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Transitional States of Acrosomal Exocytosis and Proteolytic Processing of the Acrosomal Matrix in Guinea Pig Sperm

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Abstract

In this study, we adapted a FluoSphere bead-binding assay to study the exposure and release of guinea pig sperm acrosomal components during the course of capacitation and acrosomal exocytosis. Prior to capacitation or the initiation of exocytosis, acrosomal proteins were not accessible to FluoSpheres coated with antibodies against two acrosomal matrix (AM) proteins, AM67 and AM50; during the course of capacitation and ionophore-induced acrosomal exocytosis, however, we detected the transient exposure of the solid-phase AM proteins on the surface of guinea pig sperm using the antibody-coated fluorescent beads. Several different transitional stages leading to complete acrosomal exocytosis were classified, and we propose these represent true, functional intermediates since some of the AM proteins are orthologues of mouse proteins that bind the zona pellucida of unfertilized eggs. In addition, we present evidence that implicates acrosin in the proteolytic processing of AM50 during AM disassembly. Thus, we propose that the transitional states of acrosomal exocytosis involve early binding of AM proteins to the zona pellucida (by what visually appear to be “acrosome-intact” sperm), maintenance of zona pellucida binding that coincides with the progressive exposure of AM proteins, and gradual proteolytic disassembly of the AM to allow sperm movement through the zona pellucida. We feel this “transitional states” model provides a more refined view of acrosomal function that supports a move away from the widely-held, overly simplistic, and binary “acrosome-reaction” model, and embraces a more dynamic view of acrosomal exocytosis that involves intermediate stages of the secretory process in zona pellucida binding and penetration.

Keywords

acrosin; acrosomal exocytosis; acrosomal matrix; acrosome

Introduction

The role of the acrosome in sperm-egg interactions at fertilization is somewhat enigmatic. To fertilize an egg, a sperm must first bind to the zona pellucida (ZP) with enough adhesive attraction to maintain contact while the flagellum continues to beat. On the other hand, this

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adhesive interaction cannot be permanent because the sperm must eventually be released from the initial site of binding at the outer surface of the ZP, move through this extracellular matrix, and complete fertilization. Furthermore, many proteins within the acrosome have been shown to possess ZP-binding properties (Jones and Brown, 1987; Mori et al., 1993; Cheng et al., 1994; Foster et al., 1997; Olson et al., 1983; Buffone et al., 2008b).

To complicate matters, the prevailing view of ZP binding and sperm transit has long been considered as a series of separate events that occur sequentially (reviewed by Yanagimachi (1994)). According to this paradigm, which we term the “Acrosome Reaction Model”, sperm first bind to the ZP (called “primary binding”) through a sperm surface plasma membrane receptor while the plasma and outer acrosomal membranes are structurally intact (i.e., “acrosome-intact”). Binding of the sperm surface receptor to the ZP induces the acrosome reaction, which results in the discharge of acrosomal components and the release of primary ZP binding, allowing for “secondary binding” that involves proteins associated with the inner acrosomal membrane of the sperm head. In this models, secondary ZP binding is considered to be a more transient and weaker form of adhesion that does not prohibit forward progression of the sperm through the ZP. Also, the activation and release of soluble acrosin or another protease from the acrosome is thought to promote a limited digestion of ZP glycoproteins, facilitating sperm transit through this extracellular matrix. Thus, the sequence of events in the “acrosome reaction” model is primary binding, acrosome reaction, secondary binding, limited proteolysis of the ZP (primarily by acrosin), and transit through the ZP into the perivitelline fluid adjacent to the oocyte plasma membrane.

Questions about the Acrosome Reaction Model have been raised for many years, and several studies within the last decade or so point to alternatives. First, an essential sperm surface receptor to mediate primary binding to the ZP has remained elusive. Only β 1,4-galactosyltransferase has emerged as a ZP-binding protein on the plasma membrane that exhibits receptor activity, but since sperm from galactosyltransferase-null male mice are fertile, there are likely other sperm surface receptors involved in ZP binding and signaling (Lu and Shur, 1997). Second, at least two sperm proteins with strong ZP-binding properties, sp56/ZP3R and zonadhesin, are associated with the acrosomal matrix (AM) but are not on the sperm surface *per se* (Cheng et al., 1994; Foster et al., 1997; Olson et al., 2004; Buffone et al., 2008b). Third, it has long been acknowledged that the plasma and outer acrosomal membranes are significantly altered during capacitation, and while sperm may be considered acrosome-intact by certain criteria, the membranes do not pose an ironclad barrier to the extracellular milieu. Fourth, acrosin (previously regarded as the major sperm protease responsible for limited digestion of the ZP) was found to be non-essential for fertilization in the mouse (Baba et al., 1994a). Instead of being the major ZP lysin, acrosin appears to have a role in the dispersion of the acrosomal contents during fertilization, perhaps by proteolysis of the acrosomal matrix (Yamagata et al., 1998); a role for acrosin in the remodeling of the acrosomal matrix has been suggested by several investigators (Green, 1978; Green and Purves, 1984; Hardy et al., 1991; Huang et al., 1985). And finally, a recent *in vitro* fertilization study in mice, in which the acrosomal status of sperm was monitored in real time by the fluorescence of enhanced GFP in the acrosome, showed sperm that had “acrosome reacted” *prior to binding the egg’s ZP* commonly fertilized the egg whereas GFP-containing, “acrosome intact” sperm that bound to the ZP typically did not undergo acrosomal exocytosis or fertilize the oocyte (Jin et al., 2011).

