mZP2 and mZP3 dimerize to create long filaments that are cross-linked by mZP1. In addition to being structural components, ZP glycoproteins bind

to sperm receptors, causing them to cluster and induce signal transduction events leading to the sperm AR. O-linked oligosaccharides of mZP3 are the ligands for sperm that are involved in primary binding and induction of the AR (Florman and Wassarman, 1985 and reviewed by Dell *et al.*, 1999; Shalgi and Raz, 1997; Wassarman *et al.*, 1999; Wassarman, 1999b). Site-directed mutagenesis shows that mutating Ser-332 or Ser-334 to Ala results in complete inactivation of mZP3 (Chen *et al.*, 1998), indicating the importance of O-linked oligosaccharides at these sites. The structures of the sperm-binding/AR-inducing components of the O-linked oligosaccharides have yet to be determined. However, several studies have begun to tackle the difficult problem of determining the crucial ZP sugars involved in sperm–egg interactions in vertebrates.

Examination of sperm–egg binding by analysis of ZP sugars and the use of neoglycoproteins, monosaccharides, and other polysaccharides have yielded conflicting results. The bioactivity of mZP3 is not dependent on sulfation, N-linked oligosaccharides, or sialic acid residues (Litscher and Wassarman, 1996; Liu *et al.*, 1997). However, removal of sialic acid from fixed eggs increases sperm binding, suggesting that these residues may conceal sperm binding sites (Mori *et al.*, 1997). Man-BSA, GlcNAc-BSA, and GalNAc-BSA are capable of inducing the mouse AR, whereas Glc-BSA and Gal-BSA have no effect. The same monosaccharides applied at millimolar concentrations neither induce nor block the AR (Loeser and Tulsiani, 1999), suggesting that multivalent interactions between carbohydrates of the ZP and their receptors on sperm are necessary for AR induction. Application of L-type Ca^{2+} channel blockers verapamil or diltiazem to sperm block the mZP3 induced AR. These drugs also block the AR induced by Man-BSA, GlcNAc-BSA, and GalNAc-BSA, lending support to the notion that these neoglycoproteins are affecting the same pathway as mZP3, the natural inducer. However, the G-protein blocker, pertussis toxin, which blocks the mZP3-induced AR, is ineffective at preventing the neoglycoprotein-induced AR (Loeser and Tulsiani , 1999). Because Man-BSA, GlcNAc-BSA, and GalNAc-BSA have similar affects on sperm as mZP3, this suggests that these sugars may be biologically relevant components of the oligosaccharides of mZP3. Furthermore, structural analysis of mouse ZP-derived glycans indicates the presence of terminal Man, GlcNAc, and GalNAc residues (Easton *et al.*, 2000).

Removal of Gal from the nonreducing termini of the O-linked oligosaccharides of mZP3 (by treatment with α-galactosidase) inhibits sperm binding (Bleil and Wassarman, 1988), indicating that these terminal Gal residues are essential for sperm–egg binding. Incubating sperm with the trisaccharide Gal-α-1→3-Gal-β-1→4-GlcNAc inhibits sperm binding to eggs to a greater extent than incubation with the trisaccharide Gal-β-1→4-GlcNAc-β-1→4-GlcNAc, though neither inhibit binding with high affinity (Johnston *et al.*, 1998). This would indicate that the α-Gal linkage is more important for sperm binding than the β-Gal linkage. However, the role of terminal Gal residues in sperm binding remains controversial. Another study found that sperm binding was reduced in β-galactosidase-treated eggs but not α-galactosidase-treated eggs (Mori *et al.*, 1997). Also, α-galactosyltransferase is necessary for the synthesis of terminal Gal-α-1→3-Gal residues, yet α-galactosyltransferase-null mice are fully fertile (Thall *et al.*, 1995). Furthermore, Gal residues localize to the inner portion of the ZP, indicating that the initial contact between the ZP and sperm does not involve Gal residues (Aviles *et al.*, 2000). Studies of other monosaccharides have yielded similarly confusing results. For example, addition of an α-3-fucose residue to the trisaccharide Gal-α-1→3-Gal-β-1→4-GlcNAc to form Gal-α-1→3-Gal-β-1→4[Fuc-α1→3]-GlcNAc, yields a tetrasaccharide with high inhibitory activity (Johnston *et al.*, 1998). However, there is no evidence of fucosylation of O-glycans based on structural analysis of carbohydrates from mouse eggs (Easton *et al.*, 2000). These examples highlight the difficulties of sorting out biologically relevant carbohydrates involved in sperm–egg interactions. Understanding this process is further complicated by the fact that sperm–egg binding involves both low and high-affinity ZP binding sites on sperm (Thaler and Cardullo, 1996).

ZPs in other vertebrates In pigs, acrosome-intact sperm bind to ZP proteins over the acrosomal ridge on the anterior portion of the sperm head (Burkin and Miller, 2000). The pig ZP contains ZPA, ZPB, and ZPC (homologues of mZP2, mZP1, and mZP3), and unlike mouse, ZPB (ZP1) is the sperm binding protein (Yonezawa *et al.*, 1997; Kudo *et al.*, 1998). Analysis of ZPB carbohydrates yields conflicting results. Some studies find that O-linked oligosaccharides and not N-linked oligosaccharides are responsible for sperm–egg binding (Yurewicz *et al.*, 1991, 1993), but other studies identify neutral N-linked oligosaccharides of ZPB as the sperm-binding components (Yonezawa *et al.*, 1997; Kudo *et al.*, 1998).

Among amphibians, *Xenopus laevis* has provided the most information about sperm–egg recognition. Egg VE glycoprotein, gp43 (ZPC or ZP3) contains several O-linked and two N-linked glycosylation sites, one of which is conserved from teleosts to humans (Kubo *et al.*, 1997; Yang and Hedrick, 1997). Proteolytic cleavage of gp69/64 (ZPA or ZP2) during fertilization results in removal of 27 amino acids from the N-terminus and loss of sperm binding. The N-terminal peptide may contain an O-linked glycan that is involved in the binding process (Tian *et al.*, 1999). Another analysis of *Xenopus* ZP proteins identified complex N-linked oligosaccharides of ZPC (mZP3) as the major sperm binding ligands and that sperm binding involves GlcNAc and Fuc residues. Furthermore, mixing isolated ZPA, ZPB and ZPC in a ratio of 1:4:4 (as occurs in the ZP) results in the binding of more sperm than the sum of the separate components (Vo and Hedrick, 2000). This result suggests that instead of having one sperm-binding protein, the molecules may act synergistically to bind sperm.

The chorion surrounding eggs in teleost fish is multilayered and varies in thickness and number of layers. The zebrafish (*Danio rerio*) has a chorion composed of three morphologically distinct layers and contains four major proteins (116, 97, 50, and 43 kDa; Bonsignorio *et al.*, 1996). The homologue of mZP2 was the first ZP protein identified in teleost fish (Lyons *et al.*, 1993), and others have been identified since then. Both ZP2 and ZP3 cDNA clones have been identified in zebrafish, but the relationship of the proteins of the zebrafish chorion to the ZP protein family is unknown. In zebrafish ZP3, only one putative N-glycosylation site and no O-glycosylation sites exist (Wang and Gong, 1999). Interestingly, most teleost sperm lack an acrosome. Instead of penetrating through the chorion, they reach the egg plasma membrane by swimming

through a hole in the chorion called the micropyle, implying that AR-inducing oligosaccharides may be unnecessary. In the medaka fish (*Oryzias latipes*), two groups of glycoproteins exist in the chorion, ZI-1,2 and ZI-3, whose precursors, choriogenin H and choriogenin L, correspond to ZP2 and ZP3 (Murata *et al.*, 1995, 1997). ZI-1,2 and ZI-3 are sparsely distributed throughout a broad diluted mucous area (DMA) on the surface of the chorion and within the micropyle (Iwamatsu *et al.*, 1997). It is thought that the sperm bind to ZI-1,2 and ZI-3 to maneuver across the surface of the egg through the DMA until locating the micropyle.

Sperm receptors for egg ZP glycoproteins

The evidence for mZP3 being the ligand for mouse sperm and the inducer of the AR is well supported, but the sperm receptor for mZP3 remains controversial. Candidate ZP receptors will be discussed.

Acrosin is an acrosomal protease, originally thought to be involved in digesting a passage through the ZP. Several lines of evidence suggest that acrosin binds to sulfated polysaccharides of the ZP, as well as Fuc-BSA, Man-BSA, and non-ZP polysulfate saccharides (Jones, 1991; Urch and Patel, 1991; Jones *et al.*, 1988). The function of acrosin in mice is questionable, because sperm of acrosin-null mice are still capable of penetrating the ZP and fertilization (Baba *et al.*, 1994), though not as effectively as normal sperm (Adham *et al.*, 1997). Also, recombinant boar acrosin binds to the ZP but does not block sperm penetration (Crosby and Barros, 1999). Recent evidence suggests that acrosin's proteolytic activity may function in the dispersal of the acrosomal vesicle contents after the AR (Yamagata *et al.*, 1998). Thus, the function of acrosin in the mammalian AR will require further work.

β-1→4-Galactosyltransferase (GalTase) has been extensively studied as a mammalian sperm receptor involved in sperm binding to mZP3. Agents that inhibit GalTase and addition of purified GalTase inhibit sperm-zona binding *in vitro* (reviewed by Shur *et al.*, 1998). GalTase specifically recognizes the oligosaccharides of mZP3 that have sperm-binding activity but does not interact with other mZP glycoproteins (Miller *et al.*, 1992). mZP3 is thought to elicit the AR by cross-linking or aggregating the sperm receptor on the plasma membrane (Leyton and Saling, 1989). Anti-GalTase antibodies (but not their Fab fragments) will induce the AR by aggregating GalTase on the sperm plasma membrane (Macek *et al.*, 1991). Multivalent GlcNAc-BSA is also capable of inducing the mouse sperm AR, whereas millimolar concentrations of the unconjugated sugar have no effect (Loeser and Tulsiani, 1999). Structural analysis of mouse ZP glycans demonstrates that the ligand for GalTase, GlcNAc, is only present on N-linked and not O-linked oligosaccharides (Easton *et al.*, 2000). GalTase has been localized to the anterior portion of the sperm head in several mammalian species, including guinea pig, mouse, rat, bull, pig, and rabbit (Larson and Miller, 1997). GalTase on the surface of porcine sperm binds the ZP. Unlike mouse GalTase, addition of uridine diphosphate galactose has no effect on sperm binding to the oocyte, nor does removal of zona ligands by N-acetylglucosaminidase (Rebeiz and Miller, 1999). This would argue that GalTase is not necessary for sperm–zona binding and the AR in pigs. Furthermore, GalTase-null male mice are fertile. However, *in vitro* studies show that the mutant sperm bind less mZP3 than wild type and do not undergo the AR in response to ZP3 or anti-GalTase antibodies (Lu and Shur, 1997). This points out the difficulty of correlating effects observed *in vitro* with the natural process occurring *in vivo*.

sp56 was identified on the basis of its affinity for mZP3 (Bleil and Wassarman, 1990). Furthermore, it was shown that sp56 localizes to the outer surface of the sperm head and that sperm binding glycopeptides of mZP3 can be crosslinked to sp56 (Cheng *et al.*, 1994). The cDNA sequence revealed sp56 to be a peripheral membrane protein that contains seven sushi domains and a highly basic COOH-terminal domain (Bookbinder *et al.*, 1995). AM67 is a guinea pig homologue of sp56 that localizes within the acrosome. Reexamination of the localization of sp56 in mouse sperm revealed that it was also found inside the acrosome (Foster *et al.*, 1997). Whether sp56 is exclusively internal or external remains unresolved.

Compelling evidence exists that acrosin, GalTase, and sp56 interact with carbohydrates of the ZP and are important components of sperm–egg interaction. The difficulty lies in teasing out the exact function of each of these receptors *in vivo*.

Secondary sperm receptors

Several sperm receptors have been identified that are thought to be involved in secondary binding of acrosome-reacted sperm to egg extracellular matrices. β-N-acetylglucosaminidase is released from mouse sperm during the AR, and the inhibitor, PUGNAC, prevents sperm penetration through the ZP. The glycosidase is thought to remove terminal GlcNAc, releasing the sperm so that it can move through the ZP (Miller *et al.*, 1992). In the toad *Bufo arenarum*, the enzyme is released from sperm and binds to the VE. Furthermore, inhibition of this enzyme results in inhibition of fertilization *in vitro* (Martinez *et al.*, 2000).

Hyaluronan is a glycosaminoglycan composed of the disaccharide repeat $(GlcNAc\beta\text{-}1\rightarrow4GlcA\beta\text{-}1\rightarrow3)_n$, and hyaluronidases selectively degrade the polymer. In mammals, hyaluronan is found in the cumulus matrix surrounding the ZP and the egg perivitelline layer surrounding the plasma membrane (Kan, 1990; Dandekar *et al.*, 1992; Camaioni *et al.*, 1996). PH-20 is a glycosyl phosphatidylinositol–anchored membrane protein first identified on the posterior head of guinea pig sperm. It has an N-terminal hyaluronidase domain that is used by acrosome-intact sperm to penetrate the cumulus matrix. Its C-terminal domain is thought to be involved in secondary sperm binding, but the mechanism remains unknown (Hunnicutt *et al.*, 1996). Homologues of PH-20 have been identified and localized to the same regions in sperm from several other mammalian species, including mouse, rat, human, and macaque (Thaler and Cardullo, 1995; Sabeur *et al.*, 1997; Yudin *et al.*, 1999; Seaton *et al.*, 2000).

In pigs, a ligand recognized by P-selectin is present in the ZP, and P-selectin exists on the acrosomal membrane of sperm. P-selectin is only detected by antibodies in acrosome reacted sperm, suggesting that it plays a role in sperm-egg recognition following the AR (Geng *et al.*, 1997). Removal of sialic acid (an important glycan of P-selectin ligands) from mZP3 does not affect binding to mouse sperm or the AR (Litscher and Wassarman, 1996), lending further support to the notion that P-selectin is involved in secondary binding. However, p-selectin deficient mice are fully fertile (Mayadas *et al.*, 1993).

Summary

The molecules of sperm–egg recognition in echinoderms appear to be entirely different from those of ascidians and vertebrates. An intriguing possibility is that a sea urchin REJ homologue is a mammalian sperm receptor. A testis-specific mammalian homologue of REJ, PKDREJ, of unknown function, has been cloned from mouse and human (Hughes *et al.*, 1999). The ascidian sperm–egg recognition system involves glycosidases binding to their appropriate glycosides. In vertebrates, glycosidases have also been implicated in sperm–egg binding (Martinez *et al.*, 2000), and in cleaving glycosides so that the sperm can penetrate the ZP (Miller *et al.*, 1992). Among vertebrates, evidence indicates that the ZP proteins are the crucial molecules responsible for the initial sperm–egg recognition events. However, the ZP protein family members serve different functions and are differentially glycosylated in the egg coats of different vertebrate groups. Furthermore, there is indirect evidence that carbohydrates play a role in species-specificity of sperm binding in vertebrates (Rankin *et al.*, 1998; Doren *et al.*, 1999). Although there is good support that oligosaccharides of the ZP proteins are crucial for primary sperm binding and induction of the AR in vertebrates, the identification of the ZP receptor on sperm remains uncertain. sp56, proacrosin, and GalTase are all candidate ZP receptors. The main focus of research to date has been in identifying primary binding events, but it is apparent from these data that there are many more potential factors in sperm–egg interactions leading to the fusion of two gametes. Much more work needs to be done to clearly delineate the complicated processes of sperm–egg interaction during fertilization.

Acknowledgments

Research in our laboratory is funded by NIH HD12986. We thank Eric E. Allen, Lihini I. Aluwihare, and Gary W. Moy for reviewing the manuscript.

Abbreviations

AR, acrosome reaction; ARIS, acrosome reaction–inducing substance; CRDs, C-type lectin domains; DMA, diluted mucous area; FSP, fucose sulfate polymer; GalTase, β-1→4-galactosyltransferase; REJ, receptor for egg jelly; VC, vitelline coat; VE, vitelline envelope; WGA, wheat germ agglutinin; ZP, zona pellucida.

References

Adham, I.M., Nayernia, K., and Engel, W. (1997) Spermatozoa lacking acrosin protein show delayed fertilization. *Mol. Reprod. Dev.*, **46**, 370–376.

Alves, A.P., Mulloy, B., Diniz, J.A., and Mourão, P.A. (1997) Sulfated polysaccharides from the egg jelly layer are species-specific inducers of acrosomal reaction in sperms of sea urchins. *J. Biol. Chem.*, **272**, 6965–6971.

Alves, A.P., Mulloy, B., Moy, G.W., Vacquier, V.D., and Mourão, P.A. (1998) Females of the sea urchin *Strongylocentrotus purpuratus* differ in the structures of their egg jelly sulfated fucans. *Glycobiology*, **8**, 939–946.

Aviles, M., Okinaga, T., Shur, B.D., and Ballesta, J. (2000) Differential expression of glycoside residues in the mammalian zona pellucida. *Mol. Reprod. Dev.*, **57**, 296–308.

Baba, T., Azuma, S., Kashiwabara, S., and Toyoda, Y. (1994) Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *J. Biol. Chem.*, **269**, 31845–31849.

Baginski, T., Hirohashi, N., and Hoshi, M. (1999) Sulfated O-linked glycans of the vitelline coat as ligands in gamete interaction in the ascidian, *Halocynthia roretzi*. *Dev. Growth Differ.*, **41**, 357–364.

Bleil, J.D., and Wassarman, P.M. (1988) Galactose at the nonreducing terminus of O-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity [published erratum appears in *Proc Natl Acad Sci USA* 1988 Dec;85(24):9600]. *Proc. Natl. Acad. Sci. USA*, **85**, 6778–6782.

Bleil, J.D., and Wassarman, P.M. (1990) Identification of a ZP3-binding protein on acrosome-intact mouse sperm by photoaffinity crosslinking. *Proc. Natl Acad. Sci. USA*, **87**, 5563–5567.

Bonsignorio, D., Perego, L., Del Giacco, L., and Cotelli, F. (1996) Structure and macromolecular composition of the zebrafish egg chorion. *Zygote*, **4**, 101–108.

Bookbinder, L.H., Cheng, A., and Bleil, J.D. (1995) Tissue- and species-specific expression of sp56, a mouse sperm fertilization protein [see comments] [published erratum appears in *Science* 1995 Aug 25;269(5227):1120]. *Science*, **269**, 86–89.

Brewis, I.A., and Moore, H.D. (1997) Molecular mechanisms of gamete recognition and fusion at fertilization. *Human Reprod.*, **12**, 156–165.

Burkin, H.R., and Miller, D.J. (2000) Zona pellucida protein binding ability of porcine sperm during epididymal maturation and the acrosome reaction. *Dev. Biol.*, **222**, 99–109.

Camaioni, A., Salustri, A., Yanagishita, M., and Hascall, V.C. (1996) Proteoglycans and proteins in the extracellular matrix of mouse cumulus cell-oocyte complexes. *Arch. Biochem. Biophys.*, **325**, 190–198.

Chen, J., Litscher, E.S., and Wassarman, P.M. (1998) Inactivation of the mouse sperm receptor, mZP3, by site-directed mutagenesis of individual serine residues located at the combining site for sperm. *Proc. Natl Acad. Sci. USA*, **95**, 6193–6197.

Cheng, A., Le, T., Palacios, M., Bookbinder, L.H., Wassarman, P.M., Suzuki, F., and Bleil, J.D. (1994) Sperm-egg recognition in the mouse: characterization of sp56, a sperm protein having specific affinity for ZP3. *J. Cell Biol.*, **125**, 867–878.

Crosby, J.A., and Barros, C. (1999) Effect of recombinant boar beta-acrosin on sperm binding to intact zona pellucida during *in vitro* fertilization. *Biol. Reprod.*, **61**, 1535–1540.

Dandekar, P., Aggeler, J., and Talbot, P. (1992) Structure, distribution and composition of the extracellular matrix of human oocytes and cumulus masses. *Human Reprod.*, **7**, 391–398.

Darszon, A., Labarca, P., Nishigaki, T., and Espinosa, F. (1999) Ion channels in sperm physiology. *Physiol. Rev.*, **79**, 481–510.

DeAngelis, P.L., and Glabe, C.G. (1990) Specific recognition of sulfate esters by bindin, a sperm adhesion protein from sea urchins. *Biochim. Biophys. Acta*, **1037**, 100–105.

Dell, A., Morris, H.R., Easton, R.L., Patankar, M., and Clark, G.F. (1999) The glycobiology of gametes and fertilization. *Biochim. Biophys. Acta*, **1473**, 196–205.

Dhume, S.T., and Lennarz, W.J. (1995) The involvement of O-linked oligosaccharide chains of the sea urchin egg receptor for sperm in fertilization. *Glycobiology*, **5**, 11–17.

Doren, S., Landsberger, N., Dwyer, N., Gold, L., Blanchette-Mackie, J., and Dean, J. (1999) Incorporation of mouse zona pellucida proteins into the envelope of *Xenopus laevis* oocytes. *Dev. Genes Evol.*, **209**, 330–339.

Drickamer, K. (1988) Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.*, **263**, 9557–9560.

Easton, R.L., Patankar, M.S., Lattanzio, F.A., Leaven, T.H., Morris, H.R., Clark, G.F., and Dell, A. (2000) Structural analysis of murine zona pellucida glycans. Evidence for the expression of core 2-type O-glycans and the Sd(a) antigen. *J. Biol. Chem.*, **275**, 7731–7742.

Evans, J.P. (2000) Getting sperm and egg together: things conserved and things diverged. *Biol. Reprod.*, **63**, 355–360.

