

# The role of the acrosomal matrix in fertilization

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**ABSTRACT** Mammalian sperm must have properly formed acrosomes to be fully functional in the process of binding and penetrating the zona pellucida (ZP), the extracellular matrix surrounding the egg. There is much evidence to raise doubts about the old "bag of enzymes" paradigm of acrosomal function, although this is the model that seems to prevail. We concur with other scientists that acrosomal exocytosis is not an all or none event where the acrosome is either "intact" or "reacted". As determined by transmission electron microscopy of human sperm undergoing acrosomal exocytosis, six stages can be identified, with the intermediate ones involving loss of acrosomal matrix material. In the mouse, there is a temporal relationship among four stages of acrosomal exocytosis. Numerous evidences suggest a more complex role for the acrosome in fertilization in which the acrosomal matrix is a scaffold for sperm-ZP interactions that self-regulates by a controlled disassembly mechanism.

**KEY WORDS:** *acrosome, sperm, zona binding, zona pellucida, sp56*

## Introduction

The acrosome (Gr. *akros* = extreme or tip, *soma* = body) is a Golgi-derived exocytotic organelle located at the tip of the sperm head from many mammalian species. Mammalian sperm must have properly formed acrosomes to be fully functional in the process of binding and penetrating the zona pellucida (ZP), the extracellular matrix surrounding the egg. Men or mice carrying mutations affecting the formation of the sperm acrosome are infertile or display subfertility (Dam *et al.*, 2007, Kang-Decker *et al.*, 2001, Lin *et al.*, 2007, Schill, 1991, Sotomayor and Handel, 1986).

In this review, we will address the structure and function of the particulate aspect of the acrosome, the acrosomal matrix. We will first discuss the features of the acrosomal matrix including some of its protein components. Next we will examine some of the frequently overlooked properties of the acrosomal matrix. Finally, we will incorporate these concepts into an alternative perspective for viewing the events occurring at the interface between the sperm and the zona pellucida. It is our goal to challenge the reader to consider the nature of the acrosomal matrix when developing paradigms for sperm-zona interactions. For example, does the acrosomal matrix progress through various stages (condensed, gel-like, soluble) following its exposure to the extracellular milieu? Do all assays of acrosomal status (intact, reacted, other) report on

the same property (plasma membrane integrity, presence of acrosomal components, existence of an ionic or pH gradient across the plasma membrane)? What molecules are involved in sperm-zona adhesion and where are they located?

## Acrosomal biogenesis and morphology

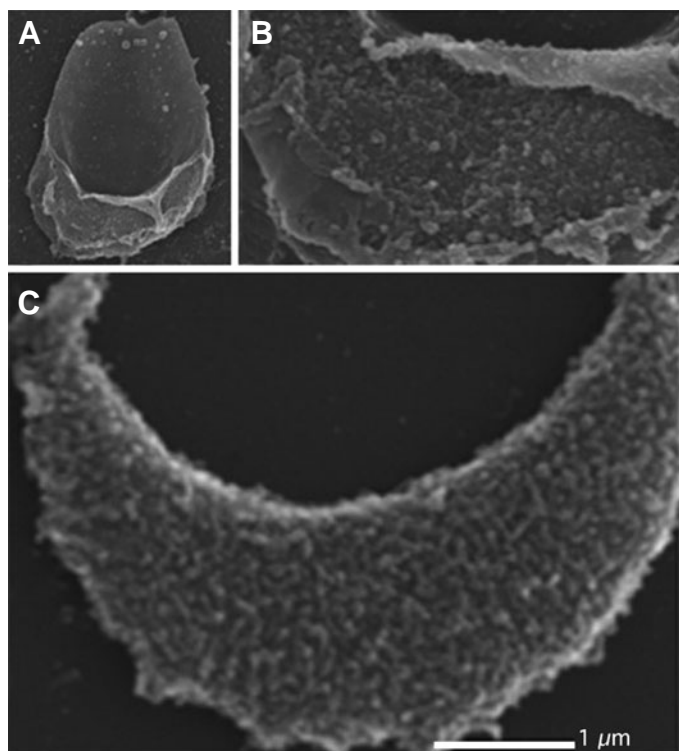
Acrosomal biogenesis begins in the late pachytene spermatocyte phase of meiosis and continues throughout the first half of spermiogenesis, the haploid phase of spermatogenic differentiation in the seminiferous tubules of the testis (Anakwe and Gerton, 1990, Escalier *et al.*, 1991, Kashiwabara *et al.*, 1990). Initially, proacrosomal vesicles are formed in the perinuclear region near the Golgi apparatus of pachytene spermatocytes. As a result of the meiotic cell divisions, these vesicles are distributed to the four daughter spermatids. In early spermiogenesis, these vesicles coalesce into a single granule that attaches to the round spermatid nucleus and continues to enlarge as Golgi-derived material is added to the developing acrosome. During the last half of spermiogenesis while the elongating spermatids are maturing, the Golgi ceases to contribute glycoconjugates to the acrosome and