Taken together, these studies support an alternative model of sperm-ZP adhesion and penetration that we have called the “Acrosomal Exocytosis Model” (Kim and Gerton, 2003). In this paradigm, capacitation-associated membrane changes allow limited exposure of acrosomal proteins to the extracellular environment; in particular, proteins of the scaffold-like acrosomal matrix might bind to the ZP. To be sure, sperm surface receptors are almost

certainly involved in sperm-ZP communication, but their main role is likely in signaling the “membrane events” of acrosomal exocytosis, including promoting the fusion and vesiculation of the plasma and outer acrosomal membranes, which may incorporate the rise in pH that triggers proacrosin activation. In addition, this model predicts that the activation of acrosin causes the proteolytic digestion of the acrosomal matrix scaffold and interrupts sperm adhesion with the ZP so that, coincident with mechanical force generated by the flagellum, this gamete is able to penetrate the ZP. As part of the Acrosomal Exocytosis Model, we propose the “transitional states hypothesis” that predicts that the acrosomal matrix is involved in each step of forming and breaking sperm-ZP interactions, from the initial binding of what appears to be an acrosome-intact sperm, proceeding through acrosomal exocytosis while passing through the ZP, and concluding as an acrosome-reacted sperm in the perivitelline space (Kim and Gerton, 2003; Gerton, 2002; Buffone et al., 2008a). In addition, we propose that the acrosomal matrix protein acrosin is responsible for the proteolytic digestion of AM50 that results in disassembly of the acrosomal matrix and the ZP-binding scaffold, enabling sperm to transit the ZP.

The guinea pig sperm acrosomal matrix is well characterized, and consists of four major proteins: AM67, AM50, proacrosin, and p32. AM67, the orthologue of the mouse ZP-binding protein sp56/ZP3R and a member of the complement regulatory superfamily, is localized in the dorsal-most region (M1) of the guinea pig sperm acrosome where the contents of the acrosome are first to be exposed during acrosomal exocytosis (Foster et al., 1997). AM50 is part of a large, disulfide-bonded complex and is localized to the ventral-most region (M3) of the 7 acrosome (Westbrook-Case et al., 1994). It is the orthologue of neuronal pentraxin II in humans and rodents (Noland et al., 1994; Reid and Blobel, 1994). Pentraxins comprise a family of binding proteins that form disulfide-bonded pentamers and decamers (Gewurz et al., 1995). The guinea pig sperm protein p32 corresponds to the proacrosin-binding protein in the boar (Baba et al., 1994b). It is particularly noteworthy that proacrosin co-localizes with AM50 in the ventral-most region (M3) of the acrosome, and a polypeptide presumed to be AM50 (based on co-migration in SDS-PAGE) was previously hypothesized to be “a physiologically relevant substrate for acrosin” (Hardy et al., 1991; Noland and Olson, 1989; Westbrook-Case et al., 1994).

In this report we provide two lines of support for the transitional states hypothesis. First, to detect intermediates of exocytosis in apparently acrosome-intact sperm, we adapted a FluoSphere-binding assay to examine the exposure of acrosomal matrix proteins to the external milieu around sperm that are undergoing capacitation and ionophore-induced acrosomal exocytosis (Kim and Gerton, 2003). The results show that acrosomal matrix proteins are accessible to the extracellular environment in capacitated cells that appear to be acrosome-intact; we also observed the continued exposure and differential release of acrosomal matrix proteins during acrosomal exocytosis. Second, we show that AM50 is proteolytically processed at sites in the protein consistent with acrosin-dependent cleavage. In addition, there are spatially and temporally relevant features to both lines of evidence that support the transitional states hypothesis.

Results

Transitional states of acrosomal exocytosis: Exposure of acrosomal matrix proteins AM50 and AM67 during spontaneous and induced acrosomal exocytosis

The FluoSphere assay was adapted from our previous mouse studies (Kim and Gerton, 2003) to examine the temporal and spatial nature of AM50 and AM67 cell surface exposure during capacitation and ionophore-simulated acrosomal exocytosis. FluoSphere binding to sperm was readily detected due to the size (1 μ m diameter) and fluorescence of the beads (Fig. 1, 2). Binding of Fluospheres coated with either anti-AM50 or anti-AM67 antibodies

(“AM50 FluoSpheres” and “AM67 FluoSpheres”, respectively) occurred almost exclusively in the acrosomal region of the head, often in discrete domains of sperm with visible acrosomes, i.e. acrosome-intact sperm (Figs. 1A–F, 2A–F). AM50 FluoSphere binding was most typically observed over the posterior and lateral portion of the acrosome (Fig. 1A–F) while AM67 FluoSphere binding was mostly observed over the anterior and dorsal portion of the acrosome (Fig. 2A–F). These patterns were frequently observed in each experimental group, and reflected the differential localization of AM50 and AM67 within the respective ventral and dorsal aspects of the acrosome (Westbrook-Case et al., 1994; Foster et al., 1997). Very little FluoSphere binding was observed on the posterior head or sperm tail, and Fluospheres coated with preimmune serum proteins did not bind to sperm at all (not shown).