Flannery, B., and Epel, D. (1998) Effects of wheat germ agglutinin on tunicate egg activation and fertilization: is there a plasma membrane sperm receptor system on *Ascidia ceratodes* eggs? *Dev. Growth Differ.*, **40**, 297–306.

Florman, H.M., and Wassarman, P.M. (1985) O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell*, **41**, 313–324.

Foster, J.A., Friday, B.B., Maulit, M.T., Blobel, C., Winfrey, V.P., Olson, G.E., Kim, K.S., and Gerton, G.L. (1997) AM67, a secretory component of the guinea pig sperm acrosomal matrix, is related to mouse

sperm protein sp56 and the complement component 4-binding proteins. *J. Biol. Chem.*, **272**, 12714–12722.

Geng, J.G., Raub, T.J., Baker, C.A., Sawada, G.A., Ma, L., and Elhammer, A.P. (1997) Expression of a P-selectin ligand in zona pellucida of porcine oocytes and P-selectin on acrosomal membrane of porcine sperm cells. Potential implications for their involvement in sperm-egg interactions. *J. Cell Biol.*, **137**, 743–754.

Hoshi, M., De Santis, R., Pinto, M.R., Cotelli, F., and Rosati, F. (1983) Is a sperm α-L-fucsidase responsible for sperm-egg binding in *Ciona intestinalis*. In J. Andre, ed., *The Sperm Cell*, Martinus Nijhoff Publishers, The Hague, pp. 107–110.

Hoshi, M., De Santis, R., Pinto, M.R., Cotelli, F., and Rosati, F. (1985) Sperm glycosidases as mediators of sperm-egg binding in ascidians. *Zool. Sci.*, **2**, 65–69.

Hoshi, M., Okinaga, T., Kontani, K., Araki, T., and Chiba, K. (1991) Acrosome reaction-inducing glycoconjugate in the jelly coat of starfish eggs. In B. Baccetti, ed., *Comparative Spermatology 20 Years After*, Raven Press, New York, pp. 175–180.

Hughes, J., Ward, C.J., Aspinwall, R., Butler, R., and Harris, P.C. (1999) Identification of a human homologue of the sea urchin receptor for egg jelly: a polycystic kidney disease-like protein. *Human Mol. Gen.*, **8**, 543–549.

Hunnicutt, G.R., Primakoff, P., and Myles, D.G. (1996) Sperm surface protein PH-20 is bifunctional: one activity is a hyaluronidase and a second, distinct activity is required in secondary sperm-zona binding. *Biol. Reprod.*, **55**, 80–86.

Iwamatsu, T., Yoshizaki, N., and Shibata, Y. (1997) Changes in the chorion and sperm entry into the micropyle during fertilization in the teleostean fish, *Oryzias latipes*. *Dev. Growth Differ.*, **39**, 33–41.

Johnston, D.S., Wright, W.W., Shaper, J.H., Hokke, C.H., Van den Eijnden, D.H., and Joziasse, D.H. (1998) Murine sperm-zona binding, a fucosyl residue is required for a high affinity sperm-binding ligand. A second site on sperm binds a nonfucosylated, beta-galactosyl-capped oligosaccharide. *J. Biol. Chem.*, **273**, 1888–1895.

Jones, R. (1991) Interaction of zona pellucida glycoproteins, sulfated carbohydrates and synthetic polymers with proacrosin, the putative egg-binding protein from mammalian spermatozoa. *Development (Cambridge)*, **111**, 1155–1164.

Jones, R., Brown, C.R., and Lancaster, R.T. (1988) Carbohydrate-binding properties of boar sperm proacrosin and assessment of its role in sperm-egg recognition and adhesion during fertilization. *Development (Cambridge)*, **102**, 781–792.

Just, M.L., and Lennarz, W.J. (1997) Reexamination of the sequence of the sea urchin egg receptor for sperm: implications with respect to its properties. *Dev. Biol.*, **184**, 25–30.

Kan, F.W. (1990) High-resolution localization of hyaluronic acid in the golden hamster oocyte-cumulus complex by use of a hyaluronidase-gold complex. *Anat. Rec.*, **228**, 370–382.

Kitazume-Kawaguchi, S., Inoue, S., Inoue, Y., and Lennarz, W.J. (1997) Identification of sulfated oligosialic acid units in the O-linked glycan of the sea urchin egg receptor for sperm. *Proc. Natl Acad. Sci. USA*, **94**, 3650–3655.

Koyota, S., Wimalasir, K.M.S., and Hoshi, M. (1997) Structure of the main saccharide chain in the acrosome reaction-inducing substance of the starfish, *Asterias amurensis*. *J. Biol. Chem.*, **272**, 10372–10376.

Kubo, H., Kawano, T., Tsubuki, S., Kawashima, S., Katagiri, C., and Suzuki, A. (1997) A major glycoprotein of *Xenopus* egg vitelline envelope, gp41, is a frog homolog of mammalian ZP3. *Dev. Growth Differ.*, **39**, 405–417.

Kudo, K., Yonezawa, N., Katsumata, T., Aoki, H., and Nakano, M. (1998) Localization of carbohydrate chains of pig sperm ligand in the glycoprotein ZPB of egg zona pellucida. *Eur. J. Biochem.*, **252**, 492–499.

Lambert, C.C. (1986) Fertilization-induced modification of chorion N-acetylglucosamine groups blocks polyspermy in ascidian eggs. *Dev. Biol.*, **116**, 168–173.

Lambert, C.C. (1989) Ascidian eggs release glycosidase activity which aids in the block against polyspermy. *Development*, **105**, 415–420.

Larson, J.L., and Miller, D.J. (1997) Sperm from a variety of mammalian species express beta1, 4-galactosyltransferase on their surface. *Biol. Reprod.*, **57**, 442–453.

Leyton, L., and Saling, P. (1989) Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. *J. Cell Biol.*, **108**, 2163–2168.

Litscher, E.S., and Wassarman, P.M. (1996) Characterization of mouse ZP3-derived glycopeptide, gp55, that exhibits sperm receptor and acrosome reaction-inducing activity *in vitro*. *Biochemistry*, **35**, 3980–3985.

Liu, C., Litscher, E.S., and Wassarman, P.M. (1997) Zona pellucida glycoprotein mZP3 bioactivity is not dependent on the extent of glycosylation of its polypeptide or on sulfation and sialylation of its oligosaccharides. *J. Cell Sci.*, **110**, 745–752.

Loeser, C.R., and Tulsiani, D.R. (1999) The role of carbohydrates in the induction of the acrosome reaction in mouse spermatozoa. *Biol. Reprod.*, **60**, 94–101.

Loeser, C.R., Lynch, C. II, and Tulsiani, D.R. (1999) Characterization of the pharmacological-sensitivity profile of neoglycoprotein-induced acrosome reaction in mouse spermatozoa. *Biol. Reprod.*, **61**, 629–634.

Lu, Q., and Shur, B.D. (1997) Sperm from beta 1, 4-galactosyltransferase-null mice are refractory to ZP3-induced acrosome reactions and penetrate the zona pellucida poorly. *Development*, **124**, 4121–4131.

Lyons, C.E., Payette, K.L., Price, J.L., and Huang, R.C. (1993) Expression and structural analysis of a teleost homolog of a mammalian zona pellucida gene [published erratum appears in *J Biol Chem* 1998 Mar 27;273(13):7782]. *J. Biol. Chem.*, **268**, 21351–21358.

Macek, M.B., Lopez, L.C., and Shur, B.D. (1991) Aggregation of beta-1, 4-galactosyltransferase on mouse sperm induces the acrosome reaction. *Dev. Biol.*, **147**, 440–444.

Martinez, M.L., Martelotto, L., and Cabada, M.O. (2000) Purification and biological characterization of N-acetyl beta-D glucosaminidase from *Bufo arenarum* spermatozoa. *Mol. Reprod. Dev.*, **57**, 194–203.

Matsuura, K., Sawada, H., and Yokosawa, H. (1993) Purification and properties of N-acetylglucosaminidase from eggs of the ascidian, *Halocynthia roretzi*. *Eur. J. Biochem.*, **218**, 535–541.

Matsuura, K., Sawada, H., and Yokosawa, H. (1995) N-acetylglucosaminidase inhibitor isolated from the vitelline coat of ascidian eggs is a candidate sperm receptor. *Biochem. Biophys. Res. Commun.*, **213**, 311–316.

Mauk, R., Jaworski, D., Kamei, N., and Glabe, C.G. (1997) Identification of a 97-kDa heat shock protein from S. franciscanus ovaries with 94% amino acid identity to the *S. purpuratus* egg surface receptor for sperm. *Dev. Biol.*, **184**, 31–37.

Mayadas, T.N., Johnson, R.C., Rayburn, H., Hynes, R.O., and Wagner, D.D. (1993) Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell*, **74**, 541–554.

Miller, D.J., Macek, M.B., and Shur, B.D. (1992) Complementarity between sperm surface beta-1, 4-galactosyltransferase and egg-coat ZP3 mediates sperm-egg binding. *Nature*, **357**, 589–593.

Mori, E., Mori, T., and Takasaki, S. (1997) Binding of mouse sperm to beta-galactose residues on egg zona pellucida and asialofetuin-coupled beads. *Biochem. Biophys. Res. Commun.*, **238**, 95–99.

Moy, G.W., Mendoza, L.M., Schulz, J.R., Swanson, W.J., Glabe, C.G., and Vacquier, V.D. (1996) The sea urchin sperm receptor for egg jelly is a modular protein with extensive homology to the human polycystic kidney disease protein, PKD1. *J. Cell Biol.*, **133**, 809–817.

Murata, K., Sasaki, T., Yasumasu, S., Iuchi, I., Enami, J., Yasumasu, I., and Yamagami, K. (1995) Cloning of cDNAs for the precursor protein of a low-molecular-weight subunit of the inner layer of the egg envelope (chorion) of the fish *Oryzias latipes*. *Dev. Biol.*, **167**, 9–17.

Murata, K., Sugiyama, H., Yasumasu, S., Iuchi, I., Yasumasu, I., and Yamagami, K. (1997) Cloning of cDNA and estrogen-induced hepatic gene expression for choriogenin H, a precursor protein of the fish egg envelope (chorion). *Proc. Natl Acad. Sci. USA*, **94**, 2050–2055.

Ohlendieck, K., and Lennarz, W.J. (1995) Role of the sea urchin egg receptor for sperm in gamete interactions. *Trends Biochem. Sci.*, **20**, 29–33.

Prasad, S.V., Skinner, S.M., Carino, C., Wang, N., Cartwright, J., and Dunbar, B.S. (2000) Structure and function of the proteins of the mammalian zona pellucida. *Cells Tiss. Organs*, **166**, 148–164.

Rankin, T.L., Tong, Z.B., Castle, P.E., Lee, E., Gore-Langton, R., Nelson, L.M., and Dean, J. (1998) Human ZP3 restores fertility in Zp3 null mice without affecting order-specific sperm binding. *Development*, **125**, 2415–2424.

Rebeiz, M., and Miller, D.J. (1999) Porcine sperm surface beta1, 4galactosyltransferase binds to the zona pellucida but is not necessary or sufficient to mediate sperm-zona pellucida binding. *Mol. Reprod. Dev.*, **54**, 379–387.

Sabeur, K., Cherr, G.N., Yudin, A.I., Primakoff, P., Li, M.W., and Overstreet, J.W. (1997) The PH-20 protein in human spermatozoa. *J. Androl.*, **18**, 151–158.

Seaton, G.J., Hall, L., and Jones, R. (2000) Rat sperm 2B1 glycoprotein (PH20) contains a C-terminal sequence motif for attachment of a glycosyl phosphatidylinositol anchor. Effects of endoproteolytic cleavage on hyaluronidase activity. *Biol. Reprod.*, **62**, 1667–1676.

Shalgi, R., and Raz, T. (1997) The role of carbohydrate residues in mammalian fertilization. *Histol. Histopath.*, **12**, 813–822.

Shur, B.D., Evans, S., and Lu, Q. (1998) Cell surface galactosyltransferase: current issues. *Glycoconj. J.*, **15**, 537–548.

Takasaki, S., Mori, E., and Mori, T. (1999) Structures of sugar chains included in mammalian zona pellucida glycoproteins and their potential roles in sperm-egg interaction. *Biochim. Biophys. Acta*, **1473**, 206–215.

Thaler, C.D., and Cardullo, R.A. (1995) *Biochemical* characterization of a glycosylphosphatidylinositol-linked hyaluronidase on mouse sperm. *Biochemistry*, **34**, 7788–7795.

Thaler, C.D., and Cardullo, R.A. (1996) The initial molecular interaction between mouse sperm and the zona pellucida is a complex binding event. *J. Biol. Chem.*, **271**, 23289–23297.

Thall, A.D., Maly, P., and Lowe, J.B. (1995) Oocyte Gal-alpha-1, 3gal epitopes implicated in sperm adhesion to the zona pellucida glycoprotein ZP3 are not required for fertilization in the mouse. *J. Biol. Chem.*, **270**, 21437–21440.

Tian, J., Gong, H., and Lennarz, W.J. (1999) Xenopus laevis sperm receptor gp69/64 glycoprotein is a homolog of the mammalian sperm receptor ZP2. *Proc. Natl Acad. Sci. USA*, **96**, 829–834.

Urch, U.A., and Patel, H. (1991) The interaction of boar sperm proacrosin with its natural substrate, the zona pellucida, and with polysulfated polysaccharides. *Development (Cambridge)*, **111**, 1165–1172.

Ushiyama, A., Chiba, K., Shima, A., and Hoshi, M. (1995) Estimation by radiation inactivation of the minimum functional size of acrosome-reaction-inducing substance (ARIS) in the starfish, *Asterias amurensis*. *Zygote*, **3**, 351–355.

Vacquier, V.D., and Moy, G.W. (1997) The fucose sulfate polymer of egg jelly binds to sperm REJ and is the inducer of the sea urchin sperm acrosome reaction. *Dev. Biol.*, **192**, 125–135.

Vacquier, V.D., Swanson, W.J., and Hellberg, M.E. (1995) What have we learned about sea urchin sperm bindin? *Devel. Growth Diff.*, **37**, 1–10.

Vilela-Silva, A.C., Alves, A.P., Valente, A.P., Vacquier, V.D., and Mourão, P.A. (1999) Structure of the sulfated alpha-L-fucan from the egg jelly coat of the sea urchin Strongylocentrotus franciscanus: patterns of preferential 2-O- and 4-O-sulfation determine sperm cell recognition. *Glycobiology*, **9**, 927–933.

Vo, L.H., and Hedrick, J.L. (2000) Independent and hetero-oligomeric-dependent sperm binding to egg envelope glycoprotein ZPC in Xenopus laevis. *Biol. Reprod.*, **62**, 766–774.

Wang, H., and Gong, Z. (1999) Characterization of two zebrafish cDNA clones encoding egg envelope proteins ZP2 and ZP3. *Biochim. Biophys. Acta*, **1446**, 156–160.

Wassarman, P., Chen, J., Cohen, N., Litscher, E., Liu, C., Qi, H., and Williams, Z. (1999) Structure and function of the mammalian egg zona pellucida. *J. Exp. Zool.*, **285**, 251–258.

Wassarman, P.M. (1999a) Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell*, **96**, 175–183.

Wassarman, P.M. (1999b) The Parkes Lecture. Zona pellucida glycoprotein mZP3: a versatile player during mammalian fertilization. *J. Reprod. Fertil.*, **116**, 211–216.

Yamagata, K., Murayama, K., Okabe, M., Toshimori, K., Nakanishi, T., Kashiwabara, S., and Baba, T. (1998) Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. *J. Biol. Chem.*, **273**, 10470–10474.

Yang, J.C., and Hedrick, J.L. (1997) cDNA cloning and sequence analysis of the *Xenopus laevis* egg envelope glycoprotein gp43. *Dev. Growth Differ.*, **39**, 457–467.

Yonezawa, N., Mitsui, S., Kudo, K., and Nakano, M. (1997) Identification of an N-glycosylated region of pig zona pellucida glycoprotein ZPB that is involved in sperm binding. *Eur. J. Biochem.*, **248**, 86–92.

Yudin, A.I., Vandevoort, C.A., Li, M.W., and Overstreet, J.W. (1999) PH-20 but not acrosin is involved in sperm penetration of the macaque zona pellucida. *Mol. Reprod. Dev.*, **53**, 350–362.

Yurewicz, E.C., Pack, B.A., Armant, D.R., and Sacco, A.G. (1993) Porcine zona pellucida ZP3 alpha glycoprotein mediates binding of the biotin-labeled M(r) 55, 000 family (ZP3) to boar sperm membrane vesicles. *Mol. Reprod. Dev.*, **36**, 382–389.

Yurewicz, E.C., Pack, B.A., and Sacco, A.G. (1991) Isolation, composition, and biological activity of sugar chains of porcine oocyte zona pellucida 55K glycoproteins. *Mol. Reprod. Dev.*, **30**, 126–134.

The text of Chapter I, in full, is a reprint of the material as it appears in Mengerink, K. J. and Vacquier, V. D. (2001) Glycobiology of sperm egg interactions in deuterostomes. *Glycobiology* 11(4): 37R-43R. The dissertation author was the primary author and the co-author listed in this publication directed and supervised the research, which forms the basis for this chapter.

CHAPTER II

suREJ Proteins: New Signaling Molecules in Sea Urchin Spermatozoa

Zygote 8 (Supplement), pp. S28–S30. © 2000 Cambridge University Press Printed in the United Kingdom

suREJ proteins: new signalling molecules in sea urchin spermatozoa

Kathryn J. Mengerink, Gary W. Moy and Victor D. Vacquier
Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093-0202, USA

In *Strongylocentrotus purpuratus*, the fucose sulphate polymer (FSP) of egg jelly induces the sperm acrosome reaction (AR; Vacquier & Moy, 1997). Protease treatment of sperm renders the cells insensitive to FSP, indicating that sperm membrane receptors mediate the signal transduction events underlying the AR. Monoclonal antibodies to a 210 kDa membrane glycoprotein induce Ca^{2+} influx into sperm and trigger the AR (Trimmer *et al.*, 1986; Moy *et al.*, 1996). Purified 210 kDa protein binds species-specifically to egg jelly and blocks AR induction by antibody (Podell & Vacquier, 1985; Moy *et al.*, 1996). FSP binds to the 210 kDa protein attached to Sepharose (Vacquier & Moy, 1997). Monoclonal antibodies localise the 210 kDa protein on the plasma membrane over the acrosome and also on the sperm flagellum. The 210 kDa protein has the attributes of a sperm receptor for egg jelly and is henceforth named suREJ1 (Moy *et al.*, 1996). We describe here the three REJ proteins found thus far in *S. purpuratus* sperm.

suREJ1

suREJ1 is 1450 amino acids and has an expected protein mass of approximately 160 kDa. Western blots of HF-deglycosylated sperm membrane proteins, probed with an IgG to a suREJ1-specific peptide, show a single reaction at approximately 160 kDa (Moy *et al.*, 1996). Glycosylation represents approximately 50% of the protein's mass (Podell & Vacquier, 1985). Beginning at the amino-terminus, suREJ1 consists of one EGF module of 40 residues, followed by two carbohydrate recognition domain (CRD) modules (120 residues each) of the C-type lectin variety. The two CRDs are divergent, being only 50% identical in amino acid sequence (Moy *et al.*, 1996). suREJ1 has one putative transmembrane segment (TMS) at its extreme carboxyl-terminus, with only 15 residues being putatively cytoplasmic. Treatment of sea urchin sperm with IgG to suREJ1 causes the translocation of the protein in the plane of the membrane, suggesting it is not strongly attached to cytoplasmic proteins (Trimmer & Vacquier, 1988). Sixty to seventy per cent of suREJ1 antigenicity becomes 200 000 *g* soluble when sperm are treated for 5 h with pH 9.1 seawater. This also shows a lack of tenacious association of suREJ1 with the sperm plasma membrane. That monoclonal antibodies to suREJ1 cause Ca^{2+} influx into sperm and induce the AR implicates this protein as an important signal transducer in sperm (Moy *et al.*, 1996).

suREJ2

One suREJ clone was found that was 82% identical to suREJ1 for 400 amino acids. The full-length sequence of this second REJ (suREJ2) is 1472 amino acids. It is quite different from suREJ1 in its amino-terminal modules. suREJ2 does not have an EGF module, but does have one CRD. Downstream of the CRD there are eight repeats of 12 residues, which are considerably hydrophobic. Next, comes an insertion of approximately 100 residues not found in suREJ1. The extreme carboxyl-terminus of suREJ2 is similar to that of suREJ1 with a single TMS and 15 residues putatively cytoplasmic. Peptide antibodies specific to suREJ2 react with one protein of approximately 150 kDa on western blots of both untreated and HF-deglycosylated sperm membrane proteins, suggesting that suREJ2 is not extensively glycosylated. Also, suREJ2 does not bind WGA lectin, whereas suREJ1 does. A peptide-specific antibody to suREJ2 localises the protein to the mitochondrial midpiece and flagellum, but not to the acrosomal region of the sea urchin sperm.

suREJ3

While sequencing suREJ2 clones, one clone was found which was neither suREJ1 nor suREJ2. Completion of this sequence shows suREJ3 is 2675 residues. From amino- to carboxyl-terminus, it has one SUEL domain (sea urchin egg lectin domain, ~105 residues; Ozeki *et al.*, 1995). Contiguous with the SUEL domain is one CRD which is 40–46% identical to the suREJ1 and

suREJ2 CRDs. Downstream of the CRD is the 100 residue insertion similar to the one found in suREJ2. A hydropathy plot of the carboxyl-terminal half of suREJ3 indicates 11 putative TMS (compared with one TMS of the other two REJ proteins). Between TMS1 and TMS2 is a domain homologous to the amino-terminal domain of lipoxygenases. An antibody to a 17mer peptide unique to the suREJ3 CRD localises the protein to the sperm head plasma membrane covering the acrosome.

