Functional consequences of cleavage, dissociation and exocytotic release of ZP3R, a C4BP-related protein, from the mouse sperm acrosomal matrix

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Summary
The acrosome is an exocytotic vesicle located on the apical tip of the sperm head. In addition to having different morphological regions, two biochemically distinct compartments can be defined within the acrosome: a particulate acrosomal matrix and a soluble partition. The domains within the acrosome participate in the release of acrosomal proteins from the sperm during exocytosis, depending on whether the proteins partition into either the soluble or matrix compartments of the acrosome. We have examined the mechanism of differential release by evaluating the solubilization of acrosomal matrix protein ZP3R (sp56) from mouse sperm during the course of spontaneous acrosomal exocytosis. Using indirect immunofluorescence and immunoblotting, we found that the ZP3R monomer is processed from 67,000 $M_r$ to 43,000 $M_r$ by proteases coincident with release from the acrosome. Sperm require a maturational step, termed capacitation, before they are competent for acrosomal exocytosis and the processing of ZP3R is dramatically reduced under non-capacitating conditions. The cleavage probably takes place in complement control protein domain (CCP) 6 or the bridge region between CCP6 and CCP7, which is not present in the guinea pig orthologue AM67. The cleaved form of ZP3R does not bind to unfertilized eggs. We have incorporated these structural considerations into a model to explain the functional consequences of acrosomal exocytosis on sperm-zona interactions.

Key words: ZP3R (sp56), Acrosomal matrix, Mouse fertilization, Sperm

Introduction
The acrosome is a membrane-bound organelle located at the anterior tip of the sperm head. It is essential for fertilization in mammals; humans and mice whose sperm lack acrosomes are infertile (Dam et al., 2007; Schill, 1991; Sotomayor and Handel, 1986). Structurally and functionally, the acrosome consists of soluble and particulate compartments. Some of the proteins described as constituents of the soluble compartment are hyaluronidase, dipeptidyl peptidase, and CRISP2 (also known as autoantigen 1 or Tpx1) (Foster and Gerton, 1996; Hardy et al., 1991). The proteins of the particulate compartment or acrosomal matrix have been studied most extensively in the guinea pig and include proacrosin, AM50 (also known as p50, apexin, Nar, and neuronal pentraxin II), AM67 (the orthologue of mouse sperm protein ZP3R; previously known as sp56), and proacrosin-binding protein (Baba et al., 1994a; Honda et al., 2002; Noland et al., 1994).

The acrosome and its contents have been implicated in sperm-ZP interactions (Yanagimachi, 1994). During mammalian fertilization, sperm bind to and then penetrate the zona pellucida (ZP), the extracellular matrix that surrounds the oocyte. It has been proposed that the initial interaction with the ZP macromolecules triggers several molecular processes in sperm that lead to acrosomal exocytosis (AE). AE allows the sperm to penetrate the ZP in a process that also requires hyperactive sperm motility. Subsequently, the fertilizing sperm interacts and fuses with the egg plasma membrane to form the zygote.

Although there are only a few ZP sulfoglycoproteins involved in these binding events (ZP1, ZP2 and ZP3 in the mouse), several sperm molecules have been proposed as ZP-binding proteins or signaling receptors on mammalian sperm, based on their affinity for the ZP (Tanphaichitr et al., 2007). Among these molecules are β-1,4 galactosyltransferase, zonadhesin, SED1, spermadhesins, and ZP3R (Bleil and Wassarman, 1990; Ensslin and Shur, 2003; Hardy and Garbers, 1995; Miller et al., 1992; Sanz et al., 1992). Without precluding a role for any candidates in sperm-ZP interactions, the binding to the ZP appears to be a redundant process since the targeted deletion of SED1, β-1,4 galactosyltransferase, or proacrosin does not result in complete male infertility (Baba et al., 1994a; Ensslin and Shur, 2003; Lu and Shur, 1997). Rather, some degree of subfertility or deregulation of acrosomal exocytosis is seen.