Several discrete conditions were observed when the visual presence or absence of the acrosome was considered along with FluoSphere binding, (Table 1). Figs. 1 and 2 show AM50 and AM67 FluoSphere binding patterns, respectively; these can be classified into 4 categories. Panels A and B show intact acrosomes with no FluoSphere binding (category 1). Panels C–D and E–F show intact acrosomes with some Fluospheres bound (category 2). Panels G–H show FluoSphere aggregates bound to sperm after the acrosome appears to be absent (category 3). Finally, panels I–J show no Fluospheres bound to sperm that appear to have completed acrosomal exocytosis (category 4).

Binding of FluoSpheres during capacitation

FluoSphere binding to uncapacitated epididymal sperm was low, although AM67 FluoSphere binding was seen in about 25% of cells (Fig. 3A, Time 0). When sperm were treated with capacitating conditions, a significant increase in FluoSphere binding was seen after five minutes, and AM67 FluoSphere binding remained slightly greater than AM50 FluoSphere binding at this time (Fig. 3A). Representative images of AM50 and AM67 FluoSphere binding to sperm can be seen in Panels C–H of Figs. 1 and 2, respectively. After 20 and 60 minutes, FluoSphere binding peaked around 90–95% of sperm for both AM50 and AM67 (Fig. 3A). Of the sperm that exhibited bead binding, the number of Fluospheres bound per sperm also increased during the time course of the experiment and peaked to approximately 25 Fluospheres per sperm at 20 and 60 minutes for both AM50 and AM67 (Fig. 3B). Control Fluospheres had almost no binding at all time points.

Binding of FluoSpheres during ionophore-induced acrosomal exocytosis

To investigate the surface exposure and release kinetics of the acrosomal matrix proteins AM50 and AM67 during acrosomal exocytosis, sperm were incubated with ionophore A23187 for periods of 0, 2.5, 5, 10, and 20 minutes in the presence of the antibody-coated beads. FluoSphere binding for both AM50 and AM67 peaked at 2.5 minutes, with about 75% of cells binding Fluospheres, although binding rapidly decreased at 5, 10, and 20 minutes following activation (Fig. 4A). Of the sperm exhibiting bead binding, the average number of AM50 and AM67 Fluospheres bound per sperm showed the highest binding at 2.5 minutes, with only a few Fluospheres bound per sperm at 5, 10, and 20 minutes (Fig. 4B). These data are consistent with other studies that observe the sequential release of AM50 and AM67 (Kim et al., 2001b), and strongly suggest that AM50 and AM67 are exposed during the initial stages of acrosomal exocytosis.

The acrosomal matrix protein AM50 is a substrate for acrosin during acrosomal matrix disassembly

Since acrosin is a trypsin-like serine protease with characteristic substrate cleavage sites, namely Arg-Xaa or Lys-Xaa (Schiessler et al., 1975), we hypothesized that if AM50 were a substrate for acrosin, then AM50_{AR} ought to be cleaved at one of these sites. To test this prediction, we determined the N-terminal amino acid sequence of AM50_{AR} from isolated

acrosomal matrices that had been incubated in pH 7.4 buffer to mimic the conditions of acrosomal exocytosis, to promote AM50 conversion to AM50_{AR}, and to activate acrosin. Three incubation times were examined (1.0 min, 2.5 min, and 5.0 min). Acrosomal matrix disassembly occurred by 1.0 min, as did the processing of AM50 to AM50_{AR} (Fig. 5A, arrowhead in lane B; Fig. 7B). N-terminal amino acid sequence analysis of AM50_{AR} excised from SDS-PAGE gels revealed two overlapping sequences, AESSKDTMGD and DTMGDLPRDP. Both of these N-terminal sequences begin after lysine residues (Lys₁₀₁ and Lys₁₀₆) found in the AM50 precursor (Fig. 5B) and are characteristic of acrosin proteolysis. Thus, acrosin is implicated in the proteolytic processing of AM50, which occurs coincident with and under the conditions that promote the disassembly of the acrosomal matrix and the dispersal of the acrosomal contents during acrosomal exocytosis. In addition, the lysine residues in these positions are conserved in neuronal pentraxin II homologues across several mammalian species (Fig. 6), suggesting possible conservation of proteolytic processing by trypsin-like serine proteases in other species and tissues.

Proteolytic processing releases AM50_{AR} from the disulfide-bonded acrosomal matrix