Relationship of suREJ proteins to PKD proteins and latrophilins

The single TMS of suREJ1 and suREJ2 aligns with the first TMS of suREJ3. Upstream from this TMS, for approximately 1000 residues, these three proteins share homology with human polycystin (huPKD1), the protein mutated in 85% of autosomal dominant polycystic kidney disease. For example, in this region suREJ1 and huPKD1 are 20% identical and 40% similar. A random jumble analysis shows the homology to be statistically significant (Moy *et al.*, 1996). huPKD1 is 4302 residues and contains a lectin-like module, leucine-rich region, cysteine-rich region, LDL receptor module and 16 repeats of approximately 100 residues specific to PKD1 called 'PKD1 repeats' (International Polycystic Kidney Disease Consortium, 1995). The 100 residue insertions found in suREJ2 and suREJ3, but not suREJ1, are homologous to the 'PKD repeats'. In suREJ3, downstream of the first TMS and for the remainder of the protein, there is significant homology to the 11 TMS of huPKD1 and other PKD family members such as PKDREJ, PKD2 and PKD-L. About 2500 residues of huPKD1 are predicted to be extracellular. huPKD1 has the characteristics of a membrane-associated carbohydrate binding protein (International Polycystic Kidney Disease Consortium, 1995; Ward *et al.*, 1996; Ibraghimov-Beskrovnaya *et al.*, 1997). huPKD1 is expressed in many human tissues (Ward *et al.*, 1996); its function remains unknown.

PKDREJ (Hughes *et al.*, 1999) is the mammalian homologue most closely related to suREJ3. This protein is slightly shorter than suREJ3. It does not have a SUEL domain, a CRD, nor any 'PKD repeats' characteristic of huPKD1, suREJ2 and suREJ3. However, PKDREJ does possess approximately 1000 extracellular residues and the 11 putative TMS characteristic of PKD proteins and suREJ3. In mouse, PKDREJ is expressed only in the testis and only during the time of sperm differentiation. These observations implicate PKDREJ as a signalling protein of mammalian sperm (Hughes *et al.*, 1999). Hydropathy plots of huPKD1, PKDREJ and suREJ3 show that the 11 TMS of these proteins are homologous. The carboxyl-terminus of suREJ3, huPKD1 and PKDREJ, comprising TMS 6-11, is homologous to huPKD2, voltage-dependent Ca^{2+} channels and TRP (transient receptor potential channels; Montell, 1997). TRPC1, a mammalian TRP channel, has been shown to associate with huPKD2 (Tsiokas *et al.*, 1998). Also, a PKD2 homologue, PKD-L, has been shown to form Ca^{2+}-activated cation channels when expressed in *Xenopus* oocytes (Chen *et al.*, 1999).

Latrophilins are G-protein coupled, 7 TMS receptors. Latrophilin-1 binds α-latrotoxin (black widow spider venom) inducing the exocytosis of synaptosomes in mammals (Sugita *et al.*, 1998; Krasnoperov *et al.*, 1997). suREJ3 shares several features with latrophilins. For example, the SUEL domain of approximately 105 residues, originally described in sea urchin eggs (Ozeki *et al.*, 1995), is the most conserved feature of latrophilins. Latrophilins are post-translationally cleaved within a conserved sequence domain in the extracellular part of the protein just before their first TMS. The three sea urchin REJ proteins possess this conserved latrophilin cleavage site as do other members of the G-protein coupled receptor family. The two cleavage products of latrophilins tightly associate on the outside surface of the cell (Sugita *et al.*, 1998; Krasnoperov *et al.*, 1997). Preliminary western blots, probed with two antibodies reacting on different sides of the suREJ3 putative latrophilin cleavage site, identify single reacting bands of different sizes, providing evidence that suREJ3 is in fact post-translationally cleaved in mature sperm.

Following the first TMS of suREJ3 are 125 cytoplasmic residues sharing homology with the amino-terminal end of lipoxygenases. Lipoxygenases comprise a large family of proteins that peroxidise lipids (Kuhn & Thiele, 1999). huPKD1 and PKDREJ also share this homology. The extracellular latrophilin cleavage site, followed by putative TMS-1, which is in turn followed by the intracellular lipoxygenase domain, indicates that the location of putative TMS-1 is correctly assigned in suREJ3 and PKD family members.

We predict that suREJ3 and mammalian PKDREJ, will prove to be key players in the induction of the sperm AR. The homology of TMS 6-11 to known channel proteins suggests that they are members of this new family of channel-forming proteins. Their close relationship with each other, and with the latrophilins, suggests that the transmembrane signalling events underlying the sperm AR are conserved among the deuterostomes.

Supported by NIH Grant HD12986.

References

Chen, X.Z., *et al.* (1999). *Nature* **401**, 383–6.
Hughes, J., *et al.* (1999). *Hum. Mol. Genet.* **8**, 543–9.
Ibraghimov-Beskrovnaya, O., *et al.* (1997). *Proc. Natl. Acad.*

Sci. USA **94**, 6397–402.
International Polycystic Kidney Disease Consortium. (1995). *Cell* **81**, 289–98.
Krasnoperov, V.G., *et al.* (1997). *Neuron* **18**, 925–37.
Kuhn, H. & Thiele, B.J. (1999). *FEBS Lett.* **449**, 7–11.
Montell, C. (1997). *Mol. Pharmacol.* **52**, 755–63.
Moy, G.W., *et al.* (1996). *J. Cell Biol.* **133**, 809–17.
Ozeki, Y., *et al.* (1995). *Exp. Cell Res.* **216**, 318–24.
Podell, S.B. & Vacquier, V.D. (1985). *J. Biol. Chem.* **260**, 2715–18.
Sugita, S., *et al.* (1998). *J. Biol. Chem.* **273**, 32712–24.
Trimmer, J.S. & Vacquier, V.D. (1988). *Exp. Cell Res.* **175**, 37–51.
Trimmer, J.S., Schackmann, R.W & Vacquier, V.D. (1986). *Proc. Natl. Acad. Sci. USA* **83**, 9055–9.
Tsiokas, L., *et al.* (1998). *Proc. Natl. Acad. Sci. USA* **96**, 3934–9.
Vacquier, V.D. & Moy, G.W. (1997). *Dev. Biol.* **192**, 125–35.
Ward, C.J., *et al.* (1996). *Proc. Natl. Acad. Sci. USA* **93**, 1524–8.

The text of Chapter II, in full, is a reprint of the material as it appears in Mengerink, K. J., Moy, G. W., and Vacquier, V. D. (2000) suREJ proteins: new signaling molecules in sea urchin spermatozoa. *Zygote* 8(4): S28-30. The dissertation author was the primary author and the co-authors listed in this publication directed and supervised the research, which forms the basis for this chapter.

CHAPTER III

suREJ3, a Polycystin-1 Protein, is Cleaved at the GPS Domain and Localizes to the Acrosomal Region of Sea Urchin Sperm

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 2, Issue of January 11, pp. 943–948, 2002
Printed in U.S.A.

suREJ3, a Polycystin-1 Protein, Is Cleaved at the GPS Domain and Localizes to the Acrosomal Region of Sea Urchin Sperm*

Received for publication, October 5, 2001, and in revised form, October 25, 2001
Published, JBC Papers in Press, November 5, 2001,DOI 10.1074/jbc.M109673200

Kathryn J. Mengerink‡, Gary W. Moy, and Victor D. Vacquier

From the Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California San Diego, La Jolla, California 92093-0202

The sea urchin sperm acrosome reaction (AR) is a prerequisite for sperm-egg fusion. This report identifies sea urchin sperm receptor for egg jelly-3 (suREJ3) as a new member of the polycystin-1 family (the protein mutated in autosomal dominant polycystic kidney disease). suREJ3 is a multidomain, 2,681-amino acid, heavily glycosylated orphan receptor with 11 putative transmembrane segments (TMS) that localize to the plasma membrane covering the sperm acrosomal vesicle. Like the latrophilins and other members of the secretin family of G-protein-coupled receptors, suREJ3 is cleaved at the consensus GPS (G-protein-coupled receptor proteolytic site) domain. Antibodies to the extracellular 1,455-residue NH_2-terminal portion identify a band at 250 kDa that shifts in electrophoretic mobility to 180 kDa upon glycosidase digestion. Antibodies to the 1,226-residue COOH-terminal portion identify a band at 150 kDa that shifts to 140 kDa after glycosidase treatment. Antibodies to both portions of suREJ3 localize exclusively to the plasma membrane over the acrosomal vesicle. Immunoprecipitation shows that both portions of suREJ3 are associated in detergent extracts. This is the first report showing that a polycystin family member is cleaved at the GPS domain. Localization of suREJ3 to the acrosomal region provides the first suggestion for the role of a polycystin-1 protein (components of nonselective cation channels) in a specific cellular process.

Substantial progress has been made in understanding the molecular mechanisms of fertilization in both echinoderms and mammals. Upon contact with the jelly layer surrounding the egg, sea urchin sperm undergo the exocytotic acrosome reaction (AR).[1] The AR involves Na and Ca^2 influx and H and K efflux. This results in increases in intracellular Ca^2 and pH values and elevation of cAMP and inositol triphosphate (1). In sea urchin sperm, suREJ1 was identified as a 1,450-amino acid type I transmembrane glycoprotein that is involved in the signaling cascade leading to the AR (2). suREJ1 binds to the fucose sulfate polymer of egg jelly, and the isolated polymer is capable of inducing the acrosome reaction in a manner similar to whole egg jelly (3, 4).

Sequence analysis of suREJ1 identified several extracellular domains including an epidermal growth factor domain, two carbohydrate recognition domains (CRDs), and the REJ module, a region of 1,000 amino acids that is homologous to only one other protein, polycystin-1 (2). Polycystin-1 is a multidomain glycoprotein of 4,303 amino acids with 11 putative TMS (5). The last six TMS are homologous to polycystin-2 and to voltage-activated calcium channels (6, 7). Human polycystin-2 is a 968-amino acid protein with six putative TMS (6). Mutations in the human polycystin-1 gene account for 85% of autosomal dominant polycystic kidney disease, whereas mutations in the polycystin-2 gene account for 15% of autosomal dominant polycystic kidney disease. This disease affects an estimated 1 in 400 to 1 in 1,000 individuals and is one of the most common hereditary disorders. All autosomal dominant polycystic kidney disease patients have renal cysts, and other manifestations include pancreatic and hepatic cysts and cardiovascular abnormalities (8, 9). Along with sequence homology to voltage-gated ion channels and transient receptor potential channels, there is accumulating functional evidence that mammalian polycystin-1 and -2 interact to form a calcium-modulated nonselective cation channel (10–12).

Several homologs of both polycystin-1 and -2 have been reported in mammals, fish, and invertebrates (7, 13, 14), demonstrating that these proteins are a new gene family whose function remains ill-defined. Recently, another human polycystin-1 homolog, hPKDREJ, has been identified. This homolog contains the 1,000-residue REJ module plus the COOH-terminal 11 TMS of polycystin-1 (15). Most interestingly, hPKDREJ is only expressed in testis, and transcripts first appear during sperm differentiation. The homology between the sea urchin sperm suREJ proteins and mammalian hPKDREJ suggests that both animal groups may share a common signaling pathway in fertilization.

While screening a sea urchin testis cDNA library with suREJ1 probes, two new homologs were discovered, suREJ2 and suREJ3. Of these, suREJ3 is unique in that it contains all 11 TMS found in mammalian polycystin-1 (5) and hPKDREJ (15).

EXPERIMENTAL PROCEDURES

DNA Sequencing—A suREJ1 clone isolated from a Lambda ZAP cDNA library of *Strongylocentrotus purpuratus* testis (2) was used for screening. Overlapping clones of suREJ3 were obtained and sequenced. After repeated rounds of screening with suREJ3 specific probes, the full-length sequence was obtained and both ends confirmed by rescreening the cDNA library.

Sequence Analysis—suREJ3 homologs were identified using BLAST (16). Specific domains and glycosylation sites were found using the

* This work was supported by National Institutes of Health Grant HD12986. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF422153.

‡ To whom correspondence should be addressed. Tel.: 858-534-2146; Fax: 858-534-7313; E-mail: kmengeri@ucsd.edu.

[1] The abbreviations used are: AR, acrosome reaction; CRD, carbohydrate recognition domain; FSW, filtered seawater; REJ, receptor for egg jelly; GPCR, G-protein-coupled receptor; GPS, G-protein-coupled receptor cleavage site; hPKDREJ, human PKDREJ; PLAT, polycystin/lipoxygenase/alpha-toxin; PKD, polycystic kidney disease; PNGase-F, protein-*N*-glycosidase-F; S/C, SUEL/CRD; SUEL, sea urchin egg lectin; suREJ, sea urchin receptor for egg jelly; TMS, transmembrane segment; TRPC, transient receptor potential channel; WGA, wheat germ agglutinin; PVDF, polyvinylidene difluoride.

ProfileScan web site www.isrec.isb-sib.ch/software/PFSCAN_form.html and the PredictProtein web site dodo.cpmc.columbia.edu/predictprotein/predictprotein.html. The signal sequence was predicted using the SignalP web site (17). Random jumble analysis was performed using the Biology Workbench web site PRSS program biowb.sdsc.edu/CGI/BW.cgi. ClustalX and ClustalW were used to make multiple sequence alignments (18), and shading was done using BoxShade www.ch.embnet.org/software/BOX_form.html. Percent identity and percent similarity (includes identical and conserved amino acid changes) were calculated using the program GeneDoc (19). TMS were predicted using hydropathy plots that were generated by the method of Kyte and Doolittle with a window of 14 amino acids (20). GenBank™ accession numbers used are as follows: suREJ3, AF422153; human polycystin-1, P98161; human polycystin-2, Q13563; hPKDREJ, XP_010050; rat latrophilin-1, T14324; *Anthocidaris crassispina* SUEL, P22031; human flamingo-1, AAG00080; and human CD97, AAB36682.

Antibody Production—Recombinant proteins were made by amplifying DNA with exact match primers that correspond to the following amino acids: Glu^{32} to Lys^{241}, SUEL/CRD; His^{1767} to Pro^{2039}, IH. The forward and reverse primers contained the restriction sites *Nde*I and *Bam*HI. The amplified DNA was digested with restriction enzymes and ligated into the expression vector pET15b (Novagen), which contains an NH_2-terminal His tag. The vector was transformed into BL21(DE3) cells (Novagen), the protein expressed by isopropyl-1-thio- -D-galactopyranoside (IPTG) induction, and purified using Ni^2 -NTA resin (Qiagen). Two milligrams of the purified protein was separated on SDS-PAGE and visualized by cupric chloride staining (21). The acrylamide-embedded protein was used for antibody production. For SUEL/CRD (S/C), chicken antibodies were raised commercially (Cocalico, Reamstown, PA) and initially purified from egg yolks as described (22). Antibodies were further affinity-purified on an S/C-conjugated Affi-Gel-10 column (Bio-Rad). Anti-IH rabbit antibodies were raised commercially (Strategic BioSolutions, Ramona, CA) and affinity-purified on an IH-conjugated Affi-Gel-10 column. (Dr. Charles G. Glabe generously provided the peptide.)

Cys-Phe^{219}-Ile-Asn-Leu-Thr-His-Gly-Gln-Trp-Ser-Trp-Arg-Asp-Cys-Glu-Asn-Arg^{235} was used to generate the R3p antibody. The peptide was attached to maleimide-activated keyhole limpet hemocyanin (Pierce) and rabbit antibodies raised commercially (Cocalico). The resulting R3p antibody was purified using the peptide conjugated to Sulfo-Link (Pierce).

Protein Preparation—All procedures were on ice or at 4 °C. Sea urchins were spawned by injection with 0.5 M KCl, and the undiluted sperm was collected with a Pasteur pipette. The sperm was resuspended in 0.45 m filtered seawater (FSW). Coelomocytes were removed by three 5-min centrifugations at 200 *g*, and sperm cells were sedimented at 5,000 *g*. For isolation of sperm heads and flagella, undiluted sperm was resuspended in 20 volumes of FSW containing 50 mM Tris-HCl, pH 6.7 and 50 mM KCl to block the AR. Resuspended sperm were homogenized with a Teflon glass homogenizer to break flagella from heads. The flagella and heads were separated by differential centrifugation for 10 min (800 *g* for heads and 5,000 *g* for flagella). Separation procedures were repeated until the samples were maximally enriched for heads or flagella, as determined by phase contrast microscopy. Whole sperm, heads, and flagella were resuspended in membrane solubilization buffer (0.15 M NaCl, 10 mM HEPES, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 g/ml of leupeptin, and 1% Nonidet P-40, pH 7.4). For immunoprecipitation, 0.5% w/v of Zwittergent 3-10 was used instead of Nonidet P-40. Solubilized protein was obtained after centrifugation at 100,000 *g* for 1 h (4 °C). Sperm membrane vesicles were made according to the pH 9 method (23).

For wheat germ agglutinin (WGA) chromatography, solubilized sperm protein was applied to a WGA-agarose column (EY Laboratories). The column was washed with wash buffer (0.15 M NaCl, 10 mM HEPES, 0.1% Nonidet P-40, pH 7.4), and the protein eluted in wash buffer containing 100 mM *N*-acetyl-D-glucosamine. SDS-PAGE was performed (24), and gels were stained with silver (25) or Coomassie Brilliant Blue.

For protein-*N*-glycosidase-F (PNGase-F) treatment of sperm protein, 40 g of WGA eluate containing 0.5% SDS and 50 mM -mercaptoethanol was boiled for 5 min. Nonidet P-40 was added to 7.5%, followed by 2.4 l of PNGase-F and 27.6 l of distilled water. The sample was incubated overnight at 37 °C. Following deglycosylation, the sample was separated on SDS-PAGE and transferred to PVDF. Western blots were then performed using S/C and IH antibodies.

Immunoprecipitation—2–10 g of affinity-purified IH antibody was incubated on ice overnight with 1 mg of Zwittergent 3-10 solubilized sperm protein in a total volume of 0.5 ml. Twenty microliters of pre-washed Pansorbin (Calbiochem) was added, and the samples were rotated at 4 °C for 1 h. The Pansorbin was sedimented at 12,000 *g* for 1 min. Supernatants were then removed, and the Pansorbin was washed three times (5 min each) with solubilization buffer. The samples were resuspended in Laemmli sample buffer, separated on SDS-PAGE, and transferred to PVDF membranes. Membranes were probed with S/C and IH antibodies, followed by horseradish peroxidase-conjugated secondary antibody (Calbiochem). Membranes were detected with SuperSignal West Dura Extended Duration Substrate (Pierce).

Immunolocalization—Freshly spawned sperm were diluted 1:100 and incubated 10 min in FSW containing 3% paraformaldehyde and 0.1% glutaraldehyde. Fixed cells were washed three times for 10 min with phosphate-buffered saline, pH 7.4. Permeabilized sperm were incubated for 10 min in phosphate-buffered saline containing 0.2% Nonidet P-40 and were then washed three times. Nonspecific sites were blocked with 3% bovine serum albumin in phosphate-buffered saline for 30 min, and were then incubated for 1 h with primary antibody in a blocking solution. This was followed by three 10-min washes, a 1-h incubation in Alexafluor 546 goat anti-rabbit IgG (Molecular Probes), and three additional washes.

RESULTS

suREJ3 Is a Multidomain Protein—The deduced amino acid sequence of suREJ3 reveals a 2,681-amino acid multidomain protein (Fig. 1). Based on Met-1 as the start site and following the 27-residue signal sequence, the presumed mature protein begins at Gly^{28}. From the NH_2 to COOH terminus, suREJ3 contains the following domains: an 84-residue sea urchin egg lectin domain (SUEL, Ref. 26), a 117-residue CRD (27), a 55-residue PKD repeat (5, 28), a 37-residue G-protein-coupled receptor proteolytic site (GPS, Ref. 29), TMS 1, a 124-polycystin/lipoxygenase/alpha-toxin domain (PLAT, Refs. 29 and 30), TMS 2–11, and a 44-residue putative cytoplasmic segment. The REJ module found in suREJ1 and polycystin-1 family members extends from Arg^{539} to Lys^{1476} (Fig. 1, *vertical lines*; 2). The extracellular portion of the 1,485 residues preceding TMS 1 contains 23 potential *N*-linked glycosylation sites, and the region between TMS 1 and 11 contains eight such sites (*asterisks*). suREJ3 contains several domains found in polycystin-1 and hPKDREJ, as well as domains unique to suREJ3 among the polycystin family members (Fig. 2).

The SUEL domain was originally described as a galactose-binding sea urchin egg lectin (26). SUEL domains are also found in fish eggs (31, 32), and latrophilins, members of the secretin family of G-protein-coupled receptors (GPCRs, Ref. 33). The SUEL domain of suREJ3 is 44–48% similar to the SUEL domain from the mammalian latrophilins. The suREJ3 CRD is a C-type lectin domain containing the six diagnostic Cys residues (27). Its specificity for carbohydrate binding is unknown. suREJ3 contains a region of 55 amino acids that have distant homology to 16 repeat sequences present in polycystin-1, called PKD repeats. A multiple sequence alignment with the 16 repeats from human polycystin-1 and suREJ3 shows 16–26% similarity; the human PKD repeats are 20–30% similar to each other. A random jumble analysis shows that the relationship is statistically significant. suREJ3, polycystin-1, and hPKDREJ all possess a REJ module of 1000 amino acids of unknown function. suREJ3 contains a PLAT domain between TMS 1 and 2, which is also present in polycystin-1 and hPKDREJ. The PLAT domain is homologous to the NH_2-terminal -barrel domain of lipoxygenases (34), enzymes that peroxidize lipids (35). Of the lipoxygenases, the suREJ3 domain is most closely related to the NH_2-terminal domain of mammalian 5-lipoxygenase (48% similar).

suREJ3 Is Cleaved at the GPS Domain—Prior to the first TMS is the GPS domain. This domain is present in several members of the secretin family of GPCRs, including latrophilins (33). Three members, latrophilin-1, hFlamingo-1, and CD97 are known to be proteolytically processed, with cleavage between the Leu and Thr residues of latrophilin-1 (Fig. 3, Refs.