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*Abbreviations used in this paper:* AM, acrosomal matrix; OAM, outer acrosomal membrane; PABP, proacrosin binding protein; ZP, zona pellucida; ZPBP, zona pellucida binding protein.

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**Fig. 1. Scanning electron micrographs of the acrosomal matrix in guinea pig sperm.** The acrosomal matrix is visible where the membranes over the acrosome appear to have been disrupted (A). (B) An enlargement of (A). An acrosomal matrix (C) isolated by the method of Hardy *et al.*, 1991 has a characteristic crescent shape and the same textured appearance as the acrosomal matrix seen within the acrosome of the cell in (A,B).

the acrosome-nucleus complex undergoes extensive morphological alterations to assume the shape that is 'characteristic for a given species' sperm. In some species, additional maturation of the acrosome occurs as the sperm transit the epididymis. In the case of a dramatic example, the guinea pig, the heads of testicular sperm entering the rete testis from the seminiferous tubules are quite planar, much like a flat hand (Fawcett and Hollenberg, 1963). As the sperm travel through the epididymis to the vas deferens, the acrosome transforms such that it resembles a cupped hand, where the fingers represent the apical segment of the acrosome and the remainder of the hand, the sperm nucleus. The acrosome forms a convex (dorsal) face with a bulge, a concave (ventral) surface, and a leading edge that tapers to a pronounced tip.

The acrosome of a mature sperm can be defined in terms of several structural compartments. Surrounding the lumen of the acrosome is the acrosomal membrane. This can be further delineated into the outer acrosomal membrane (OAM), which is in close apposition to the plasma membrane overlying the acrosome, and the inner acrosomal membrane (IAM), which is in tight association with the nuclear membrane (Eddy and O'Brien, 1994). During the course of acrosomal exocytosis, the OAM and plasma membrane fuse and are lost as hybrid membrane vesicles or as a fused membrane sheet. In some cases, an acrosomal ghost or shroud can be seen that consists of the membrane sheet or the vesicles held together by acrosomal

matrix material (Bedford, 1998, Lea *et al.*, 2001). The lumen of the acrosome contains soluble components and a particulate structure known as the acrosomal matrix (Foster *et al.*, 1997, Kim *et al.*, 2001a, Olson and Winfrey, 1994, Westbrook-Case *et al.*, 1995). As discussed below, morphological subcompartments within the acrosomal matrix of some sperm can be clearly distinguished as regions of different density by electron microscopy or zones containing different acrosomal antigens as detected by immunoelectron microscopy.