The proteolytic processing of AM50 to AM50_{AR} detected by immunoblotting occurs in a time-frame consistent with acrosomal matrix disassembly, as observed by phase-contrast microscopy. Since disulfide bonds are responsible for the multimeric nature of AM50 as well as for the acrosomal matrix as a whole, we sought to characterize the relationship between disulfide bonding and proteolytic processing of AM50 and other acrosomal matrix proteins. Two affinity-purified, peptide-specific antibodies were used to evaluate AM50 status (Fig. 7). Although we previously were unable to determine the N-terminal sequence of AM50 (Noland et al., 1994), the QDK antibody was raised against the predicted amino-terminal sequence of AM50 exposed after removal of the putative signal sequence of the preprotein (Gln-Asp-Lys-Pro-Leu-Pro-Gly-Ser-His-Phe-Val-Cys). This sequence was expected to lie within an approximately 8000 *M_r* peptide produced by the processing of AM50 to AM50_{AR}. The DTM antibody was raised against the sequence representing the new N-terminus of AM50_{AR} (Asp-Thr-Met-Gly-Asp-Leu-Pro-Arg-Asp-Pro-Ser-Arg-Val-Val-Glu-Gln-Leu-Cys, where the cysteine replaced a serine so that the peptide could be conjugated to a carrier protein for immunization). In Western blots of non-reduced acrosomal matrices, both the QDK and DTM antibodies recognized a single polypeptide complex at >200,000 *M_r* as predicted (Figs. 7A, 7B); however, the DTM antibody also detected a small amount of AM50_{AR} (42,000 *M_r*), suggesting that a proteolysis was occurring even at low pH in the presence of protease inhibitors or during the course of electrophoresis. Under reducing conditions, both antibodies detected a single band at about 50,000 *M_r* in acrosomal matrices prepared with protease inhibitors at pH 5.0 (Fig. 7A, B). Following incubation of acrosomal matrices in neutral pH to induce proteolytic processing, no QDK-immunoreactive bands were detected at 50,000 *M_r* (Fig. 7A), indicating that proteolytic processing at pH 7.4 had modified the epitope. Although this cleavage is predicted to yield an 8,000 *M_r* cleavage product, none was detected, most likely because it is difficult to detect such a small peptide by the immunoblotting protocols used in these experiments. Nevertheless, this result shows that the QDK antibody was able to detect the unprocessed form of AM50 as predicted. Further evaluation of the proteolytic processing of AM50 at pH 7.4 showed that the predicted ~42,000 *M_r* AM50_{AR} polypeptide was detectable under both non-reducing and reducing conditions with the DTM antibody (Fig. 7B). Together these findings indicate that the proteolytic cleavage of the disulfide-bonded AM50 releases the AM50_{AR} fragment as a monomer from the multimeric pentraxin complex.

AM67 is the guinea pig orthologue of the ZP-binding protein sp56/ZP3R in mice and, like AM50, is also a disulfide-bonded multimeric component of the acrosomal matrix (Foster et al., 1997). During acrosomal exocytosis, guinea pig AM67 does not appear to undergo

processing even through mouse sp56/ZP3R is processed coincident with release from the sperm (Kim and Gerton, 2003; Buffone et al., 2009). Immunoblot analysis to evaluate whether or not AM67 processing occurred in isolated acrosomal matrix's showed a single immunoreactive band at 67,000 M_r at both pH 5 and pH 7 (Fig. 8). Thus, AM67 does not appear to undergo proteolytic processing or monomerization under these conditions.

Discussion

The acrosome is essential for mammalian fertilization, yet concerns about the current models of acrosome function indicate that our understanding of this organelle is far from complete [for reviews see (Gerton, 2002; Buffone et al., 2008a)]. Although it has long been considered that the interactions between the egg ZP and the plasma membrane overlying the sperm acrosome are defining events in sperm-egg recognition and signaling, results from our laboratories and others have prompted us to reassess the prevailing Acrosome Reaction Model of acrosome function because it does not adequately explain the process (Baibakov et al., 2007; Gahlay et al., 2010; Jin et al., 2011). There are several controversies regarding this model. First, discrepancies in the literature as to the acrosomal status of sperm that bind to the ZP (acrosome-intact or acrosome-reacted) remain unresolved, possibly due to the use of static assays to characterize a dynamic and rapid process. A recent study using real-time analysis of in vitro fertilization in mouse, however, provides clear evidence that acrosome-reacted sperm are fully capable of interacting with and penetrating the ZP (Jin et al., 2011). Second, acrosomal proteins, especially those of the matrix, are likely to have a more significant and, perhaps, earlier role in sperm-ZP binding than has been appreciated. As a case in point, sperm galactosyltransferase is a surface receptor that binds to the ZP, but novel sperm plasma membrane receptors or adhesion molecules have not emerged to provide functional redundancy for the ZP-binding function of this important, but not essential, protein (Lu and Shur, 1997). On the other hand, the discovery that the ZP-binding proteins sp56/ZP3R and zonadhesin are acrosomal matrix proteins suggests the acrosomal matrix has an important role in sperm-ZP adhesion (Kim et al., 2001a; Olson et al., 2004). As a follow-up to the study of Jin et al. (2011), Hirohashi et al. (2011) showed that, following loss of the soluble EGFP from the fertilizing transgenic mouse sperm in the cumulus mass, peanut agglutinin reactivity (a marker of the acrosomal matrix) remains associated with the sperm head, indicating that the membranes over the acrosome and soluble acrosomal components are lost before the sperm encounter ZP, although particulate acrosomal matrix material persists. Third, the consensus concerning the accepted function of acrosin in fertilization has shifted from a role as a ZP lysin toward being a dispersant of acrosomal contents, perhaps by disassembly of the acrosomal matrix scaffold. The results of our study provide insights into each of these controversies and present evidence in support of an alternative model involving "transitional states" of sperm-ZP interaction.

Acrosomal status and transitional states of acrosomal matrix protein exposure

The bead-binding assay provides some important insights into the acrosomal status during the course of sperm capacitation. The finding that ostensibly "acrosome-intact" sperm bind to beads coated with antibodies against acrosomal matrix proteins, such as sp56/ZP3R in mouse sperm (Kim and Gerton, 2003) and AM50 and AM67 in this study (Figs. 1, 2), demonstrates that these components are exposed to the extracellular environment prior to an overt "acrosome reaction". Thus, it is reasonable to hypothesize that sp56/ZP3R, zonadhesin, or other acrosomal matrix proteins capable of binding to the ZP could mediate the initial adhesion of apparently acrosome-intact sperm with oocytes (Cheng et al., 1994; Foster et al., 1997; Bi et al., 2003). Consistent with this hypothesis is the finding that apparently acrosome-reacted guinea pig sperm bind to the ZP (Myles et al., 1987), and mouse sperm visualized in real-time almost invariably undergo loss of GFP (have initiated

acrosomal exocytosis) prior to binding and penetrating the ZP (Jin et al., 2011). One possibility explanation is that, under these conditions, these sperm retain exposed acrosomal matrix material that cannot be seen by light microscopy, yet is involved in egg binding.