The focus of this study, mouse sperm ZP-binding protein ZP3R is closely related to the alpha chain of complement 4-binding protein (C4BP) (Foster et al., 1997) and both proteins form high order oligomers. Each monomer of these proteins contains complement control protein domains (CCP). In the case of ZP3R, there are seven complete and one partial CCP (numbered consecutively as CCP1-CCP8); in the mouse C4BP alpha chain, there are six CCPs followed by a C-terminal extension. Ligand-binding regions of human C4BP
have been mapped to the various regions of the N-terminal domain and include Factor I, C3b, C4b, heparin and bacterial M proteins. The CCP domains of C4BP interact with proteases in the complement cascade (i.e. as a cofactor of Factor I in the proteolytic cleavage of C4b and in preventing the assembly of C3-convertase) (Blom et al., 2004).

ZP3R was initially reported to be on the surface of capacitated sperm (Bleil and Wassarman, 1990; Suzuki-Toyota et al., 1995). However, results from our laboratory demonstrated that ZP3R is a component of the acrosomal matrix since it could not be detected on the plasma membrane of non-capacitated sperm by indirect immunofluorescence and conventional immunoelectron microscopy (Foster et al., 1997); rather, it localizes to the particulate compartment of the acrosome (Kim et al., 2001a). One probable reason for the discrepancy in the localization of ZP3R in mouse sperm is the state of sperm capacitation; the protein cannot be detected on the sperm surface of uncapacitated sperm (Foster et al., 1997), but the protein appears to be on the surface of capacitated sperm (Bleil and Wassarman, 1990; Suzuki-Toyota et al., 1995). The association of ZP3R with the acrosome matrix may allow the protein to be revealed on the sperm surface after exposure to the external environment during the course of sperm capacitation. Although this has been described as a movement from the acrosome to the surface of the sperm (Wassarman, 2009), we believe it is more probable that intact ZP3R remains associated with the acrosomal matrix and is exposed to the external milieu through limited fusion of the outer acrosomal and plasma membranes in preparation for the more complete fusion of these membranes during the course of ZP-stimulated acrosomal exocytosis (Buffone et al., 2008a). As a consequence of AE, the acrosomal matrix gradually dissolves, releasing ZP3R into the surrounding environment. Following our finding that ZP3R is part of the acrosomal matrix, we have begun to reexamine the events of AE and the role of the acrosome in sperm-ZP interactions. We support the paradigm that AE occurs prior to or coincident with sperm-ZP binding, a process involving the acrosomal matrix (Kim and Gerton, 2003). At the same time, however, a mechanism must exist to enable the sperm to release from their point of attachment and move through the ZP without losing contact with the egg’s extracellular matrix.

Because ZP3R is of interest in sperm-ZP interactions, we investigated its properties during the progression of AE. In this study we found that, coincident with its release from the acrosome during the course of AE, the ZP3R monomer was cleaved to a 43,000 M<sub>r</sub> form by proteases. The process was dramatically reduced under non-capacitating conditions. The cleavage took place in complement control protein domain (CCP) 6 or the bridge region between CCP6 and CCP7, which is not present in the guinea pig orthologue AM67. The cleaved form of ZP3R did not bind to unfertilized eggs, suggesting that the function of this protein was modified during AE.

**Results**

ZP3R is processed coincident with acrosomal exocytosis and its release into the medium

Our laboratory previously demonstrated that there is a temporal relationship among several phases of acrosomal exocytosis, suggesting that these patterns represent different transitional stages leading to the eventual, complete release of acrosomal components (Kim and Gerton, 2003). Using FluoSphere beads coated with anti-CPT (an antibody directed against a peptide in CCP6), we showed that most of ZP3R is lost during the first 2 hours of spontaneous acrosomal exocytosis. In this study, we used immunofluorescence and immunoblotting of both anti-CPT and anti-VYK (an antibody directed against a peptide in the C-terminal CCP8) to expand these previous observations. We found that, after 120 minutes of incubation in capacitating conditions, ZP3R was barely detectable in sperm, consistent with our previous results (Figs 1 and 2). When the suspension of sperm undergoing spontaneous acrosomal exocytosis was centrifuged, the recovery of ZP3R in the supernatant correlated with its loss from the sperm heads (Fig. 2). In addition, we discovered that during the course of spontaneous acrosomal exocytosis, most of the 67,000 M<sub>r</sub> form of mouse ZP3R was converted to a 43,000 M<sub>r</sub> form (ZP3RAE) coincident with its release from the sperm (Fig. 2A). The band corresponding to ZP3R in intact sperm was barely observed in the supernatant.