Sea Urchin Sperm suREJ3

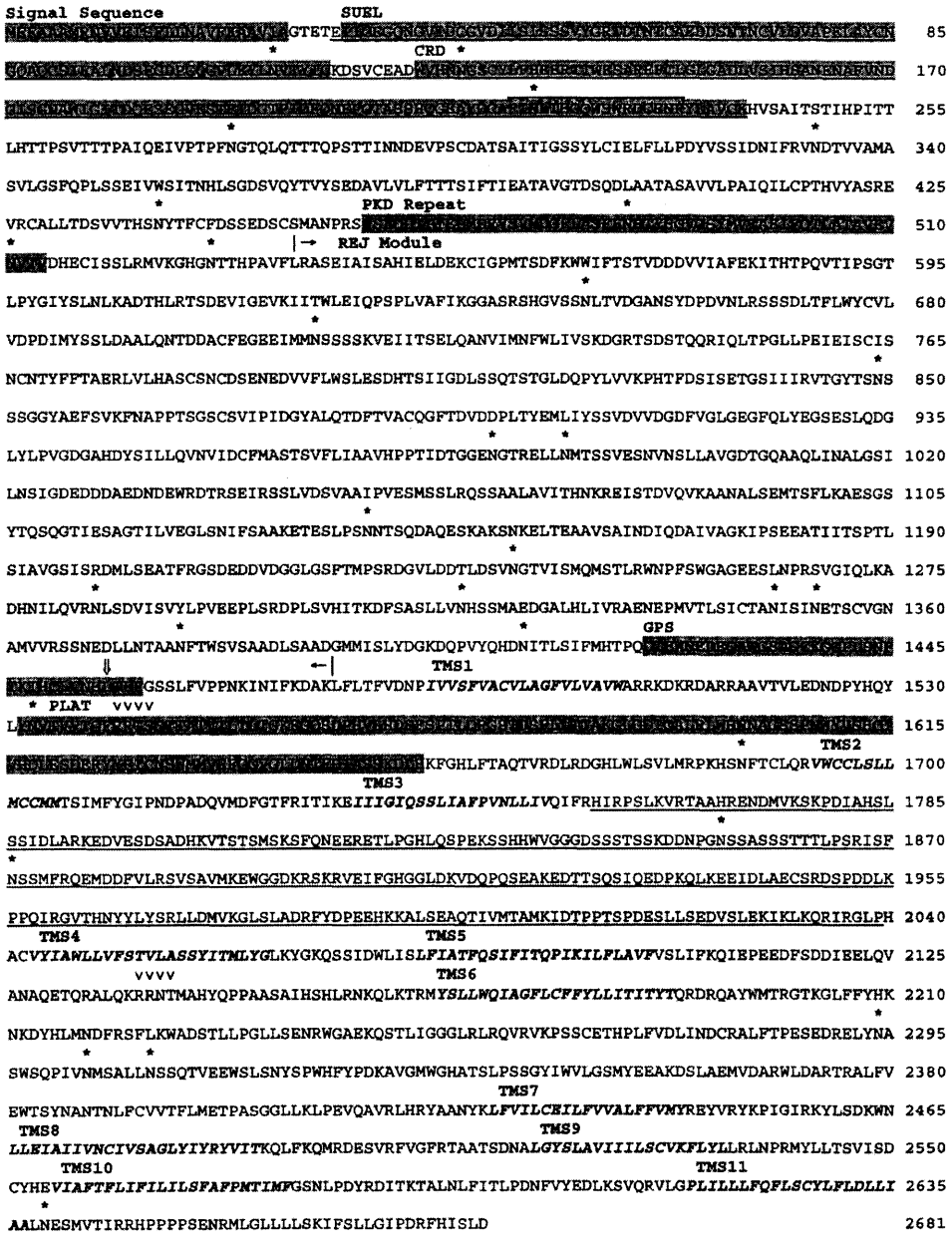


FIG. 1. **The 2,681-deduced amino acid sequence of suREJ3.** Domains are highlighted in *gray*, with the exception of the REJ module, which is denoted by *arrows* (34) above the sequence. Names of domains appear at the beginning of the sequence. The putative cleavage site within the GPS domain (Leu^{1455}) is labeled with an arrow (s). The *underlined* SUEL/CRD region denotes the bacterially expressed antigen for the S/C antibody. The *line* above 17 residues of the *CRD* shows the synthesized peptide antigen used for the R3p antibody. The *underline* between TMS 3 and 4 indicates the bacterially expressed antigen for the IH antibody. *Asterisks* (*) above the sequence indicate the Asn-linked glycosylation sites. *Arrows* (v v v v) above the sequence indicate two protein kinase A sites, both in the cytoplasmic loops. Putative transmembrane segments (TMS1–11) are *bold* in *italics* and labeled above the sequence. The GenBank™ accession number is AF422153.

36–39). If suREJ3 was cleaved at the GPS domain, the unglycosylated NH_2 portion of 1,455 amino acids would be 160 kDa and the COOH portion would be 140 kDa. Sperm membrane proteins were solubilized in Nonidet P-40, separated by SDS-PAGE, and transferred to PVDF membrane. Immunoblots were then performed. An antibody to the S/C in the NH_2-terminal region reacted with a single component at 250 kDa, whereas the IH antibody to the internal loop between TMS 3 and 4 reacted with a single band at 150 kDa (Fig. 4*A*). Digestion of the preparation with PNGase-F resulted in elec-

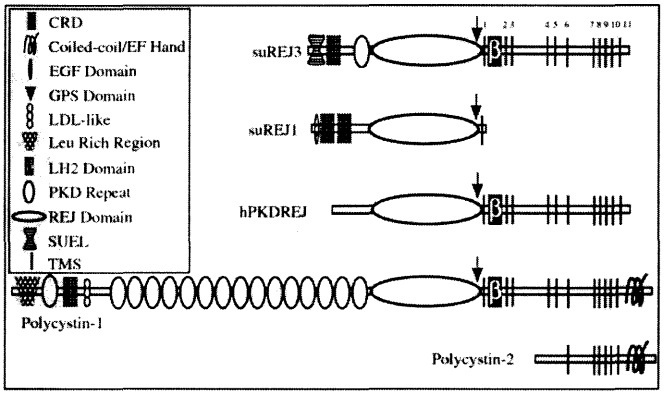


FIG. 2. **The domain structure of suREJ3, suREJ1, polycystin-1 and -2.** The key defines each domain. TMS are numbered above suREJ3. suREJ3, polycystin-1, and PKDREJ are homologous to polycystin-2 in the region with the last 6 TMS.

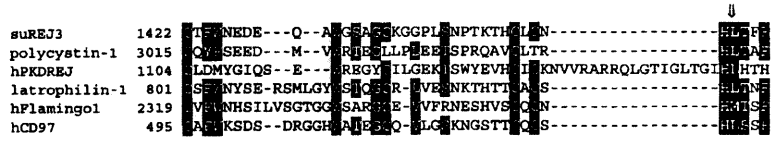


FIG. 3. **Alignments of GPS domains.** *Dashes* are included for alignment. *Black boxes* denote identity and *gray boxes* similarity. The *arrow* above the alignment indicates the GPS cleavage site. The GenBank™ accession numbers are as follows: suREJ3 (AF422153), human polycystin-1 (P98161), hPKDREJ (XP_010050), latrophilin-1 (T14324), human Flamingo-1 (AAG00080), and human CD97 (AAB36682).

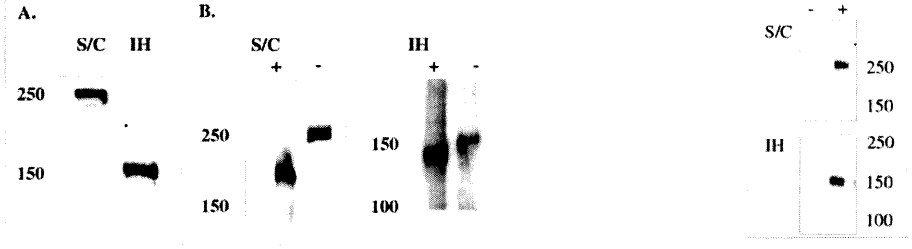


FIG. 4. **Immunoblots with suREJ3 antibodies.** *A*, immunoblot of Nonidet P-40 solubilized total sperm protein identifying the NH_2 terminus (*S/C*) and COOH terminus (*IH*) of suREJ3. The relative molecular masses are shown on the *right*. *B*, immunoblots with S/C and IH antibodies after PNGase-F digestion of WGA eluate of Nonidet P-40 solubilized sperm protein (, with enzyme; , no enzyme). Five micrograms of protein were loaded per lane.

FIG. 5. **Both halves of suREJ3 remain associated in detergent extracts of sperm heads.** IH antibody ( ) was used for immunoprecipitation with 2 mg of Zwittergent 3-10 solubilized protein from isolated sperm heads. The washed Pansorbin containing the attached antibody/antigen complex was boiled in Laemmli sample buffer. The supernatant was separated by SDS-PAGE, and transferred to PVDF membrane. The blots were probed with either the S/C or IH antibodies. Control immunoprecipitates with no IH antibody ( ) did not bind either half of suREJ3.

trophoretic mobility shifts of the S/C-reacting band to 180 kDa and the IH-reacting band to 140 kDa (Fig. 4*B*). The most likely explanation for these results is that, like the GPCRs, suREJ3 is cleaved into approximately equal halves at the GPS domain, the NH_2-terminal portion being heavily glycosylated. Association of the two halves of suREJ3 with each other was confirmed by immunoprecipitation of both proteins from Zwittergent 3-10 extracts of sperm using the IH antibody (Fig. 5). Like suREJ1, the NH_2 terminus of suREJ3 can be partially removed from the membrane by incubating sperm in pH 9.2 FSW (23), whereas the COOH terminus is exclusively associated with the membrane fraction (Fig. 7). This indicates that the interaction between the NH_2 and COOH termini is not because of disulfide bonds, but rather because the interaction is noncovalent.

suREJ3 Localizes to the Plasma Membrane Covering the Sperm Acrosomal Vesicle—Immunofluorescence on whole sperm using Fab fragments of rabbit anti-R3p and rabbit anti-IH IgG show that both halves of suREJ3 co-localize exclusively to the acrosomal vesicle region of sperm (Fig. 6). Reaction with IH antibody occurred only after treatment of the fixed sperm with 0.2% Nonidet P-40, supporting the putative intracellular location of the IH antigen. The exclusive localization to sperm heads was supported by Western blots showing that suREJ3 antigens are present in sperm heads, but not sperm flagella (Fig. 7).

DISCUSSION

The extracellular portion of suREJ3 contains three domains: SUEL, CRD, and the PKD repeat, all of which indicate a role in extracellular matrix interactions. The SUEL domain and CRD suggest a lectin-like interaction with carbohydrates, presumably from the multicomponent egg extracellular matrix known as egg jelly (3). We do not know the identity of possible ligands binding these three domains. The SUEL domain was first identified as an -D-galactose-specific lectin in eggs of the sea urchin *A. crassispina* (26). SUEL is a 105-amino acid protein that forms a homodimer that localizes to the egg cortex after fertilization. SUEL binding is calcium-independent and does not

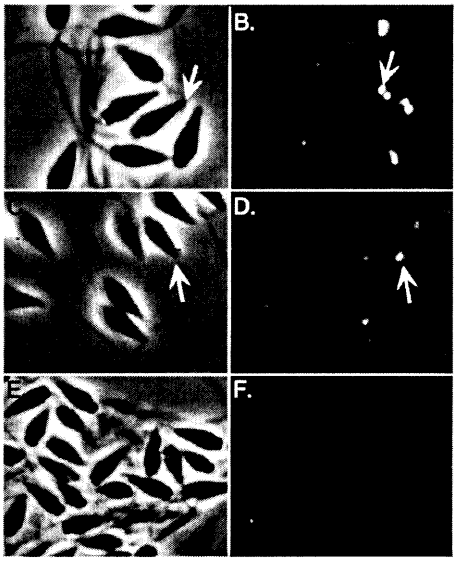


FIG. 6. **Immunolocalization of the NH_2- and COOH-terminal halves of suREJ3.** Phase contrast images are shown on the *left* (*A*, *C*, and *E*), and immunofluorescent images on the *right* (*B*, *D*, and *F*). *B*, anti-R3p Fab; *D*, anti-IH IgG; and *F*, no primary antibody. *White arrows* indicate the same sperm. Both halves of suREJ3 localize exclusively over the acrosomal vesicle. The suREJ3 antigen is present as a collar around the acrosome. In thumb-squashed preparations, it sometimes appears as two dots of fluorescence, one on each side of the acrosomal vesicle, as in *B*.

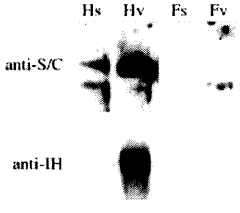


FIG. 7. **Immunoblots of isolated sperm heads and flagella membrane fractions.** Sperm heads and flagella were separated and resuspended in pH 9.2 FSW. The high pH causes the plasma membrane to bud off the cell component as tightly sealed vesicles (23). After removing the cellular debris by two 30-min centrifugations at 6,000 *g*, the resulting supernatant was centrifuged at 200,000 *g* for 1 h. The pellet-containing sperm membrane vesicles and the supernatant were separated on gels and transferred to PVDF membrane. Probing the blots with anti-S/C antibody showed that the NH_2-terminal half of suREJ3 was in both the head supernatant (*Hs*) and head vesicle fraction (*Hv*). The flagellar fraction did not react with this antibody (*Fs*, *Fv*). The IH antibody to the COOH-terminal half of suREJ3 reacted only with the sperm head vesicle preparation (*Hv*). Five micrograms of protein were loaded per lane.

require reduction. The mammalian orphan GPCRs, known as latrophilins, also contain SUEL domains. Latrophilin-1 binds -latrotoxin (black widow spider toxin) in a calcium-independent manner, resulting in massive exocytotic release of neurotransmitter (40). Comparison of rat and bovine latrophilins shows that the SUEL domain is the most conserved part of the protein, suggesting that it is important in latrophilin signaling (41). Although G-protein subunits have been found in sea urchin spermatozoa, there is no evidence that they are involved in triggering the AR (42).

The single CRD of suREJ3 has the diagnostic structure of a calcium-dependent lectin (27). suREJ1 has two NH_2-terminal-located CRDs, which are only 50% identical to each other over an alignment of 120 residues (21). The single CRD of suREJ3 is 52% identical to CRD-1 of suREJ1 and 47% identical to the suREJ1 CRD-2. The sequences of these CRDs do not disclose their sugar binding specificity. suREJ3 has only one PKD repeat, whereas human polycystin-1 has 16. PKD repeats are not found in Lov-1, the *Caenorhabditis elegans* homolog of polycystin-1 (13, 14). The most likely explanation is that duplication of PKD repeats occurred in the evolution of the vertebrates on the way to mammals. NMR studies show the PKD repeat has a -sandwich fold, similar to the immunoglobulin fold, but it is evolutionarily unrelated to IgG molecules (28). The PKD repeats from polycystin-1 mediate both cis- (on the same cell) and trans- (cell-to-cell) calcium-dependent, homotypic interactions (43). The aforementioned data suggest that the single PKD repeat of suREJ3 could act in homotypic clustering of the protein in the acrosomal region.

The REJ module was originally described as a domain of 707 amino acids found in suREJ1 and human polycystin-1 (2). The addition of other polycystin-1 homologs to the data base showed that the REJ module was 1,000 amino acids (7). This domain also occurs in the mammalian testis-specific PKDREJ. The GPS cleavage site is within this domain in suREJ3. The REJ module is not found in the secretin family of GPCRs, which have the GPS domain; therefore, possession of the REJ module is independent of possession of a GPS site. Because the GPS site is known to be extracellular, and PLAT is exclusively intracellular, the positions of these two domains support the topology presented herein for the TMS of suREJ3. Also, the IH antibody to the putative intracellular sequence between TMS 3 and 4 binds only to the acrosomal regions in sperm that have been detergent permeabilized.

Among the secretin family of GPCRs, latrophilins are unique in their relationship to suREJ3, because they contain both the SUEL and GPS domains. This report demonstrates that suREJ3 is cleaved and that the deglycosylated halves correspond to their predicted sizes based on cleavage at the GPS domain. Although we do not experimentally show the exact point of cleavage of suREJ3, based on work with other GPS-containing proteins, we believe that suREJ3 is cleaved at the consensus Leu-Thr site (Fig. 2). This is the first evidence that a polycystin-1-like protein is cleaved. The presence of this domain in suREJ1 may also explain the previous observation that 70% of suREJ1 can be removed from sperm by treatment with pH 9.2 seawater for 5 h (2). Whether mammalian polycystin-1 and hPKDREJ are also cleaved at the GPS domain remains to be demonstrated. The two halves of suREJ3, although associated in detergent extracts of sperm membranes (Fig. 5), are not as tenaciously associated as are the two halves of latrophilin-1. Unlike latrophilin-1, the two halves of suREJ3 dissociate in SDS-mercaptoethanol sample buffer without the addition of 8 M urea (39). Also, like suREJ1, the NH_2-terminal half of suREJ3 can be partially removed from sperm by treatment with pH 9.2 seawater, showing that it is a peripheral component of the cell membrane. The relationship between suREJ3 and latrophilins and the location of suREJ3 over the acrosomal vesicle suggests that suREJ3 may regulate AR exocytosis. -Latrotoxin does not induce the AR of sea urchin sperm (data not shown). This is not surprising, because black widow spider toxins show phylogenetic specificity (44). Also, recent work suggests that -latrotoxin does not activate latrophilin-1 to cause exocytosis, but instead functions by tethering to latrophilin-1 and inserting itself into the membrane to form pores (45).

In addition to the location of suREJ1 and suREJ3 on the cell membrane covering the acrosomal vesicle, the exocytosis reg-

ulatory proteins, soluble NSF attachment protein (SNAP)-25, vesicle-associated membrane protein (VAMP), and syntaxin are all exclusive to the acrosomal region of sea urchin sperm (46, 47). Antibodies to suREJ1 induce the exocytotic AR of sea urchin sperm (2). However, neither the S/C and R3p antibodies made to the extracellular NH_2-terminal half, nor the IH antibody made to the intracellular COOH-terminal half of suREJ3 induce the AR. suREJ1 is known to bind to the fucose sulfate polymer of egg jelly (2, 3). Two different isotypes of this polymer exist that differ in the placement of sulfate groups. Both are linear polymers of -L-1,3-linked fucose with a molecular mass of approximately one million (4). We do not know if the fucose sulfate polymer, or other ligands in egg jelly, bind to suREJ3. The AR always requires the fucose sulfate polymer; however, oligosaccharide chains of egg jelly glycoproteins greatly enhance the fucose sulfate polymer-induced AR, suggesting that other sperm receptor proteins are involved in the inductive mechanism (48).[2] Many scenarios are possible for the involvement of both suREJ1 and suREJ3 in the AR. They may represent redundant signaling pathways, or the two sea urchin sperm receptors could interact in the same pathway.

From the similarities of the proteins shown in Fig. 2, and the fact that polycystin-1 and -2 (11), polycystin-2L (10), and polycystin-2 from syncytiotrophoblast (12) form nonselective cation channels, it is valid to speculate that suREJ3 may form an ion channel or be a component of the regulatory apparatus of a channel. Both polycystin-2 and transient receptor potential channels (TRPCs) are excellent candidates for components of such a channel in the sperm AR. TRPCs were first described in *Drosophila* photoreception as store-operated cation channels (49). TRPC-1, a mammalian TRP homolog, associates with polycystin-2 through the COOH-terminal coiled-coil domain and also TMS regions (50). Mouse TRPC-2 has been shown to mediate the sustained increase in calcium associated with the sperm AR (51).

Unlike polycystin-1 and -2 (50, 52), sea urchin suREJ3, mammalian PKDREJ (15) and *C. elegans* Lov-1 (13) do not have a predicted coiled-coil domain in their COOH-terminal ends. Thus, these three proteins, which are all involved in male reproduction, might interact with proteins through other domains. Although clearly related to each other in their COOH-terminal halves, there is much less relationship among them in their NH_2-terminal halves. Until the discovery of the sea urchin REJ proteins, the proteins associated with gamete recognition in sea urchins have been unrelated to known mammalian proteins. The smaller genomic size of the sea urchin (800 megabases) and the characteristic of fewer duplicated genes than found in mammals may make the sea urchin a more tractable model for discovering the pathways of signal transduction leading to the deuterostome sperm AR. Further study of suREJ3 may help clarify the function of polycystin-1 and its role in the genesis of polycystic kidney disease in humans, as well as contribute to the understanding of the molecular mechanisms of sperm-egg interactions in both sea urchins and mammals.

Acknowledgment—We thank W. J. Swanson and J. R. Schulz for many important discussions.