Acrosomal status and ZP binding

The binary terminology (acrosome-intact vs. acrosome-reacted) used to describe the status of the acrosome in the Acrosome Reaction Model does not adequately describe the transitional states of acrosomal matrix protein exposure and ZP interactions. The acrosomal location and timing of bead binding suggest that acrosomal matrix proteins are exposed to the ZP in a dynamic transition with spatial and temporal features. AM67 bead binding occurred in the area over the dorsal M1 domain of the acrosome that corresponds to the leading surface of swimming sperm. This is also the area where the initial membrane events of acrosomal exocytosis occur in the guinea pig (Flaherty and Olson, 1988, 1991). AM50 bead binding occurs more posteriorly and laterally over the area corresponding to the M2/M3 domains. AM67 bead binding slightly preceded AM50 bead binding, suggesting a temporal and spatial order of exposure of the acrosomal matrix in which AM67 might bind the ZP prior to AM50 binding. This also is consistent with previous studies examining guinea pig acrosomal exocytosis at the ultrastructural level: Flaherty and Olson (1988) concluded that stable nonfusogenic domains are present in both the plasma and outer acrosomal membranes of the apical segment of the guinea pig sperm acrosome, and that membrane-associated assemblies may maintain these domains, providing direction to some of the membrane fusion events of acrosomal exocytosis.

Particulate nature and dispersal of the acrosomal matrix

Regulated secretory vesicles contain dense cores that can modulate the release of vesicular contents through various mechanisms such as “kiss and run” exocytosis and piecemeal degranulation (Crivellato et al., 2006). Results from our group and others illustrate that the acrosomal matrix is a substantial “dense-core”, scaffold-like structure in the acrosome. As such, the acrosomal matrix may be considered to regulate the retention or release of acrosomal proteins during acrosomal exocytosis and to have a role in ZP interactions. At the same time, the acrosomal matrix is highly susceptible to disassembly by proteolysis at neutral pH. These dual features (particulate nature at acidic vesicular pH, disassembly as pH increases to match the pH of the external milieu) are consistent with a model whereby sperm-ZP occurs through a transient binding interaction that is released as the matrix gradually dissolved when exposed to the neutral extracellular pH.

AM50 is a likely substrate for acrosin

AM50 is a major component of the guinea pig acrosomal matrix (Noland et al., 1994; Reid and Blobel, 1994), and there are several reports of the conversion of AM50 to a 42,000–43,000 M_r polypeptide coincident with the activation of acrosin during the acrosome reaction in cauda epididymal sperm (Westbrook-Case et al., 1994), in isolated acrosomal matrix's [a “48–50 kDa AM polypeptide” (Hardy et al., 1991)], and in isolated apical segments (Noland and Olson, 1989). While these and other reports (Green, 1978; Huang et al., 1985; Flaherty and Swann, 1993; Fraser, 1982; Perreault et al., 1982) have suggested that acrosin was the protease responsible for acrosomal matrix dispersal and/or AM50 processing, the evidence in these studies was circumstantial and no data were presented to identify the site(s) of cleavage within the AM50 polypeptide. Additional circumstantial evidence for the proteolytic disassembly of acrosomal matrix components by acrosin includes: 1) acrosomal matrices and apical segments must be isolated in low pH (5.0–5.5) and in the presence of serine protease inhibitors (Hyatt and Gwatkin, 1988; Hardy et al., 1991; Noland et al., 1994); 2) the activity of guinea pig acrosin is very low at this pH, but has peak activity from pH 7.5 to 8 (Adekunle et al., 1989); and 3) the acrosomal matrix

quickly disassembles when isolated acrosomal matrices or apical segments are incubated in buffers at physiological pH (Noland et al., 1994).

We have shown that the conversion of AM50 to a lower M_r form (AM50_{AR}) during acrosomal matrix disassembly is the result of proteolytic processing at sites consistent with acrosin-specific cleavage. This finding strongly implicates and provides direct evidence, as predicted by Yamagata et al. (1998) and others, that acrosin (or another trypsin-like serine protease) plays a central role in the disassembly of the acrosomal matrix. First, proacrosin and AM50 comprise a major portion of the acrosomal matrix and appear in SDS-PAGE to be present in similar abundance (Hardy et al., 1991; Noland et al., 1994; Westbrook-Case et al., 1994). Second, proacrosin and AM50 co-localize within the ventral-most domain (M3) of the guinea pig acrosomal apical segment (Hardy et al., 1991; Westbrook-Case et al., 1994). Third, sperm from acrosin-knockout mice release the acrosomal contents more slowly than wild-type sperm, suggesting that the acrosomal matrix does not disassemble efficiently in the absence of acrosin (Baba et al., 1994a; Yamagata et al., 1998). In fact, these studies are only the latest in a host of studies in the literature that implicate acrosin in the dispersal of acrosomal contents (Green, 1978, 1982; Fraser, 1982; Working and Meizel, 1983; Huang et al., 1985; Hardy et al., 1991). Nevertheless, the current study provides the most direct evidence for the role of acrosin in the proteolytic processing of an acrosomal matrix protein that leads to the dispersal of the acrosomal contents. The lysine residues in the cleavage sites of AM50 are conserved among the AM50/neuronal pentraxin II orthologues of several mammals, suggesting that similar proteolytic processing events could occur in the acrosomal matrix during exocytosis in sperm of other species.