The cleavage of ZP3R during spontaneous acrosomal exocytosis takes place in CCP6 or the bridge region between CCP6 and CCP7

We next set out to determine at what point in the polypeptide backbone the cleavage of ZP3R takes place. This modification was apparently due to a proteolytic cleavage between CCP6 and the CCP7.

**Fig. 1.** ZP3R is released to the supernatant during the course of spontaneous acrosomal exocytosis. Mouse sperm were incubated under capacitating conditions for up to 120 minutes. At the indicated times, sperm were immunostained with anti-ZP3R antibody (anti-CPT) and observed under the microscope to determine the presence of ZP3R in the acrosome. After 120 minutes of incubation most of the sperm lost the ZP3R protein. (A) Percentage of sperm with ZP3R staining (%). (B) Representative images showing sperm stained with anti-ZP3R antibody at 0 and 120 minutes of incubation in capacitating conditions (left and middle panels respectively) with their respective bright-field images. The right panels show a negative control (rabbit IgG). Results are expressed as the mean ± standard deviation (n=4).
Acrosomal exocytosis and ZP3R

C-terminal end of the ZP3R monomer, since anti-VYK (an anti-peptide antibody generated against the C-terminal region of the protein) no longer recognized the same band detected by anti-CPT, an antibody that binds to a more central region of the polypeptide backbone (Fig. 2B). Instead, the anti-VYK detected an ~24,000 \( M_r \) form in the supernatant resulting from the cleavage of the ZP3R monomer. Fig. 3A, is a diagram of the ZP3R monomer, illustrating the relative positions of the peptides used to generate anti-CPT and anti-VYK antibodies.

The guinea pig orthologue of mouse ZP3R, AM67, is not released as a cleaved form as a consequence of acrosomal exocytosis

Although guinea pig AM67 is most closely related to mouse ZP3R (Foster et al., 1997), it does not contain a region corresponding to the bridge between CCP6 and CCP7 of mouse ZP3R (Fig. 3B; residues 414-453). Since the guinea pig protein lacks the bridge region, we hypothesized that AM67 would not undergo proteolytic cleavage during the course of acrosomal exocytosis similar to mouse ZP3R. To test this hypothesis, we incubated guinea pig sperm under capacitating conditions for 1 hour, separated the sperm suspension into supernatant and pellet by centrifugation, and analyzed the fractions by immunoblotting with anti-VYK antibodies. As shown previously with the anti-CPT antibody, AM67 was released into the supernatant as a consequence of acrosomal exocytosis but not as a proteolytically processed form (Fig. 4).

Cleavage of ZP3R to ZP3R\(_{AE}\) requires capacitating conditions and acrosomal proteases

Sperm require a maturational step termed capacitation before they are competent for acrosomal exocytosis. Several studies have demonstrated that mouse sperm need calcium, glucose, bicarbonate and a cholesterol acceptor such as BSA or cyclodextrin in the medium for capacitation to occur in vitro (Visconti et al., 1995). We omitted the cholesterol acceptors from the medium to determine whether they were required for release and cleavage of ZP3R. The processing of ZP3R under non-capacitating conditions was greatly impeded (Fig. 5A) compared with parallel capacitating incubations with either BSA (Fig. 5B) or cyclodextrin (Fig. 5C), suggesting that the capacitation process is essential for the cleavage and release into the surrounding medium. We did not observe any difference between BSA and cyclodextrin in promoting the ZP3R cleavage and release of ZP3R\(_{AE}\), indicating that the cholesterol efflux is critical for creating conditions conducive to ZP3R processing.