REFERENCES

1. Darszon, A., Labarca, P., Nishigaki, T., and Espinosa, F. (1999) *Physiol. Rev.* **79,** 481–510
2. Moy, G. W., Mendoza, L. M., Schulz, J. R., Swanson, W. J., Glabe, C. G., and Vacquier, V. D. (1996) *J. Cell Biol.* **133,** 809–817
3. Vacquier, V. D., and Moy, G. W. (1997) *Dev. Biol.* **192,** 125–135
4. Alves, A. P., Mulloy, B., Moy, G. W., Vacquier, V. D., and Mourão, P. A. (1998) *Glycobiology* **8,** 939–946
5. Consortium, T. E. P. K. D. (1994) *Cell* **77,** 881–894
6. Mochizuki, T., Wu, G., Hayashi, S., Xenophontos, S. L., Veldhuisen, B., Saris, J. J., Reynolds, D. M., Cai, Y., Gabow, P. A., Pierides, A., Kimberling, W. J., Breuning, M. H., Deltas, C. C., Peters, D. J. M., and Somlo, S. (1996) *Science* **272,** 1339–1342
7. Sandford, R., Sgotto, B., Aparicio, S., Brenner, S., Vaudin, M., Wilson, R. K., Chissoe, S., Pepin, K., Bateman, A., Chothia, C., Hughes, J., and Harris, P. (1997) *Hum. Mol. Genet.* **6,** 1483–1489
8. Harris, P. C. (1999) *Hum. Mol. Genet.* **8,** 1861–1866
9. Calvet, J. P., and Grantham, J. J. (2001) *Semin. Nephrol.* **21,** 107–123
10. Chen, X.-Z., Vassilev, P. M., Basora, N., Peng, J.-B., Nomura, H., Segal, Y., Brown, E. M., Reeders, S. T., Hediger, M. A., and Zhou, J. (1999) *Nature* **401,** 383–386
11. Hanaoka, K., Qian, F., Boletta, A., Bhunia, A. K., Piontek, K., Tsiokas, L., Sukhatme, V. P., Guggino, W. B., and Germino, G. G. (2000) *Nature* **408,** 990–994
12. González-Perret, S., Kim, K., Ibarra, C., Damiano, A. E., Zotta, E., Batelli, M., Harris, P. C., Reisin, I. L., Arnaout, M. A., and Cantiello, H. F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1182–1187
13. Barr, M. M., and Sternberg, P. W. (1999) *Nature* **401,** 386–389
14. Barr, M. M., DeModena, J., Braun, D., Nguyen, C. Q., Hall, D. H., and Sternberg, P. W. (2001) *Curr. Biol.* **11,** 1341–1346
15. Hughes, J., Ward, C. J., Aspinwall, R., Butler, R., and Harris, P. C. (1999) *Hum. Mol. Genet.* **8,** 543–549
16. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
17. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Protein Eng.* **10,** 1–6
18. Higgins, D. G., Thompson, J. D., and Gibson, T. J. (1996) *Methods Enzymol.* **266,** 383–402
19. Nicholas, K. B., Nicholas, H. B., Jr., and Deerfield, D. W., II (1997) *EMBNEW News* **4,** 14
20. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132
21. Lee, C., Levin, A., and Branton, D. (1987) *Anal. Biochem.* **166,** 308–312
22. Gassmann, M., Thömmes, P., Weiser, T., and Hübscher, U. (1990) *FASEB J.* **4,** 2528–2532
23. Vacquier, V. D. (1986) *Methods Cell Biol.* **27,** 15–40
24. Laemmli, U. K. (1970) *Nature* **227,** 680–685
25. Morrissey, J. H. (1981) *Anal. Biochem.* **117,** 307–310
26. Ozeki, Y., Matsui, T., Masami, S., and Titani, K. (1991) *Biochemistry* **30,** 2391–2394
27. Drickamer, K. (1988) *J. Biol. Chem.* **263,** 9557–9560
28. Bycroft, M., Bateman, A., Clarke, J., Hamill, S. J., Sandford, R., Thomas, R. L., and Chothia, C. (1999) *EMBO J.* **18,** 297–308
29. Ponting, C. P., Hofmann, K., and Bork, P. (1999) *Curr. Biol.* **9,** R585–588
30. Bateman, A., and Sandford, R. (1999) *Curr. Biol.* **9,** R588–590
31. Tateno, H., Saneyoshi, A., Ogawa, T., Muramoto, K., Kamiya, H., and Saneyoshi, M. (1998) *J. Biol. Chem.* **273,** 19190–19197
32. Hosono, M., Ishikawa, K., Mineki, R., Murayama, K., Numata, C., Ogawa, Y., Takayanagi, Y., and Nitta, K. (1999) *Biochim. Biophys. Acta* **1472,** 668–675
33. Bockaert, J., and Pin, J. P. (1999) *EMBO J.* **18,** 1723–1729
34. Gillmor, S. A., Villaseñor, A., Fletterick, R., Sigal, E., and Browner, M. F. (1997) *Nat. Struct. Biol.* **4,** 1003–1009
35. Kuhn, H., and Thiele, B. J. (1999) *FEBS Lett.* **449,** 7–11
36. Usui, T., Shima, Y., Shimada, Y., Hirano, S., Burgess, R. W., Schwarz, T. L., Takeichi, M., and Uemura, T. (1999) *Cell* **98,** 585–595
37. Gray, J. X., Haino, M., Roth, M. J., Maguire, J. E., Jensen, P. N., Yarme, A., Stetler-Stevenson, M. A., Siebenlist, U., and Kelly, K. (1996) *J. Immunol.* **157,** 5438–5447
38. Sugita, S., Ichtchenko, K., Khvotchev, M., and Südhof, T. C. (1998) *J. Biol. Chem.* **273,** 32715–32724
39. Krasnoperov, V. G., Bittner, M. A., Beavis, R., Kuang, Y., Salnikow, K. V., Chepurny, O. G., Little, A. R., Plotnikov, A. N., Wu, D., Holz, R. W., and Petrenko, A. G. (1997) *Neuron* **18,** 925–937
40. Davletov, B. A., Shamotienko, O. G., Lelianova, V. G., Grishin, E. V., and Ushkaryov, Y. A. (1996) *J. Biol. Chem.* **271,** 23239–23245
41. Matsushita, H., Lelianova, V. G., and Ushkaryov, Y. A. (1999) *FEBS Lett.* **443,** 348–352
42. Cuellar-Mata, P., Martinez-Cadena, G., Castellano, L. E., Aldana-Veloz, G., Novoa-Martinez, G., Vargas, I., Darszon, A., and Garcia-Soto, J. (1995) *Dev. Growth Differ.* **37,** 173–181
43. Ibraghimov-Beskrovnaya, O., Bukanov, N. O., Donohue, L. C., Dackowski, W. R., Klinger, K. W., and Landes, G. M. (2000) *Hum. Mol. Genet.* **9,** 1641–1649
44. Schiavo, G., Matteoli, M., and Montecucco, C. (2000) *Physiol. Rev.* **80,** 717–766
45. Hlubek, M. D., Stuenkel, E. L., Krasnoperov, V. G., Petrenko, A. G., and Holz, R. W. (2000) *Mol. Pharmacol.* **57,** 519–528
46. Schulz, J. R., Wessel, G. M., and Vacquier, V. D. (1997) *Dev. Biol.* **191,** 80–87
47. Schulz, J. R., Sasaki, J. D., and Vacquier, V. D. (1998) *J. Biol. Chem.* **273,** 24355–24359
48. Keller, S. H., and Vacquier, V. D. (1994) *Dev. Growth Differ.* **36,** 551–556
49. Montell, C. (1997) *Mol. Pharmacol.* **52,** 755–763
50. Tsiokas, L., Arnould, T., Zhu, C., Kim, E., Walz, G., and Sukhatme, V. P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3934–3939
51. Jungnickel, M. K., Marrero, H., Birnbaumer, L., Lémos, J. R., and Florman, H. M. (2001) *Nat. Cell. Biol.* **3,** 499–502
52. Qian, F., Germino, F. J., Yiqiang, C., Zhang, X., Somlo, S., and Germino, G. G. (1997) *Nat. Genet.* **16,** 179–183

[2] N. Hirohashi and V. D. Vacquier, unpublished data.

The text of Chapter III, in full, is a reprint of the material as it appears in Mengerink, K. J., Moy, G. W., and Vacquier, V. D. (2002) suREJ3: a polycystin-1 protein, is cleaved at the GPS domain and localizes to the acrosomal region of sea urchin sperm. *Journal of Biological Chemistry*, 277(2): 943-948. The dissertation author was the primary author and the co-authors listed in this publication directed and supervised the research, which forms the basis for this chapter.

CHAPTER IV

An ATP-Binding Cassette Transporter is a Major Glycoprotein of Sea Urchin Sperm Membranes

AN ATP-BINDING CASSETTE TRANSPORTER IS A MAJOR GLYCOPROTEIN OF SEA URCHIN SPERM MEMBRANES

Kathryn J. Mengerink and Victor D. Vacquier

Center for Marine Biotechnology and Biomedicine

Scripps Institution of Oceanography

University of California San Diego

La Jolla, California 92093-0202

Correspondence to VDV

Telephone: (858) 534-4803

Fax: (858) 534-7313

Email: vvacquier@ucsd.edu

Running Title: Sea urchin sperm ABC transporter

SUMMARY

Sperm are terminally differentiated cells that undergo several membrane altering events before fusion with eggs. One event, the sea urchin sperm acrosome reaction (AR) is blocked by the lectin wheat germ agglutinin (WGA). In an effort to identify proteins involved in the AR induction, peptide sequence was obtained from a 220 kDa WGA-binding protein. Degenerate PCR and library screening resulted in the full-length deduced amino acid sequence of an ATP-binding cassette transporter, suABCA. The protein of 1,764 residues has two transmembrane regions, two nucleotide-binding domains and is most closely related to the human ABC subfamily A member 3 transporter (ABCA3). Sequence analysis suggests a large extracellular loop between TMS 7 and 8, with five N-linked glycosylation sites. An antibody made to the loop region binds to non-permeabilized cells, supporting that this region is extracellular. suABCA is found in sperm membrane vesicles, it can be solubilized with nonionic detergents, and it shifts from 220 kDa to 200 kDa upon PNGase-F digestion. suABCA localizes to the entire surface of sperm in a punctate pattern, but is not detected in lipid rafts. Based on its relationship to subfamily A, suABCA is most likely involved in phospholipid or cholesterol transport. This is the first investigation of an ABC transporter in animal sperm.

INTRODUCTION

As spermatocytes mature, their shape is altered from round, undifferentiated spermatocytes to long, thin, highly compartmentalized, terminally differentiated cells. Most sperm consist of a head containing an acrosomal vesicle and a nucleus, a midpiece containing mitochondria, and a long flagellum. Upon release into seawater, sea urchin sperm activate motility and before fusing with eggs, undergo the exocytotic acrosome reaction (AR)[1]. In mammals, this process is further complicated by the need to undergo capacitation before the AR can be triggered. Capacitation is a poorly understood process involving cholesterol efflux, changes in intracellular ion concentrations and tyrosine phosphorylation (1). All of these processes involve changes in membrane structure and composition (2), and the molecules involved in these changes are only now being discovered.

One of the best-studied membrane-altering events in fertilization is the sea urchin sperm AR. When sperm contact a fucose sulfate polymer contained in the jelly layer surrounding the egg, multiple fusions occur between the acrosomal vesicle and the plasma membrane of sperm resulting in exocytosis of the vesicle (3). A newly exposed membrane, covering the actin containing acrosomal process then fuses with the egg plasma membrane (4). Wheat germ agglutinin (WGA) blocks the egg jelly-induced AR of sea urchin sperm (5). One WGA-binding protein, suREJ1, has been demonstrated to be involved in triggering the AR (6), and another, suREJ3, has been implicated in this process (7). This paper identifies another major WGA-binding

protein of sea urchin sperm as an ATP-binding cassette (ABC) transporter, most closely related to human ABCA3.

ABC transporters make up one of the largest families of transmembrane proteins and have been identified in every organism. These proteins use ATP to drive the transport of a wide variety of substances across the membrane including phospholipids, amino acids, peptides, toxins, metals and antibiotics. The functional unit contains two distinct transmembrane regions, each with five to eight transmembrane spanning segments (TMS), and two distinct nucleotide-binding domains (NBD). In bacteria, each domain is a separate polypeptide, whereas in eukaryotes a single polypeptide chain can contain a half transporter (one transmembrane region and one NBD) or a full transporter with two transmembrane regions and two NBDs (8, 9).

Forty-eight human ABC transporters are divided into seven distinct families based on sequence similarity. Human subfamily A contains 12 members, which can be further subdivided into two distinct groups (10). The first group in subfamily A has seven members, all of which are implicated in phospholipid and cholesterol transport. Here we identify a sea urchin ABC transporter belonging to the subfamily A. This is the first description of an ABC transporter in animal sperm.

EXPERIMENTAL PROCEDURES

Protein Preparation and Peptide Sequencing — All procedures were on ice or at 4°C. Sea urchins, *Strongylocentrotus purpuratus*, were spawned by injection with 0.5 M KCl and the undiluted sperm collected with a Pasteur pipette. Sperm were resuspended in 0.45 μm-filtered seawater (FSW), coelomocytes were removed by three (5 min) centrifugations at 200 x g, and sperm cells sedimented at 5,000 x g (15 min). Sperm membranes were solubilized by suspending sperm pellets in 0.15 M NaCl, 10 mM HEPES pH 7.4, and 1% NP-40. Solubilized protein was obtained from the supernatant after centrifugation at 100,000 x g for 1 h. This supernatant was applied to a WGA-agarose column (EY Laboratories). The column was washed with 50 column volumes of wash buffer (0.15 M NaCl, 10 mM HEPES, 0.1% NP-40, pH 7.4) and the protein eluted in wash buffer containing 100 mM N-acetyl-D-glucosamine. SDS-PAGE was performed (11) and the gel stained with Coomassie brilliant blue. The 220 kDa band was cut excised from the gel, destained and sent to the Stanford University PAN Facility for trypsin digestion and peptide sequencing.

DNA Sequencing and Sequence Analysis — Degenerate primers were designed according to peptide 1 and peptide 4 and used to amplify a message from cDNA from an entire sea urchin. Specific primers were made to this message and 3' RACE (Ambion) with testis mRNA was performed to obtain most of the 3' end of the sequence. This product was used to screen a testis Lambda ZAP II library (Stratagene). Overlapping clones were obtained and sequenced.

suABCA homologs were identified using BLAST (12). Specific domains and glycosylation sites were found using the ProfileScan website (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html) and the PredictProtein website (http://dodo.cpmc.columbia.edu/predictprotein/predictprotein.html). The signal sequence was predicted using the SignalP website (13). TMS were predicted using hydropathy plots that were generated by the method of Kyte and Doolittle with a window of 14 amino acids (14), as well as the TMHMM website (http://www.cbs.dtu.dk/services/TMHMM-2.0/html ; 15). Multiple sequence alignments were made using Clustal W, Clustal X, and GeneDoc (16 - 18). Neighbor joining trees with bootstrap values (1,000 replicas) were made using Clustal X and viewed using TreeView (19).

Antibody Production — A portion of suABCA DNA corresponding to Ala^{961}-Pro^{1158} was ligated into the pET15b vector (Novagen), which contains an NH_2-terminal His tag, and bacterially expressed according to a previously published protocol (7). The resulting purified recombinant protein, ABCe, was separated by SDS-PAGE and negatively stained with cupric chloride (20).The protein was excised, destained, and used to make commercially raised anti-ABCe rabbit antibodies (Strategic BioSolutions). The antibodies were subsequently affinity purified on an ABCe-conjugated Affigel-15 column (Bio-Rad).

Other Methods— Sperm membrane vesicles (SMVs) were made according to the pH 9 method (21). For protein-N-glycosidase-F (PNGase-F) treatment of sperm protein, 40 μg WGA eluate containing 0.5% SDS and 50 mM β-mercaptoethanol was

boiled 5 min. NP-40 was added to 7.5%, followed by 2.4 µl PNGase-F and 27.6 µl distilled water. The sample was incubated overnight at 37°C. Following deglycosylation, the sample was separated on SDS-PAGE, transferred to PVDF and western blots performed using ABCe antibody. Lipid rafts were made following a previously described protocol (22). Briefly, 200 µl dry sperm were solubilized with 1 ml of 1% Triton X-100 in solubilization buffer (SB;10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 5 mM EDTA), incubated on ice for 20 min and homogenized with 10 strokes of a Dounce homogenizer. The cell debris was removed by a 5 min, 1,300 x g centrifugation. The supernatant was mixed with equal volume of 85% (w/v) sucrose in SB. Two milliliters of the resulting supernatant were layered successively with 6 ml 30% sucrose in SB and 3.5 ml 5% sucrose in SB. The lipid rafts were obtained by ultracentrifugation at 200,000 x g at 4°C for 18 h, and 1 ml fractions collected. Protein was separated by SDS-PAGE, and gels were stained with silver (23), Coomassie brilliant blue, or transferred to PVDF for immunoblotting.

Immunofluorescence — Freshly spawned sperm were diluted 1:100 and incubated 10 min in FSW containing 3% paraformaldehyde and 0.1% glutaraldehyde. Fixed cells were washed three times for 10 min with PBS pH 7.4. For washing, cells were sedimented by a 5 min centrifugation at 1,000 x g and resuspended in PBS. Permeabilized sperm were incubated 10 min in PBS containing 0.2% NP-40 and then washed three times. Nonspecific sites were blocked with 3% bovine serum albumin in PBS for 30 min, and then incubated for 1 h with primary antibody in blocking solution. This was followed by three 10 min washes in PBS, a 1 h incubation in

Alexafluor 546 goat anti-rabbit IgG (Molecular Probes), and three additional washes before viewing by epifluorescence.

RESULTS

Peptide Sequencing and Sequence Analysis — Several high molecular weight proteins are enriched in WGA eluates of solubilized sperm protein when compared to the starting material, including suREJ1 and suREJ3 (6, 7). The 220 kDa band is enriched in the eluate and can also be detected in the starting material (Fig. 1). To purify this protein, a WGA-eluate was separated by SDS-PAGE and the gel stained with Coomassie brilliant blue. The 220 kDa protein was excised and trypsin digestion and peptide sequencing performed. Three peptide sequences were obtained (Fig. 2). Peptides 1 and 3 were used to design degenerate primers, and the primers used to amplify a partial sequence from cDNA. The initial PCR product was then used to obtain the full-length sequence by a combination of screening a testis cDNA library and 3' RACE with testis cDNA. Using BLAST to search GenBank identified this protein as be a new member of the ABC transporter superfamily, named suABCA.

suABCA is a 1,764 amino acid full ABC transporter, containing two NBDs and two transmembrane regions of 6 TMS each (Fig. 2). SignalP predicts a signal sequence from Met^{1} - Arg^{44}. All three peptides were found in the deduced suABCA amino acid sequence. The NBDs of suABCA contain the subfamily A signature sequences, including the Walker A motif, the #50 sequence, the "hot spot", the ATS/C region, the Walker B motif and the Switch #162 sequence (Fig 3A; (24,25). Within all of these

regions, the two homologous domains of suABCA vary by only three amino acids. Also, suABCA is conserved in the additional ABCA regions 100-130 amino acids downstream of the Walker B motif, varying by only two amino acids.

A Kyte-Doolittle plot (Fig. 3B) indicates that suABCA has 12 predicted TMS, with the first TMS located within the putative signal sequence. The TMS are also predicted by TMHMM (15). Between TMS 1 - 2 and TMS 7 - 8 are large, putative extracellular loops of 158 and 242 residues, respectively. The first loop contains three potential N-linked glycosylation sites, and five are predicted in the second loop (Fig 3D). Two of the peptide sequences are found in the second loop, and each includes an N-linked glycosylation site. In both cases, the asparagine could not be determined by Edman degradation, most likely due to possession of oligosaccharide chains.

The human ABC transporter subfamily A is divided into two groups based on phylogeny (10). Human ABCA1, 2, 3, 4, 7, and 12 fall into one group. Multiple sequence alignments of suABCA to this group show the sea urchin transporter to be most similar to member 3 (Fig. 3C). Also, suABCA is similar in size to human ABCA3 (1,764 compared to 1,704 amino acids) and, like human ABCA3, contains a shorter extracellular loop between TMS 1 - 2 in comparison to the other members of this group. In summary, suABCA has a large glycosylated extracellular loop in the first transmembrane region and a second larger glycosylated loop in the second transmembrane region. Six TMS are predicted in each transmembrane region; however TMS1 may be cleaved after the signal sequence (Fig. 2).

Immunoblots — Anti-ABCe antibody reacts with a band at the expected size of 220 kDa in an NP-40 extract of whole sperm (Fig. 4A). The antigen is enriched in sperm membrane vesicles and is not present in the supernatant of the vesicle preparation. A second band, appearing in the SMV sample is most likely a breakdown product. suABCA is highly enriched in the WGA eluate. Lanes N, V, and S contain 10 μg protein, while lane W contains 0.5 μg protein. The density of the band in lane W is similar to that of the SMV preparation, indicating that the WGA eluate provides approximately a 20-fold enrichment of the suABCA transporter. Based on amino acid sequence alone, suABCA should be ~195 kDa. Treatment of WGA-binding proteins with PNGase-F, followed by anti-ABCe immunoblotting demonstrates that suABCA shifts in relative mass from 220 kDa to 200 kDa (Fig. 4B), confirming N-linked glycosylation.

Localization of suABCA — Immunofluorescence using the anti-ABCe antibody localizes the antigen to the entire surface of sperm in a punctate pattern (Fig. 5A). This pattern and level of fluorescence was seen in both nonpermeabilized and permeabilized cells, and was not altered by time of fixation. Also, decreasing the concentration of antibody resulted in the same punctate pattern with lower levels of fluorescence. Because of the punctate pattern and possible role of suABCA in phospholipid or cholesterol transport, lipid rafts were isolated and tested for the presence of suABCA (Fig 5B). A sucrose cushion was floated on top of the detergent extracted supernatant and the sample ultracentrifuged. This resulted in a lipid-dense band in fraction 4, which has been characterized previously as the glycosphingolipid-

rich raft fraction (22). Fractions taken from top to bottom show that suABCA is mostly solubilized by detergent and is not detected in the lipid raft fraction.