Proteolytic processing of AM50 disrupts the disulfide-bonded acrosomal matrix complex

Previous studies have shown that during acrosomal exocytosis, AM50_{AR} is released from a multimeric disulfide-bonded complex [Fig. 8, Hardy et al. (1991); Fig. 5, Westbrook-Case et al. (1994)]; our immunoblots using an antiserum against an AM50_{AR} peptide (DTM) has confirmed these findings (Fig. 7). The coincident and rapid occurrence of acrosomal matrix disassembly, AM50 processing by acrosin, and release of AM50_{AR} suggest that the integrity of AM50 multimers is important for the structure of the acrosomal matrix. These data, together with the identification of the proteolytic cleavage sites of AM50 at Lys₁₀₁ and Lys₁₀₆ in this study, indicate that two of the three cysteine residues that occur in the upstream N-terminal portion of AM50 are involved in intermolecular disulfide bonds that are important in maintaining the structure of the AM50 multimeric complex and, perhaps, the structure of the entire acrosomal matrix (Fig. 9). AM50_{AR} is released slowly during acrosomal exocytosis; intact AM50 remains associated with sperm, and only the processed form, AM50_{AR}, is released (Kim et al., 2001b). Previous work from Westbrook-Case et al. (1994) demonstrated that AM50_{AR} binds to an affinity column linked to the serine protease inhibitor p-aminobenzamidine, suggesting that AM50_{AR} remains associated with proteases after the acrosome reaction. Together these data suggest that proteolytic processing of AM50 during acrosomal exocytosis results in the release of AM50_{AR} from sperm and contributes to a partial, if not total, disassembly of the acrosomal matrix.

AM67 is part of a large disulfide-bonded complex that is not affected by proteolytic processing during disassembly of acrosomal matrices isolated from guinea pig sperm. This is consistent with findings during acrosomal exocytosis that show AM67 does not appear to undergo proteolytic processing even though it is readily released from sperm during acrosomal exocytosis (Kim et al., 2001b; Buffone et al., 2009). Taken together, these results indicate that AM67 is released from the acrosomal matrix in a disulfide-bonded multimeric form.

Conclusions

The results of this study support the Acrosomal Exocytosis Model, which holds that the acrosome goes through several transitional states that are not clearly discernable by light microscopy but, at the molecular level, involve a series of transient interactions between acrosomal proteins exposed on sperm surface with the external milieu that may involve an egg ZP. In this model, sperm capacitation involves dynamic, intermediate states of interaction between the outer acrosomal and the plasma membranes, resulting in the progressive presentation of the acrosomal matrix to the external milieu. The exposure of the acrosomal matrix occurs spontaneously at a slow rate, but its emergence may be accelerated following the interaction of sperm surface signaling molecules with the ZP and the promotion of more extensive fusion of the outer acrosomal and the plasma membranes. The exposed acrosomal matrix components then enhance the attachment of sperm to the ZP, leading to the stable binding of the sperm to the ZP as acrosomal exocytosis proceeds. Thus, acrosomal exocytosis leads to the progressive exposure of acrosomal matrix proteins that become a *de facto* extracellular matrix on the surface of the sperm head. The dynamic interactions of this newly exposed sperm extracellular matrix with the egg ZP govern sperm-egg recognition and penetration of mammalian sperm. Finally, a serine protease that cleaves after lysine residues is involved in the proteolytic disassembly of the acrosomal matrix that occurs during acrosomal exocytosis. Acrosin is the likely protease, given its abundance and location in the acrosomal matrix, kinetics, and substrate specificity.

Materials and Methods

Preparation of Sperm

Retired male breeder Hartley strain guinea pigs were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Sperm were recovered from the caudae epididymides, washed twice by centrifugation for 5 min at $300 \times g$, and resuspended at a concentration of 2×10^7 sperm/ml in modified MCM (25 mM NaHCO_3 , 112 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl_2 , 20 mM sodium lactate, 1 mM sodium pyruvate, 2 mM CaCl_2 , pH 7.2) (Barros, 1974; Mújica et al., 1991). In some experiments, acrosomal exocytosis was induced by 5 μM ionophore A23187.

FluoSpheres® binding assay

Yellow-green and red fluorescent FluoSpheres® sulfate microspheres, diameter 1.0 μm , were obtained from Molecular Probes (Invitrogen, Inc., Eugene, OR). To coat the FluoSpheres, 1 ml of pre-immune serum, anti-AM50 antiserum (Noland et al., 1994), or anti-AM67 antiserum (Foster et al., 1997) was added to 2 ml phosphate buffer (50 mM phosphate buffer, pH 7.4, containing 0.9% NaCl), respectively, and a 1.5-ml aliquot of 2% aqueous suspension of microspheres was added to each mixture. The mixtures were incubated at room temperature overnight. The tubes were then centrifuged to separate the protein-labeled microsphere particles from the unreacted protein ($3,000\text{--}5,000 \times g$ for 20 minutes). The pellets were resuspended in 50 mM $1\times$ phosphate-buffered saline (PBS) by gentle vortexing. The centrifugation and resuspension steps were repeated twice for a total of 3 washes. The protein-conjugated microspheres were resuspended in 4.5 ml of 50 mM phosphate buffer, pH 7.4, containing 0.9% NaCl and 1% BSA. Sodium azide was added to a final concentration of 2 mM and the FluoSpheres were stored at 4°C .