### Operational definition of "acrosomal matrix"

Originally, the use of the term "acrosomal matrix", referred to the electron dense material within the lumen of the acrosome. However, as studies were performed to determine the biochemical nature of the acrosome, the operational definition of the acrosomal matrix has become the particulate, membrane-free component of the acrosome that remains following treatment of sperm with the non-ionic detergent Triton X-100 (Fig. 1). For biochemical characterization, acrosomal matrices have been dislodged from guinea spermatozoa and hamster by shaking the cells in a pH 5.2 buffer containing Triton X-100 and protease inhibitors, and enriching the particulate matrices on a glass-bead column (Huang *et al.*, 1985, Hyatt and Gwatkin, 1988). Under these circumstances, a morphologically intact but membrane-less acrosomal matrix can be isolated free of the remaining sperm heads and tails. The same extraction conditions can be applied to mouse sperm to demonstrate the presence of particulate acrosomal matrix within these smaller acrosomes; under these conditions the acrosomal matrix remains laminated on the apical aspect of the sperm head (Kim *et al.*, 2001a). "The ability to isolate acrosomal matrices enabled several laboratories to begin characterizing their protein composition and to study how the matrices behave once they are exposed to the extracellular milieu.

### Protein composition of the acrosomal matrix

The composition of the acrosomal matrix is limited in the number of different proteins present. Proteases, glycosidases, and various binding proteins can be found there. In fact, several ZP binding proteins are present within the acrosomal matrix. Most acrosomal matrix proteins appear to have orthologues in other species. Below we present a brief overview of some of the more commonly studied or better known acrosomal matrix components.

#### *Proacrosin*

The serine protease zymogen proacrosin is the quintessential acrosomal protein. For many years, the active enzyme, acrosin, was thought to be an essential zona lysin that enables the sperm to penetrate the ZP. Creation of mice null for the proacrosin gene demonstrated that this protease is not required for fertilization (Baba *et al.*, 1994a). However, the penetration of the ZP by sperm lacking proacrosin is impeded, apparently due to a delay in dispersing the acrosomal matrix (Adham *et al.*, 1997, Yamagata *et al.*, 1998b). Thus, acrosin may process other proteins in the acrosome and on the membranes during acrosomal exocytosis (Honda *et al.*, 2002). Acrosin has also

been characterized as a ZP-binding protein (Howes and Jones, 2002, Jones and Brown, 1987, Moreno *et al.*, 1998, Mori *et al.*, 1995, Richardson and O'Rand, 1996, Williams and Jones, 1991).

#### Proacrosin binding protein (PABP)

Also known as sp32, this protein has been identified in a number of species (Yi and Polakoski, 1992). It has been most extensively studied in porcine sperm where it significantly accelerates the autoactivation of proacrosin at a basic pH *in vitro* and alters the activation pathway of proacrosin. When PABP is present, a 49-kDa acrosin intermediate accumulates from the activation of the 55- and 53-kDa proacrosins, instead of a 43-kDa acrosin intermediate (Baba *et al.*, 1994b).

#### AM67/sp56/ZP3R

Originally described by Bleil, Wassarman, and colleagues, sp56 is a binding protein for ZP3, one of the three ZP proteins (Bleil and Wassarman, 1990, Bookbinder *et al.*, 1995, Cheng *et al.*, 1994, Cohen and Wassarman, 2001). In the mouse, the gene encoding sp56 has been assigned the symbol *Zp3r* (for ZP3 receptor). ZP3R/sp56 is an oligomeric member of the complement regulatory protein family (Bookbinder *et al.*, 1995) and is more closely related to the guinea pig acrosomal matrix protein AM67 than it is to mouse complement 4-binding protein (Foster *et al.*, 1997). Initially, ZP3R/sp56 was characterized as a plasma membrane protein but subsequent studies demonstrated that this protein, like guinea pig AM67, is intra-acrosomal (Fig. 2) and a component of the acrosomal matrix (Cheng *et al.*, 1994, Foster *et al.*, 1997, Kim *et al.*, 2001a, Suzuki-Toyota *et al.*, 1995).

#### AM50/p50/apexin/NPTX2

Three laboratories independently identified AM50/apexin from guinea pig sperm (Noland *et al.*, 1994, Reid and Blobel, 1994, Westbrook-Case *et al.*, 1994). Additional studies in the human and rat identified the orthologues (neuronal pentraxin 2 [NPTX2] or Narp) from neuronal tissues (Hsu and Perin, 1995, Tsui *et al.*, 1996). Pentraxins are multimeric proteins that usually form decamers and come in two basic flavors: classical pentraxins (C-reactive protein, serum amyloid P-component) and long pentraxins (PTX3, AM50). They have been implicated in innate immunity, inflammation, matrix deposition, and female fertility (Garlanda *et al.*, 2005). As a consequence of acrosomal exocytosis, AM50 monomers are processed from a 50,000  $M_r$  monomer to a 43,000  $M_r$  monomer (Kim *et al.*, 2001b, Westbrook-Case *et al.*, 1995).