DISCUSSION

WGA blocks the egg jelly induced AR of sea urchin sperm (5), and there are approximately 10 major WGA-binding proteins visible on a silver-stained gel. One or more of these proteins must play a role in AR induction. suREJ1 was the first WGA-binding protein shown to be a receptor for the egg jelly fucose sulfate polymer, a known AR (3, 6). Also, the NH_2- and COOH-terminal halves of suREJ3 bind WGA and are implicated in the AR, due to their location on the plasma membrane covering the acrosomal vesicle (7).

The 220 kDa (previously reported as 190 kDa) WGA-binding protein to be an ABC transporter belonging to the human subfamily A transporters. Twelve members of this subfamily have been identified from human, which are all full transporters with two transmembrane regions and two NBDs (25). Available data shows that the members of subfamily A are involved in cholesterol or phospholipid transport (10). Members of subfamily A that cluster with suABCA include ABCA 1, 2, 3, 4, 7 and 12, and, of these, suABCA is most similar to human ABCA3 (Fig. 3C). ABCA3 is found associated with the lamellar bodies of lung alveolar type II cells, and is thought to play a role in phospholipid transport during surfactant production (26, 27). The best-studied member of this subfamily is ABCA1, mutations in which cause the autosomal recessive disorder, Tangier disease. Patients with Tangier disease have virtually no

plasma high density lipoprotein (HDL), they accumulate cholesterol in macrophages, and they have a high incidence of atherosclerosis (28, 29). Accumulating evidence shows ABCA1 transports cholesterol and phospholipids to lipid-poor apolipoproteins, such as HDL (28,29). It is tempting to speculate that suABCA has a similar lipid-transporting function.

Mammalian sperm must lose cholesterol during capacitation in order to become competent to undergo the AR. In guinea pig sperm, cholesterol distribution is expanded over the acrosomal region during capacitation (2). While it is known that bovine serum albumin, cyclodextrins, and HDL are capable of acting as cholesterol acceptors (1), there has been no mechanism described for regulated cholesterol extrusion from sperm membranes. An ABC subfamily A transporter would be the ideal protein to perform such a task.

Anti-ABCe was made against the region between TMS 7 and 8. This region is thought to be a regulatory region in ABCA1 (25), and several models have been proposed describing the topology of this region in subfamily A members (24, 30). With ABCA1, data support a model for an intracellular location with the hydrophobic region (TMS 7) sticking into the membrane but not passing through (24). In ABCA4, evidence indicates that the region corresponding to TMS 7 passes through the membrane, so that the region between TMS 7 - 8 is extracellular. Like ABCA4, our data support an extracellular location of this region. Twenty kDa of the relative molecular mass of suABCA can be accounted for by N-linked glycosylation and five N-linked sites are predicted in this region. Two sites are present in the sequenced

peptides and asparagine residues could not be identified via Edman degradation. This indicates that they are glycosylated. Also the anti-ABCe antibody made against this putative extracellular region binds nonpermeabilized cells, and the level of binding does not change after permeabilization. Because this region may be a regulatory in some ABCA members (25), anti-ABCe was dialyzed into FSW and applied to live sperm. No differences could be detected in swimming behavior, cell agglutination or acrosome reactions (data not shown).

suABCA has a distinct punctate pattern of immunofluorescence. To test the possibility that suABCA was present in specific membrane microdomains, anti-ABCe immunoblots were performed on isolated lipid rafts. Rafts have previously been isolated from sea urchin sperm, and shown to be enriched in glycosphingolipids but not cholesterol (22). Several sea urchin membrane proteins such as the speract receptor, suREJ1, adenylate cyclase, and guanylate cyclase have been detected in the raft fraction but suABCA is not (31). Likewise, human ABCA1 does not associate with sphingomyelin/cholesterol-rich lipid rafts (32).

Immunofluorescence of testis homogenates shows that the protein is present in a similar pattern on all immature spermatogenic cells (data not shown). A potential role for suABCA could be involved in the early stages of membrane structuring to create the terminally differentiated mature spermatozoa.

Although, many proteins and carbohydrates have been identified as important players in sperm-egg interactions (4), the membrane alterations during fertilization are poorly understood. One interesting question raised by this work is why suABCA is

such an abundant membrane protein present on the entire surface of the sperm. Sperm might alter their membranes during spermatogenesis, motility, the acrosome reaction and ultimately sperm-egg fusion.

This research has implications beyond sperm physiology. Membrane vesicles can be isolated from sperm as right-side-out vesicles by the pH 9 method (33) or as a mixture of right-side-out and inside-out vesicles by nitrogen cavitation (34). suABCA remains tightly associated with SMVs. SMV preparations can be combined with methods such as WGA chromatography or affinity chromatography to enrich for suABCA-containing lipid vesicles. The abundance of suABCA may offer an excellent opportunity to study the biochemical properties of these transporters in their native environment.

REFERENCES

1. Visconti, P. E., Westbrook, V. A., Chertihin, O., Demarco, I., Sleight, S., and Diekman, A. B. (2002) *J. Reprod. Immunol.* **53**, 133-150
2. Bearer, E. L., and Friend, D. S. (1990) *J. Electron Microsc. Tech.* **16**, 281-297
3. Vacquier, V. D., and Moy, G. W. (1997) *Dev. Biol.* **192**, 125-135
4. Mengerink, K. J., and Vacquier, V. D. (2001) *Glycobiology* **11**, 37R-43R
5. Podell, S. B., and Vacquier, V. D. (1984) *J. Cell Biol.* **99**, 1598-1604
6. Moy, G. W., Mendoza, L. M., Schulz, J. R., Swanson, W. J., Glabe, C. G., and Vacquier, V. D. (1996) *J. Cell Biol.* **133**, 809-817
7. Mengerink, K. J., Moy, G. W., and Vacquier, V. D. (2002) *J. Biol. Chem.* **277**, 943-948
8. Croop, J. M. (1998) *Methods Enzymol.* **292**, 101-116
9. Klein, I., Sarkadi, B., and Varadi, A. (1999) *Biochem. Biophys. Acta* **1461**, 237-262
10. Dean, M., Hamon, Y., and Chimini, G. (2001) *J. Lipid Res.* **42**, 1007-1017
11. Laemmli, U. K. (1970) *Nature* **227**, 680-685
12. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403-410
13. Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997) *Protein Eng.* **10**, 1-6
14. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105-132

15. Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E. L. L. (2001) *J. Mol. Biol.* **305**, 567-580

16. Higgins, D. G., Thompson, J. D., and Gibson, T. J. (1996) *Methods Enzymol.* **266**, 383-402

17. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25**, 4876-4882

18. Nicholas, K. B., Nicholas, H. B., Jr., and Deerfield, D. W., II. (1997) *EMBNEW News* **4**, 14

19. Page, R. D. (1996) *Comput. Appl. Biosci.* **12**, 357-358

20. Lee, C., Levin, A., and Branton, D. (1987) *Anal. Biochem.* **166**, 308-312

21. Vacquier, V. D. (1986) *Methods Cell Biol.* **27**, 15-40

22. Ohta, K., Sato, C., Matsuda, T., Toriyama, M., Lennarz, W. J., and Kitajima, K. (1999) *Biochem. Biophys. Res. Commun.* **258**, 616-623

23. Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307-310

24. Schmitz, G., and Langmann, T. (2001) *Curr. Opin. Lipidol.* **12**, 129-140

25. Broccardo, C., Luciani, M.-F., and Chimini, G. (1999) *Biochem. Biophys. Acta* **1461**, 395-404

26. Mulugeta, S., Gray, J. M., Notarfrancesco, K. L., Gonzales, L. W., Koval, M., Feinstein, S. I., Ballard, P. L., Fisher, A. B., and Shuman, H. (2002) *J. Biol. Chem.* **277**, 22147-22155

27. Yamano, G., Funahashi, H., Kawanami, O., Zhao, L. X., Ban, N., Uchida, Y., Morohoshi, T., Ogawa, J., Shioda, S., and Inagaki, N. (2001) *FEBS Lett.* **508**, 221-225

28. Lin, G. (2002) *Biochem. Biophys. Res. Commun.* **291**, 727-731

29. Oram, J. F., and Lawn, R. M. (2001) *J. Lipid Res.* **42**, 1173-1179

30. Bungert, S., Molday, L. L., and Molday, R. S. (2001) *J. Biol. Chem.* **276**, 23539-23546

31. Ohta, K., Sato, C., Matsuda, T., Toriyama, M., Vacquier, V. D., Lennarz, W. J., and Kitajima, K. (2000) *Glycoconj. J.* **17**, 205-214

32. Mendez, A. J., Lin, G., Wade, D. P., Lawn, R. M., and Oram, J. F. (2001) *J. Biol. Chem.* **276**, 3158-3166

33. Podell, S. B., Moy, G. W., and Vacquier, V. D. (1984) *Biochim. Biophys. Acta* **778**, 25-37

34. Ward, G. E., Moy, G. W., and Vacquier, V. D. (1986) *J. Cell Biol.* **103**, 95-101

35. Podell, S. B., and Vacquier, V. D. (1985) *J. Biol. Chem.* **260**, 2715-2718

FOOTNOTES

[1] The abbreviations used are: ABC, ATP-binding cassette; ABCA, ABC subfamily A; ABCe, bacterially expressed protein; AR, acrosome reaction; FSW, filtered seawater; HDL, high density lipoprotein; TMS, transmembrane segment; WGA, wheat germ agglutinin.

[2]GenBank Accession Number AF529424

ACKNOWLEDGEMENTS

This work was supported by NIH grant HD12986. We thank Yi-Hsien Su and Brian J. Hillier for providing testis RNA and whole sea urchin cDNA, and Anna T. Neill for reviewing the manuscript.

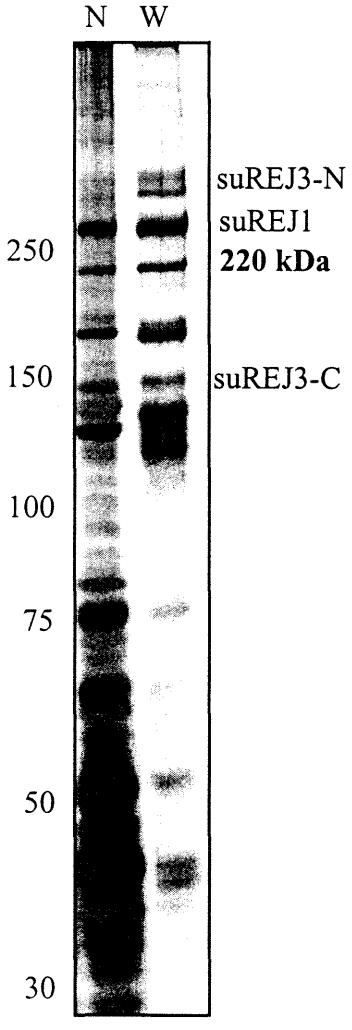


Fig. 1. The 220 kDa protein was purified by WGA chromotography. Silver stained gel of NP-40 solubilized sperm proteins (N) and WGA-binding proteins (W). Previously identified proteins are labeled to the right in regular text, and the 220 kDa of the current study is labeled in bold text. suRE3-N, NH2-terminal portion, and suREJ3-C, COOH-terminal portion of suREJ3 (7). The apparent molecular mass of these proteins vary, depending on the type of SDS-PAGE performed. Thus, the 220 kDa protein has been referred to as the 190 kDa protein, and suREJ1 as the 210 kDa protein in previously published articles (6, 31, 33, 35).

Signal Peptide/TMS1 * 85
MGRRLNQFGLLLWKNFILQFRRPIGTTFEILVPIACASLLLLARNLIKIDEKCFTTFDEEFVDTGHGFSLDDLSTIEGCIANGTS
* * 170
CPAIAFYPNNTVTFPIMELVSGISGFPLSTRNFSSASEMGAIVTEGDLEYYAAVEFDMDYDATEIPTVMKYTIRLPHDIASFGSW
TMS2 TMS3 255
FTELNIFPWLGLGPSRINNYKKRFILIQNIIDRVVIAAQAYVYTSNPAVFIAAQNMEYGMQQFPYPAYTDDRFIISIESLMPLLL
TMS4 * TMS5 340
FLSFIYGAGSITRELTFEKECRLKESMKMMGLANWMHWLAWFIKYFVYLLIPTILILVIVVVGNIFPNSSIVILLIYFVLWMVAT
TMS6 425
IAWSFLVSCFFSRARLGLIFGMVLWFLNYLPMFFLDFKSSSDATKTAVCLLSNTCMGEGVLVLARYELKGEGAQFSNIGESPSEG
510
STFSMGSVFGMLILDTVLYLLITWYVEGVYPGTYGIPKPFYFPFQPSYWCGYKPTKVDVNPDGDDVVEGPGQTQPAHANHEDEPT
* * NBD1 595
NLEAGITISNLTKVYKSSVGSKLAVDNLSVSMFKGQITALLGHNGAGKTTTMSILTGLYTPSSGKALVNGHSILDDMEGVRQSLG
680
LCPQHNVLFDRLTVKEHLKFFIRLKGKSGPDADAEINQMIEDLQLVDKTNTLSTKLSGGMKRKLSCAIALIGGSEIVILDEPTSG
765
MDPYARRATWDLLLKYKAGRTMVLTTHFMDEADLLGDRIAIMADGQLRASGSSLFLKNRFGIGYHLTLVQNEKVDMNSIQHMIKN
* peptide 1 850
HIPNASLESRVGSEIDYILPRESSSTFKDLFTQLENERGPLGIDSFGVSVTTMEEVFMKVGEMVDEEEANGGGMMRRRSSLIPPP
TMS7 935
RPAQADPVIYTNGEAGVKDPNVKISLFGINSGQSALVTGIFLKFLQFKAIFIKRFLCALRDKKSVITQFILPIVFVILGIVLLKT
* peptide 2 * 1020 *
SSDPTDDRARLLNLKNISEYAPSGSAKVFYADLTGSEHFEYLEALMESLTVNPVNITSDLMNAMDDNAGNLINGVSRTSNSECCN
* 1105
YTNMVLSEYCQEYLYNDGAGRTVCDDVTTFGYYNCPTCVADTDEDVTDCSVGANSSTVTTDTLYLSNYILGIADTQNFFYDNVAS
* peptide 3 TMS8 1190
YILSTTPGGNTKLTIGYSNQGLHIPAEALNGAANIMLKYFTNDSFAIETINYPLPRNAQSQEDAAVASTEVFYFALLLLFGLAFL
TMS9 TMS10 1275
TASFILFIVNEKQTKSKHLQFVSGLDTITYWLSNYCWDVINYLFIWIIIIILVAASSVDAYTGENLDSFVVVLLLFGLAAISFVY
* TMS11 1360
LFSLLFNSSVIAYALTAFALSLIGMGSLIAVFILEILEEESAAKYTDYIFNLLPTHALARSIMFIATNDAIRTSCESSKLAREQC
* * TMS12 1445
ANSNVSYALNNLDWKQPGIGLNCTYLALEAIIYLLLTIIIELGFGYSCCVSNYFKDSGLPQDPDVAEEKILVDNTDPHDSKYAVI
NBD2 1530
IKNLAKVYRGKRTPAVDKLSVTIPKGECFGLLGVNGAGKTTTFGMLTGDVRMSAGTAYMGGYDIQTQRRKVQQRIGYCPQFDALV
1615
ERLTGREVLMLFARLRGIPSNQMISVVDHTIDHLNLNKWQDKLCGTYSGGNKRKLSTAVALVGNPPIVLLDEPTSGMDPKARRYL
1700
WDSLTSIMEGGRSIILTSHSMEECEALCTRLAIMVNGQFKCLGSTQHLKSRFGTGYTLIIKTSTPALIAPTKAFVAEGFEGAVLL
* 1764
EQHQQLVHYQVDSGTTNWSFIFGLLEENKERLGIVDYSVSQTTLEQVFIXFAKDQHVDPRAKXE

Fig. 2. The deduced amino acid sequence of suABCA. The gray box denote domains or regions corresponding to peptide sequences, with names listed above. TMS are labeled above the sequence and are bolded and italicized. Asterisks indicate potential Asn-linked glycosylation sites. The region expressed to make the antibody, ABCe, is underlined.

A.

NBD		Walker A#1	#50	Hot Spot	ATS/C	Walker	Switch #162
1	con	GQXXXXLGHNGAGKTTT	GXCPQXN	LTVXEHXXFY	LSGGMXRK	LDEPTXGXDP	LXTHXMDEAXXLGDR
	su	.....	.....	.....F	.....	.....	.....
2	con	GECFGLLGVNGAGKSTT	GYCPQFD	LTGREXL	YSGGXKRK	LDEPTTGMDP	LTSHSMEECEALCXR
	su	.....T..	.....	.....	.....	....S.....	.....

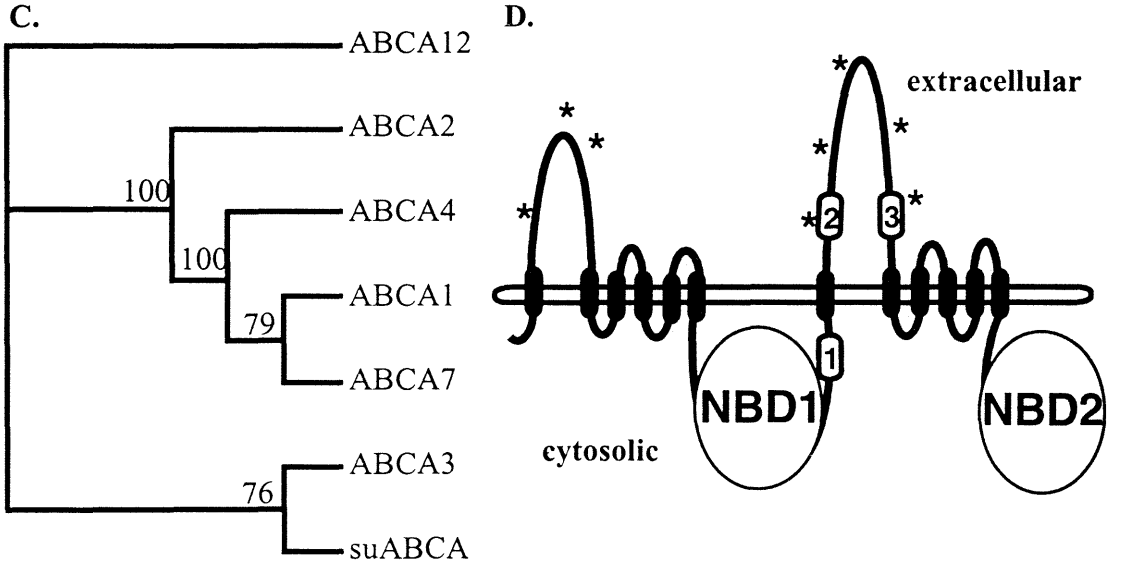


Fig. 3. The sea urchin ABC transporter belongs to human subfamily A.
A. Alignments of suABCA with the subfamily A-specific NBD sequence. Bolded residues are in all ABC transporters, Xs indicate any amino acid. The top line is the consensus sequence (con) and the second is suABCA (su). Dots denote identity. **B.** Kyte-Doolittle hydrophilicity plots. Predicted TMS are numbered beneath peaks. The black bars indicate two extracellular loops and NBDs are shaded gray. **C.** A neighbor joining tree of suABCA and human subfamily A members, based on multiple sequence alignments spanning the entire length of all proteins. Bootstrap values are at nodes. GenBank accession numbers are as follows: ABCA1, AAD49849; ABCA2, Q9BZC7; ABCA3, XP_028843; ABCA4, NP_000341; ABCA7, AAK00959; ABCA12, AAK54355; suABCA, AF5294242. **D.** The predicted membrane topology, the three peptide sequences (boxed), and potential N-linked sites (*).