Sperm suspension droplets (100 μl) were placed on Petri dishes and covered by mineral oil. Aliquots (10 μl) of each antibody-coated FluoSphere suspension (prewashed three times with PBS to remove azide from the storage medium) were added to each sperm droplet and were incubated for 0, 5, 20, and 60 min. At each time point, 5 μl aliquots of each sperm-

FluoSphere suspension were placed on glass slides and covered by coverslips. The numbers of FluoSpheres bound per sperm were determined by phase-contrast and fluorescence microscopy.

Isolation of acrosomal matrices

Acrosomal matrices were prepared as described by Hardy et al. (1991). Briefly, cauda epididymal sperm were treated with a detergent buffer (50 mM sodium acetate containing 0.1 M NaCl, 0.625% Triton X-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM p-aminobenzamidine, pH 5.2) to solubilize membranes, and the extracted sperm were forced through a 26G needle to dislodge the acrosomal matrices from the sperm heads. After the sperm remnants (acrosomal matrices and membrane-free sperm) were filtered on a 0.2–0.3 mm glass bead column, the acrosomal matrices were collected from the flow-through fraction whereas the membrane-free sperm remained trapped on the column.

Characterization of acrosomal matrix disassembly

After several washes with acetate-buffered saline (50 mM sodium acetate containing 0.1 M NaCl, pH 5.0), acrosomal matrices were centrifuged at $12,000 \times g$ for 5 min and resuspended in either acetate-buffered saline containing protease inhibitors (control for no processing) or incubated in Tris-buffered saline (50 mM Tris-HCl containing 0.1 M NaCl, pH 7.4) containing 2 mM CaCl_2 (to allow processing of AM50). Samples were incubated at 37°C for designated time periods, followed by incubation on ice and the addition of protease inhibitors to final concentrations of 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM p-aminobenzamidine. The results were evaluated by SDS-PAGE and detection by standard silver staining.

N-terminal microsequence analysis of AM50_{AR}

Isolated acrosomal matrices were incubated Tris-buffered saline for 30 min at 37°C, subjected to non-reducing SDS-PAGE, and transferred to PVDF membrane in basic CAPS electroblotting transfer buffer (10 mM 3-[cyclohexylamino]-1-propane sulfonic acid (Sigma C2632), adjusted to pH 11 with NaOH and containing 10% methanol). Strips of membrane containing AM50_{AR} were sent for amino terminal microsequencing to Dr. John Leszyk (University of Massachusetts Medical Center, Worcester, MA).

Abbreviations

AM	acrosomal matrix
ZP	zona pellucida

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Figure 1. Surface exposure of the acrosomal matrix protein AM50 during capacitation in guinea pig sperm

Paired phase-contrast (A, C, E, G, I) and fluorescence (B, D, F, H, J) images of guinea pig sperm incubated with FluoSpheres coated with anti-AM50 antibodies (AM50 FluoSpheres). In uncapacitated sperm, AM50 FluoSpheres did not bind sperm (A and B), but following treatment in capacitating conditions AM50 FluoSpheres were typically bound to the posterior and lateral aspects of the acrosome (C–F). In sperm that lack apparent acrosomes, some cells contained aggregates of AM50 FluoSpheres (G and H) while others had no AM50 FluoSphere binding (I and J).

Figure 2. Surface exposure of the acrosomal matrix protein AM67 during capacitation in guinea pig sperm

Paired phase-contrast (A, C, E, G, I) and fluorescence (B, D, F, H, J) images of guinea pig sperm incubated with FluoSpheres coated with anti-AM67 antibodies (AM67 FluoSpheres). In uncapacitated sperm, AM67 FluoSpheres did not bind sperm (A and B) but following treatment in capacitating conditions AM67 FluoSpheres were typically bound to the anterior and dorsal aspects of the acrosome (C–F). In sperm that lack apparent acrosomes, some cells contained aggregates of AM67 FluoSpheres (G and H) while others had no AM67 FluoSphere binding (I and J).

Figure 3. Temporal analysis of FluoSphere binding to guinea pig sperm during incubation in capacitating conditions

Cauda epididymal sperm were incubated with antibody-coated FluoSpheres [AM50, AM67, or preimmune serum (control), as indicated] in capacitating medium for 0, 5, 20, and 60 minutes, and the percentage of sperm with bound FluoSpheres (A) and, for the sperm exhibiting bead binding, the average number of FluoSpheres bound per sperm (B) were determined. Preimmune FluoSphere controls showed almost no binding to sperm. At time 0, about 25% of sperm had AM67 FluoSphere binding while less than 5% of sperm had AM50 FluoSphere binding. The percentage of AM50 and AM67 bound FluoSpheres increased dramatically during incubation in capacitating conditions for 5, 20, and 60 minutes, and peaked around 95% for both at 20 and 60 minutes. Likewise, the number of AM50 and AM67 FluoSpheres bound per sperm increased over the incubation time and peaked at about 25 beads per sperm at 20 and 60 minutes.