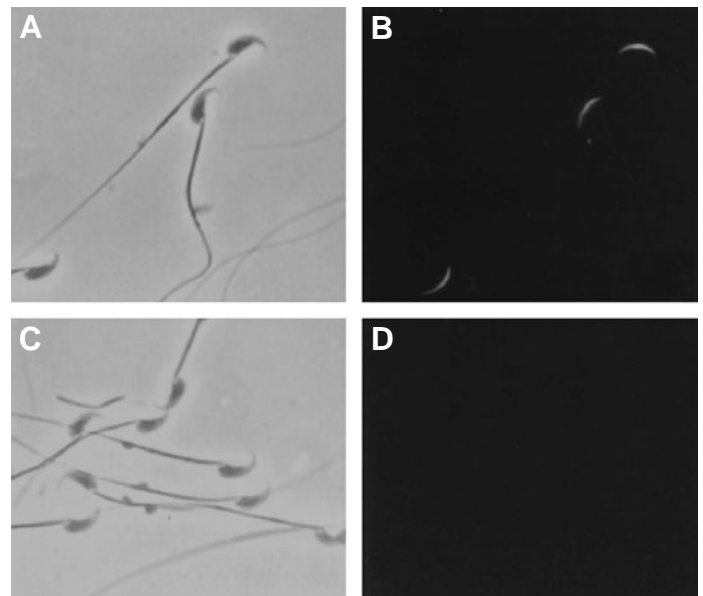
#### ZBP1/sp38

Zona pellucida binding protein 1, ZBP1/sp38, was first identified by Baba and co-workers (Mori *et al.*, 1993, Mori *et al.*, 1995) and purified from detergent extracts of porcine epididymal sperm. This protein also demonstrated the ability to bind the pig ZP in a calcium-dependent manner. The binding of sp38 to ZP could be inhibited by proacrosin, suggesting that these two proteins competitively interact with ZP during early steps of fertilization. Subsequently, the bovine orthologue has been isolated and characterized from a specialized domain of the acrosomal matrix, the inner acrosomal membrane extracellular

coat, and shown to possess properties similar to the porcine protein (Yu *et al.*, 2006). More recently, the mouse gene for ZBP1 and its paralogue ZBP2 have been identified and mice null for these genes have been created (Lin *et al.*, 2007). ZBP1-null male mice are sterile while ZBP2-null male mice are subfertile. The defects in the germ cells are not limited to their ability to bind and penetrate the ZP; sperm from the null mice have dysmorphic acrosomes and heads, as well as other defects in the seminiferous tubules, indicating that these proteins have important functions during spermiogenesis. ZBP2 paralogs are also found in amphibians, birds, and other mammals (Lin *et al.*, 2007).

#### Zonadhesin

Zonadhesin was initially identified and isolated from boar sperm as a membrane protein that binds in a species-specific manner to the ZP of the egg (Hardy and Garbers, 1995). Subsequently, cDNAs or genes have been partially or completely sequenced from mouse, human, rabbit, hamster, and non-human primates (Bi *et al.*, 2003, Gao and Garbers, 1998, Gao *et al.*, 1997, Hardy and Garbers, 1995, Herlyn and Zischler, 2007, Lea *et al.*, 2001, Olson *et al.*, 2004). Testicular porcine zonadhesin is extractable with non-ionic detergents and would not be considered a component of the acrosomal matrix by the operational definition listed above (Bi *et al.*, 2003). In round and elongating spermatids, zonadhesin localizes to the developing outer acrosomal membrane. However, during the course of sperm transit the epididymis, most of the protein becomes incorporated into an extraction-resistant fraction, thus meeting the criteria for being an acrosomal matrix protein. Subsequent immunoelectron microscopy studies localized zonadhesin to