Processing of ZP3R to ZP3R\(_{AE}\) alters the oligomer stability

We recently demonstrated that recombinant ZP3R monomers form a high order oligomeric protein containing six to eight disulfide-linked monomers (Buffone et al., 2008b). Native ZP3R was also found in a high order oligomer (Fig. 6A). After treating ZP3R with and a cholesterol acceptor such as BSA or cyclodextrin in the medium for capacitation to occur in vitro (Visconti et al., 1995). We omitted the cholesterol acceptors from the medium to determine whether they were required for release and cleavage of ZP3R. The processing of ZP3R under non-capacitating conditions was greatly impeded (Fig. 5A) compared with parallel capacitating incubations with either BSA (Fig. 5B) or cyclodextrin (Fig. 5C), suggesting that the capacitation process is essential for the cleavage and release into the surrounding medium. We did not observe any difference between BSA and cyclodextrin in promoting the ZP3R cleavage and release of ZP3R\(_{AE}\), indicating that the cholesterol efflux is critical for creating conditions conducive to ZP3R processing.

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increasing concentrations of dithiothreitol (DTT), the complex was broken down into a series of smaller oligomers, suggesting that the complete oligomer consisted of at least six monomers (Fig. 6A). By contrast, oligomeric ZP3R AE displayed a series of oligomeric bands in the absence of DTT (Fig. 6B). In addition, the ZP3R AE complex was dissociated into monomers using a fourfold more dilute concentration of DTT (250 μM, Fig. 6B) than is required for ZP3R (1 mM).

Benzamidine blocks the proteolysis of ZP3R but not its release
The inclusion of a protease inhibitor mixture in the medium decreased the processing of the ZP3R monomer as well as the release of ZP3R AE from the sperm relative to controls (Fig. 7A), suggesting that acrosomal protease activity may inactivate a tethering protein and cause the release of ZP3R AE from the acrosomal matrix (Fig. 7B). We suspected that this cleavage may be mediated by acrosin, and cleavage of ZP3R was indeed inhibited when a single competitive inhibitor of trypsin and trypsin-like proteases such as acrosin (1 mM benzamidine) was included in the incubation medium (Fig. 7C). Benzamidine did not prevent the release of the uncleaved ZP3R to the surrounding medium, indicating that other proteases are involved in the disassembly of the acrosomal matrix and that proteolysis of ZP3R is not a prerequisite to its release from the acrosomal matrix. Even after incubating the sperm in the presence of the protease inhibitor mixture for 120 minutes, the amount of ZP3R AE did not increase as occurs in the absence of the inhibitors (Fig. 7B). We confirmed this observation by immunofluorescence, showing that ZP3R remains associated with the sperm in the presence of the protease inhibitor mixture but is released to a greater extent when the only included protease inhibitor is benzamidine (Fig. 7D).

Proteolysis decreases the stability of ZP3R oligomers
To determine whether the processing of ZP3R to ZP3R AE has an impact on oligomerization, proteins extracted from sperm or recovered from the supernatant around sperm incubated for 2 hours under capacitating conditions were analyzed by immunoblotting under non-reducing conditions. As shown in Fig. 8A, protein extracted from sperm ran as a high order oligomer with a molecular weight much greater than 250,000. After incubation for 120 minutes under capacitating conditions, this protein was essentially depleted from the sperm (Fig. 8A, lane 2). As shown above (Fig. 7C), uncleaved ZP3R was released into the supernatant when 1 mM benzamidine was included in the incubation medium; however, the protein maintained a similar oligomerization state as the protein extracted from non-capacitated sperm (Fig. 8B, left lane). In the supernatant recovered from sperm incubated in the absence of benzamidine, a ladder of different sized oligomers was detectable when analyzed by non-reducing SDS-PAGE (Fig. 8B, right lane). The high order molecular weight complex released into the medium under capacitating conditions, contains both the N-terminal fragment (Fig. 8B, right lane) as well as the C-terminal fragment of the monomer (Fig. 8C) as detected by anti-CPT and anti-VYK antibodies, respectively. For reference purposes, the SDS-PAGE gel of the same samples run under reducing conditions is shown in Fig. 8D. We conclude that processing of ZP3R to ZP3R AE decreases the ability of the monomers to maintain the original oligomeric structure.