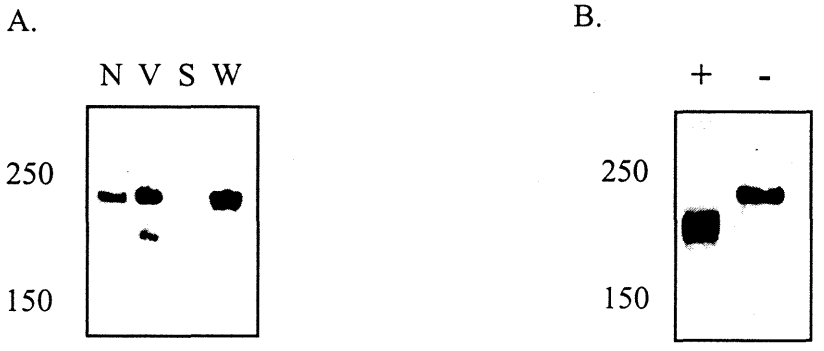
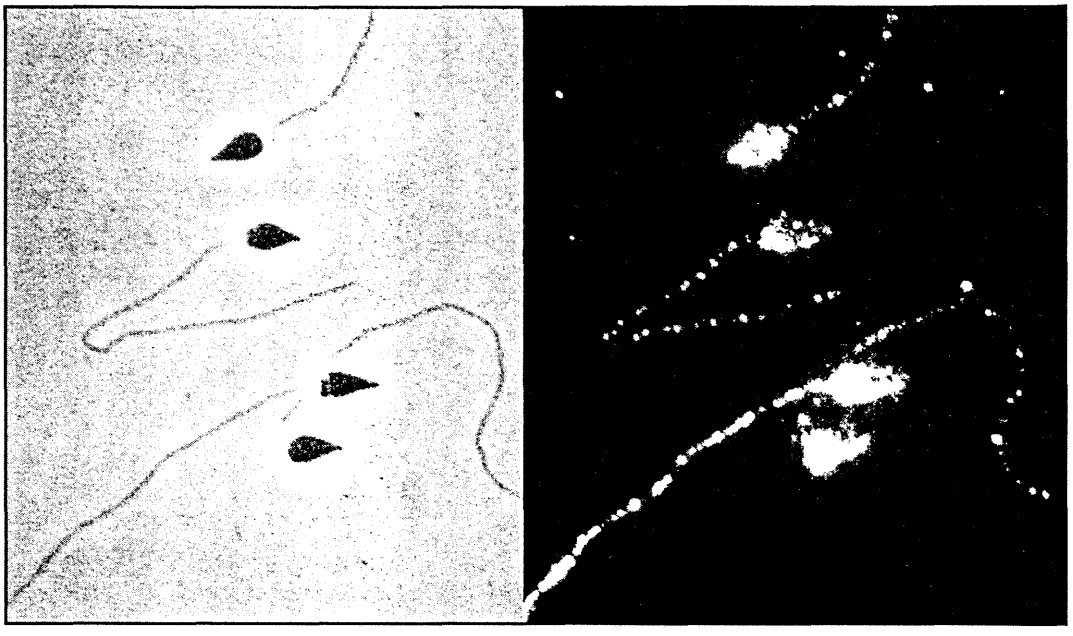


Fig. 4. Immunoblots using anti-ABC. A. T, NP-40 solubilized sperm protein; V., solubilized sperm membrane vesicles; S, supernatant of sperm membrane vesicles; W., wheat germ agglutinin eluate. T, V, W, 10 mg per lane. W, 0.5 mg per lane. **B.** PNGase-F-treated solubilized sperm protein (+) versus the untreated control (-).

A.



B.

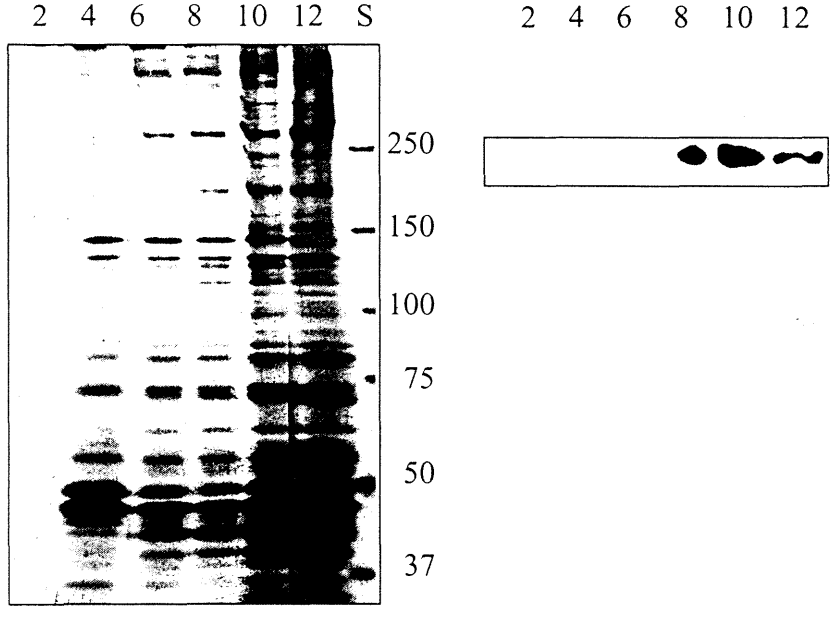


Fig. 5. suABCA is present in a punctate pattern all over sperm but is not detected in lipid rafts. A. Phase contrast image of sperm on left and the same sperm on the right showing immunofluorescence of anti-ABCe. B. Anti-ABCe immunoblot of fractions taken from the sucrose gradient of the lipid raft experiment. The first panel is a silver-stained gel and the second panel is the immunoblot. Fraction 4 contains the lipid rafts, fraction 6, 8 and 10 are part of the sucrose gradient, and fraction 12 is the solubilized sperm protein.

The text of Chapter IV, in full, is a manuscript submitted to *The Journal of Biological Chemistry* on July 18, 2002. The dissertation author was the primary author and the co-author listed in this publication directed and supervised the research, which forms the basis for this chapter.

CHAPTER V

Perspectives

INTRODUCTION

Wheat germ agglutinin (WGA) is a lectin capable of blocking the egg jelly induced acrosome reaction (AR). Previous work demonstrated the WGA-binding protein, suREJ1, was a receptor for egg jelly and inducer of the AR (1). The focus of this thesis has been to identify and characterize other sperm membrane proteins that bind to WGA in an effort to identify additional receptors involved in the sea urchin AR.

suREJ3

Chapter III identifies suREJ3 as a WGA-binding protein that is implicated in the AR. suREJ3 is proposed to be a component of an ion channel based on its relationship to polycystin-1. Most likely, it interacts with a homolog of polycystin-2. Gary W. Moy and Anna T. Neill have demonstrated that polycystin-2 is present in testis and localizes to the acrosomal region of mature sperm (unpublished data). The localization appears the same as suREJ3, though this needs to be confirmed. One way to do this would be to label suREJ3 and polycystin-2 antibodies with two distinct sizes of immunogold beads and and co-localize using transmission electron microscopy. Also, co-immunoprecipitation of the complex would be ideal but a much harder experiment. The COOH-terminal suREJ3 antibody, anti-IH, was able to co-immunoprecipitate the NH_2-terminus of suREJ3, using Zwittergent 3-10 to solubilize isolated sperm heads. This experiment was unsuccessful with the nonionic detergent, NP-40. Another gentle detergent such as CHAPS may be better suited for maintaining

a complex. To date, there are no known compounds that specifically block the polycystin channels. This limits the ability to investigate channel activity *in vivo*. If the native complex could be isolated, it could be reconstituted in lipid vesicles and tested for ion channel activity. Also, co-expression of suREJ3 and polycystin-2 in mammalian cells could be another way to examine ion channel activity.

suREJ3 has many distinct domains. Extracellularly, suREJ3 is composed of a sea urchin egg lectin domain (SUEL), a carbohydrate recognition domain (CRD), a PKD repeat domain, a REJ domain, and a GPS cleavage site. Within the transmembrane region of suREJ3, there is a lipoxygenase domain implicated in protein-protein interactions. Expressing the domains separately may be a way to determine function.

Both the SUEL domain and the CRD should interact with carbohydrates and are ideal regions for interaction with the egg jelly. The SUEL domain of suREJ3 was bacterially expressed using the pET15 vector (Novagen), containing an NH_2-terminal His-tag. Preincubation of the egg jelly with the purified SUEL inhibited the egg jelly's ability to induce the AR. This was also the case when purified fucose sulfate polymer was substituted for egg jelly. The same experiment using the purified expressed SUEL/CRD region did not have the same effect. This indicates that SUEL's egg jelly blocking function was lost when expressed in conjunction with the CRD. Further experiments should be undertaken to characterize this phenomenon. Also, the affinity for various carbohydrates should be tested using bacterially expressed SUEL, CRD, and the combined SUEL/CRD.

suREJ3 is post-translationally cleaved to form an extracellular NH2-terminus and a COOH-terminal transmembrane region. The cleavage most likely occurs at the GPS, but the exact site of cleavage is not known. One way to determine where the protein is cleaved would be to attempt to get NH_2-terminal sequence from the COOH-terminal end of suREJ3 (suREJ3-C). This could be difficult due to its large size (140 kDa). Another approach would be to make antibodies on flanking sides of the site to verify cleavage. Peptides of the COOH-terminal regions of suREJ1 and suREJ2 were synthesized in hopes of producing antibodies to the COOH-terminal regions. Unfortunately, the peptides were incredibly hydrophobic and we were unable to attach them to keyhole limpet hemocyanin for antibody production.

Between the first and second transmembrane domain is the PLAT domain. This domain is a beta barrel region and is thought to associate with other proteins. The PLAT domain was expressed as a GST-fusion using a pGEX vector (Novagen). This is an ideal system for doing pull-down assays. Excess amounts of purified PLAT-GST fusion protein could be added to a sperm extract in an attempt to isolate binding partners. Large amounts of the fusion protein were produced but the protein was insoluble in non-denaturing conditions. This was most likely due to the formation of inclusion bodies. Conditions need to be altered to enrich for soluble protein.

While expression studies can be very informative, there are many challenges associated with them. Drawbacks of bacterial expression include difficulties in obtaining soluble protein, lack of appropriate glycosylation, and protein misfolding.

Use of a eukaryotic expression system such as mammalian cell culture or insect cell culture may alleviate some of these issues.

suABCA

suABCA is the first ABC transporter identified from animal sperm and is found in great abundance. It is a full transporter containing two transmembrane regions and two nucleotide binding domains. Based on its relationship to the human subfamily A transporters, it is most likely involved in phospholipid or cholesterol transport. Efforts should be aimed at investigating its function.

One possibility is to isolate and reconstitute suABCA in lipid vesicles. Several such protocols have been described for bacterial and eukaryotic ABC transporters (2-5). The first step would be protein purification. WGA chromatography can be used as a first step to enrich for suABCA. One drawback to this method is that most ABC transporter purification protocols use reducing agents in the solubilization buffer, and reducing agents destroy WGA activity. Affigel-Blue (BioRad) is a matrix that binds many enzymes including some ATPases. Solubilized sperm protein was applied to an Affigel-Blue column, but suABCA did not attach. Another possibility is to use immunoaffinity chromatography. The suABCA antibody, anti-ABCe, can be attached to Affigel-10 or Protein-A resin and used to purify suABCA. Yet another option is ion exchange chromatography. Once the protein is isolated, it would be reconstituted in lipid vesicles, the vesicles loaded with ATP, and the transporter tested for ATPase activity under varying conditions. For example, endogenous activity could be

compared to activity when egg jelly is present. Substances that lead to cholesterol loss during capacitation, such as bovine serum albumin, high-density lipoprotein or cyclodextrins could also be tested. If they induce ATPase activity, they could be added to sea urchin sperm and the sperm tested for cholesterol loss.

Another possibility would be to express suABCA in a mammalian cell culture system, and measure loss of phospholipids and cholesterol from the cell when compared to control cells.

CONCLUSIONS

Identifying WGA-binding proteins from sea urchin sperm has allowed us to examine a subset of glycosylated surface proteins that may be involved in the AR. There are approximately ten WGA-binding proteins visible in silver stained gels. Investigating these proteins has resulted in the identification of two new proteins, suREJ3 and suABCA. Another putative REJ protein has been identified from WGA eluate of solubilized sperm, suREJX (see Appendix A), and peptide sequence has been obtained from a fourth (122 kDa protein). The genome of *Strongylocentrotus purpuratus* is slated to be sequenced in the next year. With this additional information, identifying suREJX, the 122 kDa protein and the remaining WGA-binding proteins' deduced amino acid sequences should be possible by peptide cleavage and mass spectroscopy. The remaining challenge will be to determine the functions of these molecules in an effort to increase our understanding of the molecular processes leading to the fusion of sperm with eggs.

REFERENCES

1. Moy, G. W., Mendoza, L. M., Schulz, J. R., Swanson, W. J., Glabe, C. G., and Vacquier, V. D. (1996) *J. Cell Biol.* **133**, 809-817

2. Ambudkar, S. V., Lelong, I. H., Zhang, J., and Cardarelli, C. (1998) *Methods Enzymol.* **292**, 492-504

3. Ames, G. F., Nikaido, K., Wang, I. X., Liu, P. Q., Liu, C. E., and Hu, C. (2001) *J. Bioenerg. Biomembr.* **33**, 79-92

4. Hall, J. A., Davidson, A. L., and Nikaido, H. (1998) *Methods Enzymol.* **292**, 20-29

5. Liu, C. E., and Ames, G. F. (1997) *J. Biol. Chem.* **272**, 859-866

APPENDIX A

Identification of Another suREJ Molecule in Sea Urchin Sperm, suREJX

SUMMARY

Wheat germ agglutinin (WGA) is a lectin that binds N-acetyl-D-glucosamine and sialic acid. When sea urchin sperm are preincubated in WGA, they are unable to undergo the egg jelly induced acrosome reaction (AR). The AR is a necessary prerequisite for sperm-egg fusion. WGA binds several high molecular weight sperm proteins, including suREJ1 and suREJ3. Both are members of the polycystin protein family and are implicated as receptors in the AR. This research identifies another suREJ molecule, suREJX, by peptide mapping. suREJX is localized to the entire surface of sperm. Like suREJ1 and suREJ3, it is partially released from the membrane when sperm are treated with pH 9.2 seawater and can be highly enriched by elution from a WGA column (although, not separated from other proteins).

INTRODUCTION

The acrosome reaction (AR) is an exocytotic event and a necessary precursor to fertilization in most animals (1). In sea urchins, the AR consists of the exocytosis of the acrosomal vesicle and the polymerization of actin to form the acrosomal process. A new membrane is exposed, which fuses with the plasma membrane of the egg. Many signal transduction events are involved in the AR including efflux of K^+ and H^+, influx of Ca^{2+} and Na^+, increase in adenylyl cyclase activity, increase in cAMP and inositol 1,4,5-trisphosphate concentrations, and an increase in protein kinase activity (2). The AR is induced when a sperm receptor for egg jelly 1 (suREJ1) contacts a fucose sulfate polymer in the jelly layer surrounding the egg (3).

suREJ1 is a single pass transmembrane receptor with several extracellular domains that are implicated in cell-cell, cell-extracellular matrix interactions (4). Two additional homologs have been identified from sea urchin testis, suREJ2 and suREJ3, and all are members of the larger polycystin family (5). suREJ3 has 11 putative transmembrane domains, and has been implicated in the AR (6). suREJ1 and suREJ3 bind to the lectin wheat germ agglutinin (WGA), and preincubation of sperm in WGA can block the AR in sea urchins (7). Several other high molecular weight sperm proteins bind WGA, and it is of interest to determine if they are also involved in the AR. In an effort to characterize the WGA-binding proteins, peptide maps were performed using in-gel protease digestion with Lys-C. This resulted in the identification of another potential suREJ homolog from sperm, named suREJX.

EXPERIMENTAL PROCEDURES

Peptide Maps

An 135 μg WGA eluate was incubated at room temperature for 10 min with 1 mCi I^{125} and one iodobead (Sigma). The sample was precipitated and the pellet washed with 90% acetone. The resulting pellet was resuspended in 675 μl Laemmli sample buffer (8) and separated on a 5% gel. An autoradiogram of the dried gel was used to cut out labeled bands. The gel slices were then proteolytically digested with Lys-C (Sigma, St. Louis, MO) within a second gel according to a previously described protocol (9). The digested peptides were separated by SDS-PAGE, the gel dried and the peptides detected by autoradiography.

Protein Preparation and Immunoblots

All procedures were on ice or at 4°C. Sea urchins were spawned by injection with 0.5 M KCl and the undiluted sperm collected with a Pasteur pipette. The sperm was resuspended in 0.45 μm-filtered seawater (FSW). Coelomocytes were removed by three (five min) centrifugations at 200 x g, and sperm cells sedimented at 5,000 x g. Sperm heads and flagella were separated according to a previously described protocol (6). Whole sperm, heads, and flagella were resuspended in membrane solubilization buffer (0.15 M NaCl, 10 mM HEPES, 1 mM PMSF, 2 μg/ml leupeptin, 1% NP-40,

pH 7.4). Sperm membrane vesicles were made according to the pH 9.2 method (10). Briefly, sperm were resuspended in 20 mM Tris pH 9.2, 10 mM benzamidine in FSW and incubated overnight at 4°C. Cell debris was removed by centrifuging at 5,000 x g, 20 min (twice). SMVs were collected by ultracentrifugation at 100 kg for 1 hour, and the resulting supernatant was retained as 100 kg soluble protein. For wheat germ agglutinin (WGA) chromatography, solubilized sperm protein was applied to a WGA-agarose column (EY Laboratories), the column was washed with wash buffer (0.15 M NaCl, 10 mM HEPES, 0.1% NP-40, pH 7.4) and the protein eluted in wash buffer containing 100 mM N-acetyl-D-glucosamine. SDS-PAGE was performed (8) and gels stained with silver (11) or Coomassie brilliant blue.

Following separation by SDS-PAGE some samples were transferred to PVDF for immunoblotting. J18/5 tissue culture supernatant was diluted 1:500 and used as the primary antibody, followed by horse radish peroxidase-conjugated secondary goat anti-mouse (Calbiochem), and detected with SuperSignal West Dura Extended Duration Substrate (Pierce).

Immunolocalization

Freshly spawned sperm were diluted 1:100 and incubated 10 min in FSW containing 3% paraformaldehyde and 0.1% glutaraldehyde. Fixed cells were washed three times for 10 min with PBS pH 7.4. Permeabilized sperm were incubated 10 min in PBS containing 0.2% NP-40 and then washed three times. Nonspecific sites were blocked with 3% bovine serum albumin in PBS for 30 min, and then incubated for 1 h

with primary antibody in blocking solution. This was followed by three 10 min washes, a 1 h incubation in Alexafluor 546 goat anti-mouse IgG (Molecular Probes), and three additional washes. A nonspecific monoclonal antibody, T8/40, was used as a negative control.

RESULTS

suREJX is a greater than 250 kDa sperm glycoprotein that binds WGA. The precise molecular mass has not been determined, due to the lack of protein standards larger than 250 kDa. Peptide maps demonstrate that proteins suREJX , suREJ3-N (the extracellular N-terminus of suREJ3) and suREJ1 have similar maps, while the ABC transporter, the 170 kDa protein, and the 122 kDa protein have maps that are quite distinct (Fig 1). Some differences can be detected between suREJ1 and the other suREJ maps, suggesting suREJ1 is less similar. Concentrated WGA eluate was separated by SDS-PAGE and the gel Coomassie stained. The suREJX band was excised and sent to the Stanford PAN facility for trypsin digestion and peptide sequencing. Unfortunately, peptides were unable to be sequenced due to heavy glycosylation and low yield.

An antibody specific to suREJX, J18/5, was identified from a pool of monoclonal antibodies created by Jim Trimmer (12). Western blots show that suREJX is highly enriched in WGA eluates and is partially released from sperm during SMV

preps (Fig. 2). PNGase-F was used remove N-linked glycosylation sites from WGA-binding proteins in an effort to determine the molecular weight based on protein only. Unfortunately, J18/5 does not recognize the deglycosylated molecule (data not shown).

suREJX localizes to the entire surface of the sperm (Fig. 3). This is further supported by the presence of suREJX in heads and flagellar protein, as determined by immunoblots (data not shown).

DISCUSSION

Based on peptide maps, suREJX, is a new member of the suREJ family of proteins. Whle suREJ3-N and suREJX maps appear identical, all evidence indicates that the two are separate polypeptides. The suREJX antibody, J18/5, does not cross-react with suREJ3-N. Also suREJ3-N antibodies (anti-S/C and R3p) recognize glycosylated and deglycosylated suREJ3-N (6); however, they do not recognize either glycosylated or deglycosylated suREJX. This eliminates the possibility of differences based on glycosylation only. Also, suREJ3-N localizes to the acrosomal region, while suREJX is found all over sperm. Ideally, the deduced amino acid sequence should be obtained for suREJX. Attempts to purify enough suREJX for peptide sequencing have been unsuccessful. WGA chromatography can enrich for it, but other proteins are also enriched. Also, immunoaffinity chromatography has proven unsuccessful. The sea urchin genome is slated to be complete in the next year. This should make finding the

sequence feasible by peptide digestion followed by mass spectroscopy, though heavy glycosylation may interfere.

suREJX is partially released from sperm when treated with pH 9.2 FSW. This indicates that suREJX is a peripheral membrane protein. suREJ1 and suREJ3-N are also partially released from sperm with high pH treatment (4,6). A GPS cleavage site occurs before the first transmembrane domains in all suREJs, as well as polycystin-1 proteins (13). It is known that suREJ3 is cleaved at this site (6), and the release from sperm at high pH indicates suREJ1 and suREJX are also cleaved.

suREJX is found on the entire surface of sperm. This pattern is distinct from that of other suREJs. suREJ1 is found on the tip of the sperm surrounding the acrosomal vesicle and the entire length of the tail (14), while suREJ3 is found only on the region surrounding the acrosomal vesicle (6). The function of suREJX is unknown. Preliminary experiments indicate that J18/5 is causes Ca^{2+} influx into the cell in a manner similar to the ionophore ionomycin (R. Cardullo, unpublished data). It is not known if this antibody is specifically interacting with suREJX or nonspecifically disrupting the membrane to cause this response.

In sum, suREJX is a putative suREJ protein based on peptide mapping. It localizes on the entire sperm surface and is partially released from sperm by high pH treatment, indicating it to be a peripheral membrane protein. Based on preliminary results from Dr. Richard Cardullo, it is an excellent candidate for involvement in the sea urchin AR.