**Fig. 2. Indirect immunofluorescence of paraformaldehyde-fixed and live mouse sperm.** Shown are corresponding phase-contrast (A,C) and immunofluorescence (B,D) micrographs of mature mouse sperm immunostained with anti-sp56 monoclonal antibody 7C5. Paraformaldehyde-fixed sperm (A,B) showed bright acrosomal fluorescence localization of sp56 (B), whereas live unfixed sperm (C,D) showed no immunofluorescence (D). Reproduced from Foster *et al.* (1997).

the perimeter of the acrosome in intact ejaculated boar spermatozoa while in sperm with disrupted acrosomal membranes, the protein was detected at the leading edge of acrosomal matrix (Bi *et al.*, 2003).

### SP10

SP-10 is a testis-specific protein that is initially expressed post-meiotically in round spermatids (Herr *et al.*, 1990, Kurth *et al.*, 1991, Kurth *et al.*, 1993) where it is localized in the acrosome and is a component of the acrosomal matrix (Foster and Herr, 1992, Olson *et al.*, 1997). In humans a 45,000  $M_r$  precursor is proteolytically processed to polymorphic forms ranging from 18,000-34,000  $M_r$  in mature sperm (Foster *et al.*, 1994, Herr *et al.*, 1990, Herr *et al.*, 1992). SP-10 antibodies interfere with sperm binding to the zona pellucida in bovine *in vitro* fertilization, suggesting that SP-10 functions in sperm-zona pellucida interactions (Coonrod *et al.*, 1996).

### Other acrosomal matrix proteins

In addition to the proteins summarized above, there are several other proteins that have been described as components of the acrosomal matrix. These include, but are not limited to, the following proteins. SLLP1 has been identified as a unique, non-bacteriolytic, c (chicken or conventional type) lysozyme-like protein present in the acrosome of human sperm and it has been proposed as a potential receptor for the egg oligosaccharide residue N-acetylglucosamine (Mandal *et al.*, 2003). N-acetylglucosaminidase has been isolated from a purified acrosomal matrix fraction of hamster spermatozoa (NagDas *et al.*, 1996a). In the mouse, it facilitates sperm penetration through the zona (Miller *et al.*, 1993b). SAMP14, a member of the Ly-6/urokinase-type plasminogen activator superfamily of receptors, localized to outer and inner acrosomal membranes as well as the acrosomal matrix of ejaculated human sperm (Shetty *et al.*, 2003). It remains exposed on the inner acrosomal membrane following exocytosis. Equatorial segment protein (ESP) is a biomarker for a specialized subcompartment of the acrosomal matrix, localizing to the equatorial segment of ejaculated human sperm (Wolkowicz *et al.*, 2003). ESP can be traced through all stages of acrosome biogenesis; it has recently been proposed to be involved in sperm/egg binding and fusion at the level of the oolemma (Wolkowicz *et al.*, 2007). This subset of studies concerning proteins of the acrosomal matrix illustrate the diversity of protein functions within this important structure.

### Compartmentalization within the acrosomal matrix

The acrosomal matrix itself is compartmentalized into different domains containing distinct subsets of proteins. Topographically, specific acrosomal proteins have been localized to defined domains within the acrosome (Foster *et al.*, 1997, Olson and Winfrey, 1994, Olson *et al.*, 1988, Tanii *et al.*, 1994, Westbrook-Case *et al.*, 1994). This has been shown mainly in species with large acrosomes (guinea pig and hamster) but has also been seen in small acrosomes (mouse and human). Thus, guinea pig sperm represent an attractive model for defining the structure and function of individual acrosomal domains.