ZP3R AE does not bind to the ZP of unfertilized eggs
We hypothesized that cleavage of ZP3R and release of ZP3R AE from the sperm during the course of acrosomal exocytosis correlates

Fig. 6. Oligomerization of ZP3R before and after acrosomal exocytosis is dependent upon disulfide bonds. Mouse sperm were incubated under capacitating conditions and, at the indicated times, the incubation mixture was separated into a pellet (sperm) and supernatant (medium) and analyzed by 7% SDS-PAGE with amounts of DTT ranging from 0 to 5 mM in the sample buffer. (A) ZP3R from the sperm pellet before the incubation. (B) ZP3R AE recovered from the medium after the capacitating incubation. Each lane contained 1 μg of protein (n=3).

Fig. 5. ZP3R is processed only in conditions supporting capacitation. Mouse sperm were incubated for up to 240 minutes under non-capacitating condition (A) and capacitating conditions in the presence of 3 mg/ml BSA (B) or 5 mM 2-OH-propyl-β-cyclodextrin (C). At the indicated times, the incubation mixture was separated and the supernatant analyzed by 10% SDS-PAGE and immunoblotting with anti-ZP3R (anti-CPT). Each lane contains 1 μg of protein. When the sperm were incubated for 30 minutes in the presence of calcium ionophore (1 μM ionomycin a potent stimulator of the acrosomal exocytosis), the maximum amount of released ZP3R was observed (B, last lane).
with the loss of the ability of the protein to bind to ZP. To test this hypothesis, the biological activities of ZP3R and ZP3R$_{AE}$ were assessed by evaluating their respective abilities to bind to unfertilized eggs. Since the native proteins are difficult to purify from mouse sperm, we used an immunoadsorption procedure to isolate ZP3R and ZP3R$_{AE}$ from complex protein mixtures prepared from sperm. The starting material for uncleaved ZP3R was an acid extract of sperm, and as the source for ZP3R$_{AE}$ we used the supernatant from sperm. The starting material for uncleaved ZP3R was an acid extract of sperm, and as the source for ZP3R$_{AE}$ we used the supernatant from sperm. The starting material for uncleaved ZP3R was an acid extract of sperm, and as the source for ZP3R$_{AE}$ we used the supernatant from sperm.

**Discussion**

The status of the acrosome in fertilization and the role of the acrosomal matrix

Although the acrosome has long been recognized to be important for fertilization, the role of this exocytotic organelle in sperm-zona pellucida interactions is not completely understood. Based on studies by Saling et al. (Saling et al., 1979; Saling and Storey, 1979), many investigators believe that the outer acrosomal and plasma membrane must be intact for sperm to recognize and bind to the ZP. Following what has been referred to as primary binding, the Acrosome Reaction Model posits that the outer acrosomal membrane (OAM) and plasma membrane (PM) fuse, releasing the contents of the acrosome (Yanagimachi, 1994). It is then believed that the inner acrosomal membrane mediates secondary binding of the sperm to the ZP. Although the idea that sperm must be 'acrosome-intact' to bind to the ZP has become a predominant model for sperm-egg interactions, we believe that it is virtually impossible to identify the fertilizing sperm at the instant of sperm-zona interaction and to define the nature of its acrosome at that specific point. Furthermore, there are several reports in the literature of mammalian sperm that are capable of binding to the ZP and/or fertilizing an egg following acrosomal exocytosis (Huang et al., 1981; Kuzan et al., 1984; Morales et al., 1989).