Figure 4. Temporal analysis of FluoSphere binding to guinea pig sperm during ionophore-induced acrosomal exocytosis

Cauda epididymal sperm were treated with calcium ionophore A23187 and incubated with antibody-coated FluoSpheres (AM50, AM67, or preimmune serum as indicated) for 0, 2.5, 5, 10, and 20 minutes. The percentage of sperm with bound FluoSpheres (A) and, for the sperm exhibiting bead binding, the average number of FluoSpheres bound per sperm (B) were determined. Preimmune FluoSphere controls showed almost no binding to sperm. At time 0, 20–25% of sperm had AM50 and AM67 FluoSphere binding. The percentage of AM50 and AM67 bound FluoSpheres increased dramatically to about 75% after 2.5 minutes and diminished sharply afterward to 10–15% at 20 minutes. Likewise, the number of AM50 and AM67 FluoSpheres bound per sperm increased dramatically to 10–15 FluoSpheres/sperm at 2.5 minutes, and diminished sharply afterward to about 2 FluoSpheres/sperm by 20 minutes.

Figure 5. Proteolytic processing of AM50 during acrosomal matrix disassembly occurs at acrosin- specific sites

A) Isolated acrosomal matrices containing AM50 (< in lane A) were incubated for one minute in pH 7.4 buffer lacking protease inhibitors, and the conversion of AM50 to AM50_{AR} (> in lane B) was detected by SDS-PAGE (silver-stained gel). B) Schematic model of the AM50 protein. Microsequencing of AM50_{AR} detected two acrosin-specific cleavage sites after K-101 and K-106, indicated by asterisks (*) in the sequence above the cleavage site (dotted line) in the model. This cleavage site implicates acrosin in AM50 processing and acrosomal matrix disassembly, and results in a 42-kDa fragment (AM50_{AR} as observed in the gel in lane B) and a presumed 8-kDa fragment that has not been observed. Also shown are the antigenic sites of peptide antibodies generated against AM50_{AR} (DTM) and the N-terminal region of AM50 (QDK) that is cleaved during proteolytic processing.

Amino Acid #	101	106
Guinea Pig	AGKA----	ESSKDTMGDL
Human	GGKARGAGATG	KDTMGDL
Chimp	GGKARGAGATG	KDTMGDL
Rhesus Monkey	GGKARGTGATG	KDTMGDL
Mouse	GGKARG---	TGKDTMGDL
Rat	GGKARGTGATG	KDTMGDL
Consensus	GGKARG*	GATGKDTMGDL

Figure 6. Aligned sequences of AM50 orthologues

The lysine residues at positions 101 and 106 in guinea pig AM50 are conserved among species, suggesting that neuronal pentraxin II might be processed by a trypsin-like serine protease in other species sperm and tissues.

Disulfides	Non-Reduced		Reduced	
pH Incubation	5	7	5	7

Mr

216-

78-

43-

Disulfides pH Incubation	Non-Red		Reduced	
	5	7	5	7

Mr

216-

78-

43-

Figure 7. Proteolytic processing of the acrosomal matrix protein AM50 during acrosomal matrix disassembly shows that the processed form, AM50AR, is released from the disulfide-bonded AM50 multimeric complex

Acrosomal matrices were isolated in pH 5 buffer containing protease inhibitors (lanes 5), and were incubated in pH 7.4 buffer lacking protease inhibitors (lanes 7). Samples were subjected to SDS-PAGE under non-reducing conditions (Non-Reduced) or reduced with 2-mercaptoethanol (Reduced). Blots were probed using a peptide antiserum generated towards the N- terminus of AM50 (A; QDK) or the N-terminus of AM50AR (B; DTM).

Disulfides	Reduced	
	5	7
pH Incubation

Figure 8. The acrosomal matrix protein AM67 does not appear to be processed nor released from the disulfide-bonded acrosomal matrix complex during acrosomal matrix disassembly in guinea pig sperm

Acrosomal matrices were isolated in pH 5 buffer containing protease inhibitors (lane 5), and were incubated in pH 7.4 buffer lacking protease inhibitors (lane 7). Samples were subjected to SDS-PAGE under reducing conditions with 2-mercaptoethanol and blots were probed using a peptide antiserum generated towards AM67.

Figure 9. Model proposing how proteolytic processing of AM50 during acrosomal matrix disassembly may release AM50AR from the disulfide-bonded complex

Data from our study and others indicate that AM50 is a disulfide-bonded multimer and that it is processed during acrosomal exocytosis into a monomer that is released from the disulfide-bonded complex. The AM50 polypeptide contains multiple cysteines that appear to be involved in maintaining the disulfide-bonded complex, but only three of these cysteines lie on the N-terminus side (i.e. upstream) of the proteolytic cleavage site. Thus, it appears that at least one of these cysteines must be involved in intermolecular disulfide bonds that maintain the AM50 multimer, and may be responsible for the structural integrity of the entire acrosomal matrix as an intact complex.

Table

Category	Visible acrosome?	FluoSpheres bound to acrosome?	Interpretation
1	Yes	No	Plasma and/or outer acrosomal membranes “intact”
2	Yes	Yes	Plasma and outer acrosomal membrane <u>not</u> “intact”; AM protein surface exposure
3	No	Yes	Acrosomal exocytosis; AM proteins retained
4	No	No	Release of AM proteins