Like other mammalian sperm, the guinea pig sperm acrosome consists of an equatorial segment that delimits the posterior

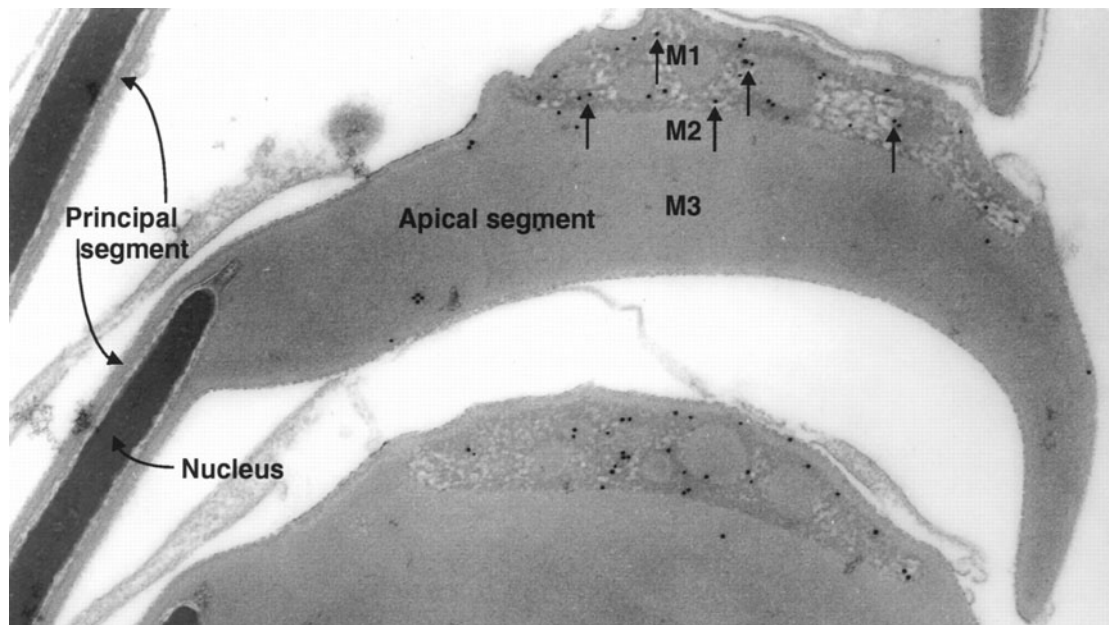
boundaries of the acrosome and encircles the nucleus, a principal segment (a thin, perinuclear region), and an apical segment that extends beyond the nucleus (Eddy and O'Brien, 1994). However, in the guinea pig, the apical segment is much larger and compartmentalized than in most mammals (Fawcett, 1975a, Fawcett, 1975b, Fawcett and Hollenberg, 1963). Three zones of differing electron density can be differentiated in the apical segment of epididymal sperm: the electron-lucent dorsal bulge (M1), a zone of intermediate electron density (M2), and the most electron-dense region (M3) (Olson *et al.*, 1987) (Fig. 3). The soluble acrosomal enzyme dipeptidyl peptidase II and matrix component AM67 are localized in the M1 zone (Foster *et al.*, 1997, Talbot and DiCarantonio, 1985) while proacrosin is localized almost exclusively in the most electron-dense regions (M2 and M3) of the acrosome (Hardy *et al.*, 1991). Westbrook-Case *et al.* (1994) published the initial characterization of AM50, a protein of the apical segment of guinea pig sperm. This protein is restricted to the ventral domain (M3) of the acrosomal matrix and is proteolytically processed during the acrosomal exocytosis. Autoantigen 1, a soluble acrosomal protein, is distributed throughout the whole acrosome with a slightly higher concentration in the denser regions (Hardy *et al.*, 1991). Both autoantigen 1 and proacrosin are found in the principal segment, but neither is present in the equatorial segment of the acrosome.

In other species's sperm with smaller acrosomes, it may be difficult to see different domains without using immunological location techniques at the level of the electron microscope. For example, porcine and human sperm have smaller acrosomes that do not exhibit the prominent domains seen by transmission electron microscopy in guinea pig sperm. By immunoelectron microscopy, zonadhesin localizes to the perimeter of the acrosome in intact ejaculated boar spermatozoa or cauda epididymal hamster sperm, and to the leading edge of acrosomal matrix overlying cells with disrupted acrosomal membranes (Bi *et al.*, 2003, Olson *et al.*, 2004). On the other hand, SP-10 is most abundant within the principal segment and posterior bulb of the equatorial segment of the human sperm acrosome (Foster *et al.*, 1994).