Taking these points into consideration, our laboratory supports an alternative view that we call the Acrosomal Exocytosis Model (Buffone et al., 2008a; Gerton, 2002). In this model, sperm capacitation leads to a metastable state of plasma membrane and outer acrosomal membrane fusion whereby acrosomal matrix components may be transiently exposed to the external milieu. In this model, initial fusion of the OAM and PM may occur without the complete loss of acrosomal components because, while some proteins are relatively soluble, other materials are found within the particulate acrosomal matrix. In support of this concept, specific acrosomal components of guinea pig sperm undergoing acrosomal exocytosis in response to calcium ionophore A23187 are differentially released depending on whether they are components of the soluble or particulate compartments (Hardy et al., 1991; Kim
is proteolytically processed to AM50\textsubscript{AR} as a consequence of its release from guinea pig sperm, consistent with the results of other studies (Westbrook-Case et al., 1994). As we have shown here, the monomer that comprises the ZP3R multimer is cleaved to a lower molecular weight form upon release from mouse sperm undergoing acrosomal exocytosis. This proteolytic event may be mediated, in part, by acrosin which appears to function in the dispersion of the matrix during acrosomal exocytosis in the mouse (Yamagata et al., 1998).

Consequences of the structural homologies of ZP3R and C4BP

The proteolytic cleavage of ZP3R has profound implications for the stability of the mouse sperm acrosomal matrix. ZP3R is a member of the complement regulatory superfamily. Other than guinea pig AM67 and rat ZP3R, mouse ZP3R is most closely related to the alpha chain of complement 4-binding protein (Foster et al., 1997; He et al., 2003). Both of these proteins form high order oligomers (six to eight monomers of ZP3R per protein complex and seven alpha and one beta subunits in human C4BP) that contain both intra- and inter-chain disulfides (Kask et al., 2002). The monomers of each protein contain CCP domains. In the case of ZP3R, there are seven complete and one partial CCP; in the mouse C4BP alpha chain, there are six CCPs followed by a C-terminal extension and these are held together by non-covalent (presumably hydrophobic) interactions (Kaidoh et al., 1981; Kristensen et al., 1987). As shown for human C4BP, these regions contain two cysteine residues each and an amphipathic alpha-helix region, which is required to polymerize the molecule within the endoplasmic reticulum (Kask et al., 2002). Ligand-binding regions of human C4BP have been mapped to the various regions of the N-terminal domain (Blom et al., 2004).

Based on its oligomeric structure and the sequence homology of the ZP3R monomer to the alpha subunit of C4BP (Bookbinder et al., 1995; Foster et al., 1997), we propose that ZP3R forms a similar spider-like structure with the C-termini oriented toward the center of the complex (Fig. 10). Coupling this model with our results using two peptide-specific antibodies, anti-CPT and anti-VYK, which target CCP6 and CCP8, respectively, and our analysis of the products released by acrosomal exocytosis, we conclude that proteolysis of ZP3R occurs in the latter part of CCP6 or in the bridge region between CCP6 and CCP7 (Fig. 10) because anti-VYK no longer recognizes the same band detected by anti-CPT. A corresponding bridge region is not present in guinea pig AM67, the orthologue of mouse ZP3R, which is interesting since our analysis of the material released from guinea pig sperm during acrosomal exocytosis established that AM67 is released but is not cleaved (Kim et al., 2001b) (Fig. 4). Thus, cleavage of AM67 is not a prerequisite for its release from guinea pig sperm during the course of acrosomal exocytosis.

Capacitation, processing and release of ZP3R from sperm

As is shown in Fig. 7, the cleavage of mouse ZP3R was inhibited in the presence of benzamidine, a competitive inhibitor of trypsin and trypsin-like enzymes, such as acrosin. This inhibitor did not prevent the release of mouse ZP3R to the surrounding medium. Therefore, as was found previously for guinea pig AM67, proteolysis of mouse ZP3R is not a prerequisite for its release from sperm undergoing acrosomal exocytosis. However, when we used a broad-range protease inhibitor mixture, both the cleavage and release of mouse ZP3R were reduced, suggesting the active
participation of non-benzamidine-inhibited acrosomal proteases or processes in the processing of ZP3R. In addition, the processing of ZP3R in mouse sperm was reduced under conditions that do not support capacitation (Fig. 5). Consequently, we hypothesize that some of the events associated with capacitation, such as the rise in intracellular pH, lead to the activation of acrosomal proteases or processes involved in cleaving and/or releasing ZP3R and other matrix proteins.