REFERENCES

1. Mengerink, K. J., and Vacquier, V. D. (2001) *Glycobiology* **11**, 37R-43R

2. Darszon, A., Beltran, C., Felix, R., Nishigaki, T., and Trevino, C. L. (2001) *Dev. Biol.* **240**, 1-14

3. Vacquier, V. D., and Moy, G. W. (1997) *Dev. Biol.* **192**, 125-135

4. Moy, G. W., Mendoza, L. M., Schulz, J. R., Swanson, W. J., Glabe, C. G., and Vacquier, V. D. (1996) *J. Cell Biol.* **133**, 809-817

5. Mengerink, K. J., Moy, G. W., and Vacquier, V. D. (2000) **8**, S28-S30.

6. Mengerink, K. J., Moy, G. W., and Vacquier, V. D. (2002) *J. Biol. Chem.* **277**, 943-948

7. Podell, S. B., and Vacquier, V. D. (1984) *J. Cell Biol.* **99**, 1598-1604

8. Laemmli, U. K. (1970) *Nature* **227**, 680-685

9. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102-1106

10. Vacquier, V. D. (1986) *Methods Cell Biol.* **27**, 15-40

11. Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307-310

12. Trimmer, J. S. (1987) in *Scripps Institution of Oceanography*, pp. 273, University of California, San Diego, La Jolla

13. Bockaert, J., and Pin, J. P. (1999) *EMBO J.* **18**, 1723-1729

14. Trimmer, J. S., Trowbridge, I. S., and Vacquier, V. D. (1985) *Cell* **40**, 697-703

A. Starting MateBialLys-C Peptide Maps

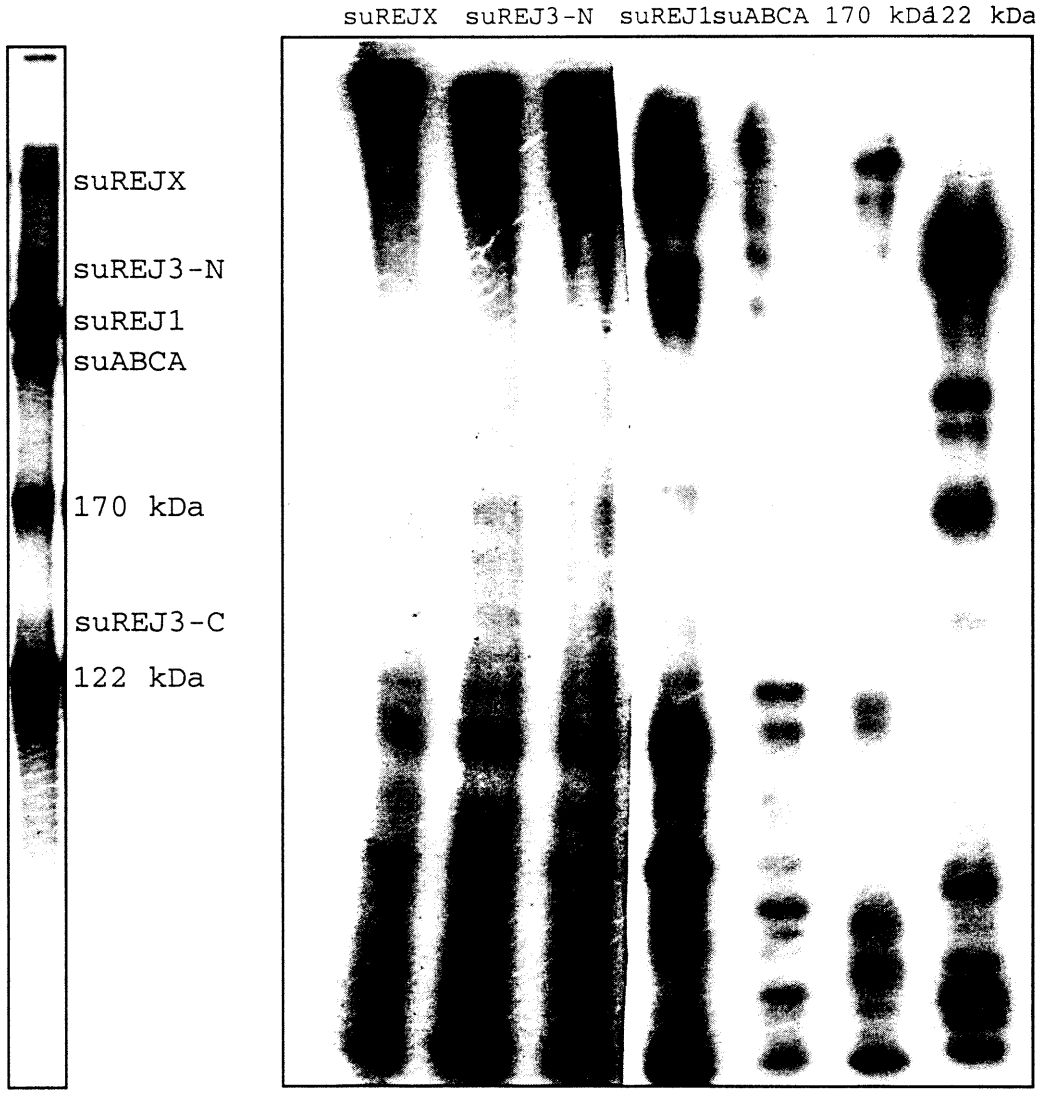


Figure 1. Peptide maps of WGA-binding sperm proteins. A. Autoradiogram of I-125 labelled WGA eluate, used for peptide maps. Proteins are labelled on right. Bolded proteins indicate mapped proteins. The band appearing at the top of the gel is the stacking/running gel interface. **B.** Autoradiogram of Lys-C in-gel peptide digests. suREJ3-N appears as a doublet in the starting material, and both bands were mapped.

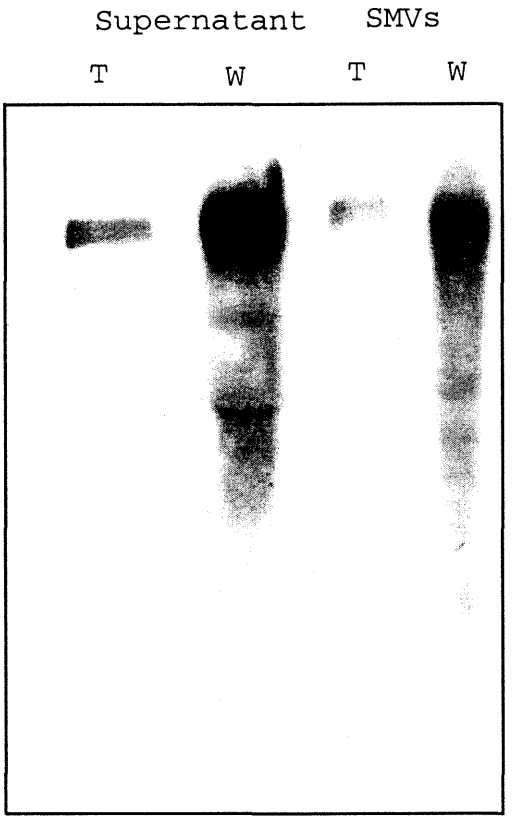


Figure 2. suREJX is partially released into seawater upon treatment of sperm with pH 9.2 medium. SMVs were prepared by incubating sperm overnight in 20 mM Tris pH 9.2 in FSW. The cell debris was removed by low speed centrifugation, followed by ultracentrifugation at 100 kg to sediment the SMVs. SMVs protein was solubilized by 1% NP-40. Aliquots of solubilized SMVs and supernatant (T) were saved. The rest of the samples used to isolate WGA-binding proteins (W) 0.5 ug protein was loaded per lane, SDS-PAGE performed, the gel transferred to PVDF and immunoblots using J18/5 at 1:500 dilution performed.

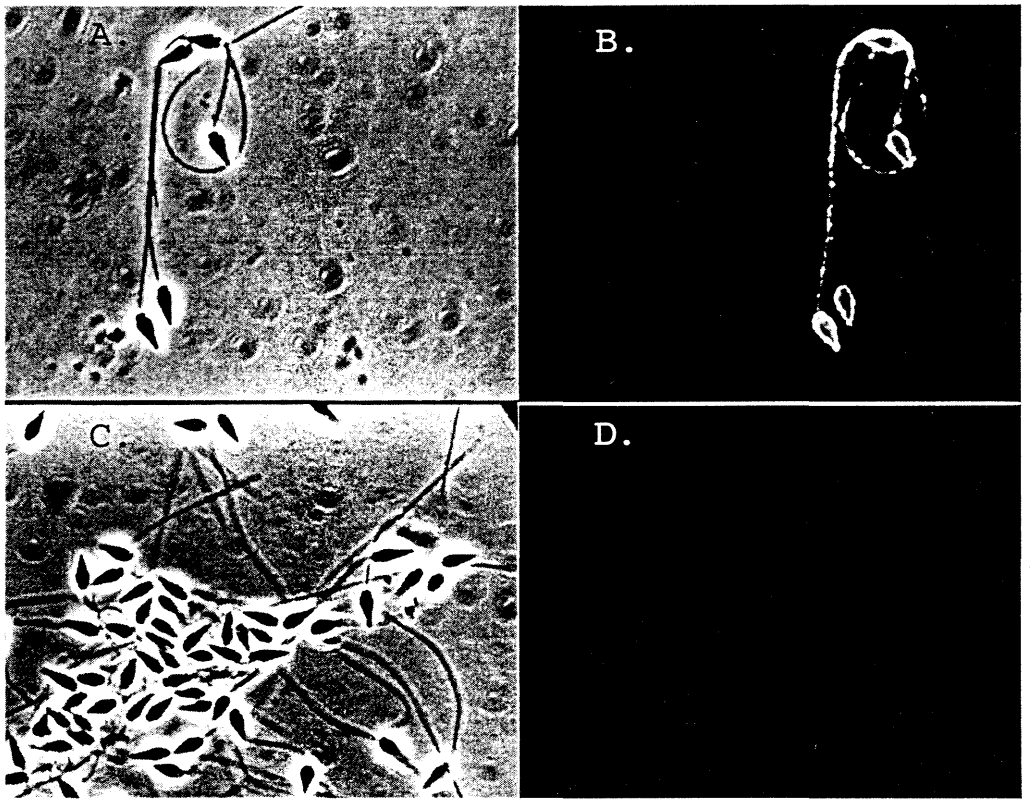


Figure 3. Immunofluorescence of sperm with J18/5. Panels A and C are phase-contrast images of the immunofluorescence in Panels B and D, respectively. A/B were incubated with J18/5 followed by goat anti-mouse secondary and C/D are negative controls using a nonspecific primary antibody, T8/40.

APPENDIX B

suREJs in Eggs and Embryos

INTRODUCTION

The polycystin family is best known for polycystin-1 and polycystin-2, proteins whose mutations result in human autosomal dominant polycystic kidney disease. While cystic kidneys are the most apparent aspects of this disease, many other organs and tissues can be affected including liver, pancreas and vascular system (1). In fact, most polycystin proteins are expressed in several to most tissues (2-4) and are developmentally regulated (5-7). Only one member, PKDREJ, is restricted in expression to the testis (8) and another member, PKD1L1, is found only in human testis and heart tissue (9). In an effort to understand this new class of proteins, it is of interest to know if the suREJ proteins are exclusive to the testis or if they function in other embryonic and/or adult processes. Polycystin-1 family members have been implicated in cell-cell and cell-extracellular matrix interactions, as well as being involved in cell differentiation (7).

There are many advantages to using sea urchin sperm to study the biochemical function of these molecules, including the ability to easily get large amounts of a single cell type. Also, unlike the polycystin proteins, the suREJ1 and suREJ3 are expressed at a fairly high level. One disadvantage, though, is that sperm are terminally differentiated cells that can only live for a relatively short period of time in seawater. A better system for studying cell-cell or cell-extracellular interactions may be sea urchin embryos. They are well-studied models that can be easily cultured to pluteus stages, and many techniques have been developed for this system. Thus, as a

preliminary investigation into the possibility of using sea urchin embryos, Northern blot analysis and PCR were used to identify suREJ transcripts in eggs and embryonic stages.

EXPERIMENTAL PROCEDURES

PCR

All primers were obtained from Gary Moy. The universal primers were primer p11 (forward) and primer H (reverse). Other primers used include, 6A-A (forward) and 6A-B (reverse), which amplify across the PKD repeat in suREJ2 and suREJ3: 5B (forward) and 5A (reverse), which are suREJ3 specific, and p30 (forward) and p41 (reverse), which are suREJ1 specific.

Northern Blot

Total RNA was isolated using a CsCl cushion and mRNA isolated using an Oligotex midiprep kit (Qiagen). RNA was separated on a 1.2% agarose gel in MOPS/formaldehyde buffer without ethidium bromide and transferred to Hybond-N. For the egg northern blot, a 540 bp piece of suREJ2's REJ domain (Ala^{1075} - Thr^{1241}) was used to make a random primed probe (Ambion kit). For the embryo northern blot, the probe was made to the suREJ2 CRD region.

RESULTS AND DISCUSSION

Partial suREJ2 transcripts were amplified from an ovary library, egg library, egg cDNA, and gastrula cDNA using both suREJ2-specific primers and universal suREJ primers (Table 1). The universal REJ primers are exact matches to all three known suREJ sequences and should amplify all three suREJ transcripts. However, only suREJ2 was amplified. When these primers are used with testis cDNA as the template, suREJ1 is amplified. This may mean that suREJ2 is the most abundantly expressed suREJ in sea urchin embryos.

Northern blot analysis shows that suREJ2 is expressed in both sea urchin eggs and embryos (Fig 1). The suREJ2 transcript is approximately 8.5 kb. This is similar in size to the suREJ1 transcript of 7.4 kb (10). suREJ2 seems to be most highly expressed in unfertilized eggs through early cleavage stage embryos. The message declines but is still present in gastrula and pluteus stage embryos. Interestingly, there are two transcripts of approximately the same size. The smaller band decreases as embryogenesis proceeds, and the larger band increases. This may be a splice variant or of another REJ transcript. A band of approximately 4.2 kb is expressed ubiquitously throughout the developmental time series. This may also be a splice variant or another homologue. It acts as a control for mRNA load on the blot, as well. Northern blots have not been performed with adult tissue. PKD1 expression is down regulated during differentiation and increases during cell proliferation in myeloid erythroleukemia K562 cells, suggesting a role for polycystin in cell proliferation (11).

It is possible that suREJ2 has a similar function, which is why it is most highly expressed during early cleavage and is down regulated during gastrulation.

PCR analysis using suREJ3-specific primers reveals that suREJ3 is expressed in both ovary and blastula cDNA libraries (Table 1). Partial suREJ1 sequence has also been amplified from the ovary library. An suREJ3-specific probe was used to probe the same blots used for the suREJ3 Northern, but no bands were visible after exposure for several days. These result conflict with the PCR data. The lack of signal could be due to low expression levels, or the PCR products could be from contaminating sources. suREJ1 has been successfully amplified only from egg cDNA. To date, suREJ1 probes have not been used to probe egg or embryo blots.

In conclusion, solid evidence is presented for the existence of suREJ2 expression in embryos, and based on PCR analysis, suREJ1 and suREJ3 are also present. Techniques to be tried include *in situs* to determine if expression is cell-type specific, the production of morpholinos to knock out expression and examine the phenotype, and microinjection experiments. Embryos may be an ideal system for studying this poorly understood protein family.

REFERENCES

1. Gabow, P. A. (1993) *N. Engl. J Med.* **329**, 332-342

2. Peters, D. J. M., van de Wal, A., Spruit, L., Saris, J. J., Breuning, M. H., Bruijn, J. A., and de Heer, E. (1999) *J. Pathol.* **188**, 439-446

3. Calvet, J. P. (1998) *J. Nephrol.* **11**, 24-34

4. Wu, G., Hayashi, T., Park, J.-H., Dixit, M., Reynolds, D. M., Li, L., Maeda, Y., Cai, Y., Coca-Prados, M., and Somlo, S. (1998) **54**, 564-568

5. Ward, C. J., Turley, H., Ong, A. C. M., Comley, M., Biddolph, S., Chetty, R., Ratcliffe, P. J., Gatter, K., and Harris, P. C. (1996) *Proc. Natl. Acad. Sci., U.S.A.* **93**, 1524-1528

6. Markowitz, G. S., Cai, Y., Li, L., Wu, G., Ward, L. C., Somlo, S., and D'Agati, V. D. (1999) *Amer. J. Phys.* **277**, F17-F25

7. Bukanov, N. O., Husson, H., Dackowski, W. R., Lawrence, B. D., Clow, P. A., Roberts, B. L., Klinger, K. W., and Ibraghimov-Beskrovnaya, O. (2002) *Hum. Mol. Genet.* **11**, 923-936

8. Hughes, J., Ward, C. J., Aspinwall, R., Butler, R., and Harris, P. C. (1999) *Hum. Mol. Genet.* **8**, 543-549

9. Yuasa, T., Venugopal, B., Weremowicz, S., Morton, C. C., Guo, L., and Zhou, J. (2002) **79**, 376-386.

10. Moy, G. W., Mendoza, L. M., Schulz, J. R., Swanson, W. J., Glabe, C. G., and Vacquier, V. D. (1996) *J. Cell Biol.* **133**, 809-817

11. Aguiari, G., Piva, R., Manzati, E., Mazzoni, E., Augello, G., Chiari, E., Moretti, S., Neri, L. M., and del Senno, L. (1998) *Exp. Cell Res.* **244**, 259-267

	Egg cDNA	Egg Library	Blastula Library	Gastrula Library
suREJ1 (specific)	X	-	-	-
suREJ2(specific/universal)	X	X	X	X
suREJ3 (specific)	-	X	X	-

Table 1. Identification of suREJ transcripts via PCR amplification. Exact match primers to either a single suREJ (specific) or all suREJs (universal) were used to amplify suREJ DNA from the above sources. The Xs indicate that the correct sequence was amplified from the template, and the dashed (-) indicate PCR reactions that failed to amplify products.

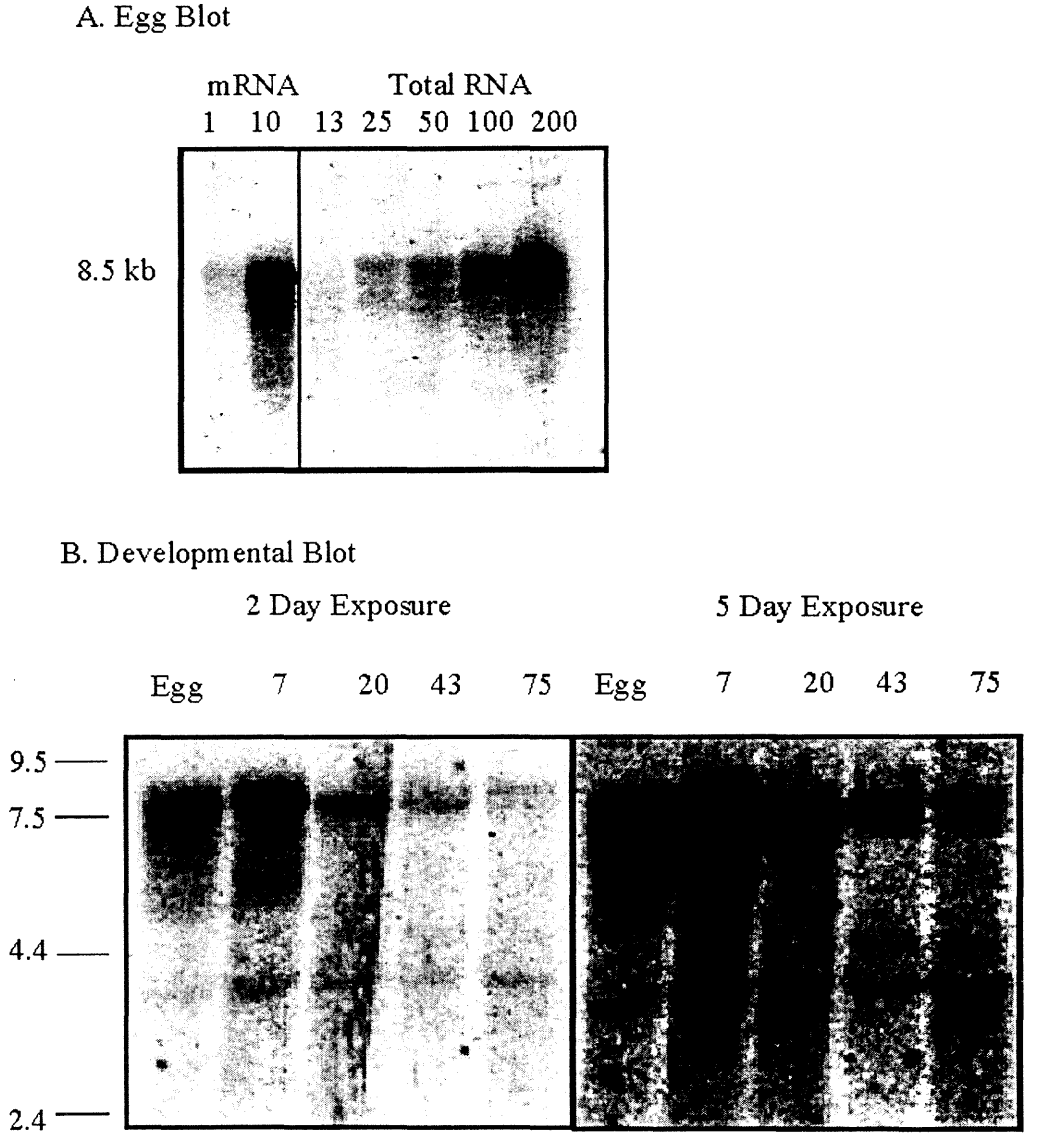


Figure 1 Northern Blots with REJ2 probe. A. Egg Blot. Total RNA was isolated from eggs and run on a gel (13 - 200 μg). mRNA was isolated from the total and run on the same gel (1 and 10 μg). An 8.5kb band was detected. **B.** Developmental Blot. mRNA was isolated from several stages of embryos (7 hour, 32 cell stage; 20 hour blastulae; 43 hour gastrulae; and 75 hour plutei). The RNA was run on a gel, transferred to a blot and probed. An egg sample was loaded as a positive control. Two different exposures are shown. The 8.5 kb band appears early in development and decreases through time. A band just above this increases from gastrulae to plutei. A band at 4.2 kb appears to be expressed at a constant rate.