Structural alterations may have functional consequences

Once attached, how might a sperm pass through the zona? ZP3R is a zona pellucida-binding protein (Bleil and Wassarman, 1990; Buffone et al., 2008b). Once a sperm attaches to the zona, there must be a mechanism that enables the sperm to move through the zona without losing its grip. The fact that the cleaved form of mouse ZP3R does not bind to unfertilized eggs (Fig. 9) leads us to the following model. We envision that a capacitated sperm encountering an egg binds to the zona via transiently exposed acrosomal matrix proteins, not a plasma membrane ZP-binding protein. Simultaneously, signaling molecules on the sperm surface interact with ligands in the ZP, stimulating signal transduction and leading to an accelerated rate of OAM and PM fusion (ZP-stimulated acrosomal exocytosis) compared with spontaneous acrosomal exocytosis. The newly exposed, particulate acrosomal matrix components assist the penetration of the spermatozoon through the ZP by the restricted disassembly of this egg vestment, either enzymatically or stoichiometrically. At the periphery of the exposed acrosomal matrix, ZP3R is processed and loses its ability to bind to the ZP. The tethering of the sperm to the zona is transiently lost until fresh, underlying (but intact) ZP3R oligomers are exposed on the surface of the sperm and come into contact with the zona. Release of the peripheral matrix proteins might be mediated by proteolysis. Processing of acrosomal matrix proteins could result from proteolytic cleavage by acrosin or other trypsin-like proteases. As a consequence of the cyclical binding, proteolysis coupled with loss of binding followed by renewed binding mediated by freshly exposed ZP3R, the sperm, propelled by its flagellum, would be able to ratchet its way through the zona.

Materials and Methods

Preparation of culture media and spermatozoa

The solution used for the preparation and culture of mouse sperm was a modified Krebs-Ringer bicarbonate medium (HMB-Hepes buffered), as described by Lee and Storey (Lee and Storey, 1986). This medium was first prepared in the absence of calcium, bovine serum albumin (BSA), NaHCO3, and pyruvate, sterilized by filtration (0.2 µm tissue culture filter unit; Naigene) and frozen at −20°C in aliquots for single use. The working ‘complete’ medium was prepared by adding CaCl2 (1.7 mM), pyruvate (1 mM), NaHCO3 (25 mM), and BSA (3 mg/ml), followed by gassing with 5% CO2, 95% air to pH 7.3. Uncapped cauda epididymal sperm were collected from mature (8–week old) CF-1 mice (Charles River, Wilmington, MA) by placing minced caudal epididymes in 1 ml of HJM medium without Ca2+, BSA and NaHCO3. After 5–10 minutes, the sperm were washed in 1 ml of HMB-Hepes buffered medium by centrifugation at 800 g for 10 minutes at room temperature. The sperm were resuspended to a final concentration of 2 × 107 sperm/ml. For the time-course experiments, aliquots of sperm were incubated for different periods of time, the sperm were spun down for 5 minutes at 5,000 g at each time point, and the medium was collected to evaluate the release of ZP3R. The pellet containing the sperm was then used for immunoblotting analysis or immunofluorescence. In some experiments, 1 mM benzamidine or EDTA-facilitated protease inhibitors (catalog number 1187358001, Roche) were added to the medium according to the manufacturer’s instructions for samples with high proteolytic activity. This mixture inhibits a broad spectrum of serine and cysteine proteases but not metallo- and aspartic proteases. Guinea pig spermatozoa were obtained and stimulated to undergo acrosomal exocytosis as previously described (Kim et al., 2001b).

Extraction of sperm protein

Cauda epididymal mouse sperm were washed twice by centrifugation for 5 minutes at 300 g and resuspended in PBS (5 × 107 sperm/ml). After the final wash the sperm pellet was resuspended with 200 µl of extraction buffer (phosphate-buffered saline containing 1% Triton X-100, 1% sodium deoxycholate and protease inhibitor mixture containing 1 mg/ml leupeptin, 1 mg/ml aprotinin and 100 µM p-aminobenzamidine, pH 7.3). The sperm suspension was placed on a rocking platform for 1 hour at 4°C. The sample was then centrifuged at 14,000 g for 15 minutes at 4°C. The supernatant was separated and stored at −20°C until used. The protein concentration of the extract was determined by the bicinchoninic acid assay (Pierce).

Fluorescent bead binding assay

Red fluorescent Fluosphere sulfate microspheres, diameter 1 µm, were obtained from Molecular Probes, Inc. Affinity-purified anti-CPT antibody against mouse ZP3R was dissolved in 1 ml phosphate-buffered saline (PBS: 50 mM phosphate buffer, pH 7.4, containing 0.9% NaCl) at a final concentration of 2 mg/ml mixed with 2 ml of 2% aqueous suspension of microspheres, and incubated at room temperature for 12 hours. The mixtures were centrifuged to separate the antibody-labeled microsphere particles from unreacted protein (3,000–5,000 g for 20 minutes). The pellet was resuspended in PBS by gentle vortexing. The washing step was repeated twice more (total of three washes). The antibody-conjugated microspheres were resuspended in 3 ml of PBS containing 1% BSA to block the non-specific sites and incubated in the BSA solution for 1 hour at room temperature. Sodium azide was added to a final concentration of 2 mM, and the Fluospheres were stored at 4°C. The anti-CPT-coated beads were then incubated for 1 hour at room temperature with a sperm protein extract containing the non-cleaved protein (10 mg/ml in PBS). Alternatively, the beads were incubated with cleaved ZP3R (10 mg/ml in PBS) recovered after a 2-hour incubation of mouse sperm under capacitating conditions. The beads were then washed twice with PBS and resuspended in 100 µl of PBS. For the binding assay, mouse netaglobin-II-arrested eggs were mixed with 100 µl of protein-conjugated Fluospheres for 1 hour at 37°C in 5% CO2. The eggs were then washed three times in PBS containing 0.1% BSA, 0.01% polyvinyl alcohol and examined by phase-contrast and fluorescence microscopy.

Acid extraction of sperm proteins

Cauda epididymal mouse sperm were washed twice by centrifugation for 5 minutes at 300 g and resuspended in PBS (5 × 107 sperm/ml). After the final wash, the sperm pellet was resuspended in two volumes of acid extraction buffer (1% glacial acetic acid with protease inhibitor mixture containing 1 mg/ml leupeptin, 1 mg/ml aprotinin, 100 µM p-aminobenzamidine, pH 3.0) to 1 volume of sperm pellet. The sperm suspension was placed on a rocking platform for 1 hour at 4°C and then centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant was then dialyzed with several changes against 1 mM HCl, pH 3.0 at 4°C for 12–24 hours. The protein concentration of extract was determined by the BCA assay (Pierce).
Indirect immunofluorescence

Mouse sperm obtained from caudae epididymides were washed twice by centrifugation for 5 minutes at 300 g. resuspended in PBS, and placed onto polylysine-coated coverslips. Sperm were then incubated with PBS containing 4% paraformaldehyde for 15 minutes at room temperature and then permeabilized with methanol. After washing in PBS, coverslips were incubated in PBS containing 10% normal goat serum (blocking buffer) for 30 minutes at room temperature, then with primary antibody (200 nM for anti-CPT) diluted in blocking buffer for 1 hour at 37°C. Following a washing step (three times, 3.5 minutes in PBS), coverslips were incubated with FITC-conjugated goat anti-rabbit IgG secondary antibody diluted 1:2,500 in blocking buffer. Blots were developed using enhanced chemiluminescence and analyzed on a STORM system (Molecular Dynamics, Sunnyvale, CA).

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