### How acrosomal matrix structure influences physiological function

#### Acrosomal matrix stabilization

The existence of an acrosomal matrix comprised of proteins in different domains suggests that these proteins interact with each other to form the particulate complex. This is supported by studies of the biochemical structure of the bull and hamster sperm acrosomal matrix. In one investigation using bovine spermatozoa, a complex of the acrosomal matrix in association with the outer acrosomal membrane was isolated and it was demonstrated that this complex could bind proacrosin in a dose-dependent manner (NagDas *et al.*, 1996b). Concentrating on proacrosin and N-acetylglucosaminidase of the hamster sperm acrosomal matrix, NagDas *et al.* (1996a) examined how these enzymes interacted with the acrosomal matrix. Both hydrolases could be extracted from the matrices with high salt (0.5 M NaCl). The residual acrosomal matrix preparation after this step retained the property to bind proacrosin and N-acetylglucosaminidase and, in a blot overlay, proacrosin bound to proteins with molecular weights of 29,000 and 24,000. These experiments suggest that



**Fig. 3. Immunoelectron microscopic localization of AM67 in the M1 (dorsal bulge) domain of guinea pig sperm.** The immunogold particles (arrows) were found solely within the M1 (dorsal bulge) domain of the lumen of the acrosome. In general, the gold particles were excluded from spherical bodies within this region. No staining was observed in the M2 (intermediate zone) or M3 (ventral zone) region. Reproduced from Foster *et al.* (1997).

the acrosomal matrix proteins contain information for self-assembly into a large complex within the acrosome. Hardy *et al.* (1991) also recognized the disulfide-bonded nature of some of the acrosomal matrix polypeptides. Specifically, proteins such as AM50 and AM67/sp56 also form high molecular weight oligomers that contain monomers with intermolecular and intramolecular disulfide bonds (Foster *et al.*, 1997, Noland *et al.*, 1994). As a result of the oligomeric nature of these proteins, they are predicted to be multivalent and may be able to help stabilize the acrosomal matrix by non-covalent cross-linking of protein components.

The genetic deletion of specific acrosomal matrix proteins can have a detrimental effect on the structure of acrosome and the function of the sperm (Lin *et al.*, 2007). When the *Zpbp1* and *Zpbp2* genes were disrupted in mice, males lacking ZPBP1 were sterile, with abnormal round-headed sperm morphology and no forward sperm motility. The absence of ZPBP1 prevents proper acrosome compaction, acrosome fragmentation, and disruption of the Sertoli-spermatid junctions. Males null for ZPBP2 are subfertile; their sperm exhibit aberrant acrosomal membrane invaginations, and reduced ability to penetrate zona pellucida.

#### **Acrosomal matrix expansion and dispersion**

The stability of the acrosomal matrix is dependent upon its sequestration from the extracellular milieu. The interior pH of the acrosome is acidic (Meizel and Deamer, 1978, Nakanishi *et al.*, 2001) and this serves to maintain proteases such as proacrosin in an inactive state (Brown and Harrison, 1978, Honda *et al.*, 2002, Noland *et al.*, 1989). As mouse sperm undergo capacitation, the acrosomal pH starts to alkalize (Nakanishi *et al.*, 2001). Whether this is related to the initial swelling or decondensation of the acrosomal matrix seen in the early phases of acrosomal exocytosis has yet to be determined (Nagae *et al.*, 1986, Yudin *et al.*, 1988). When the pH of the acrosome increases during the course of acrosomal exocytosis, proacrosin becomes activated. The conversion of proacrosin to acrosin apparently is then involved in the proteolytic processing of some of the acrosomal

matrix components since acrosin-null mice exhibit a delayed dispersal of the acrosomal matrix (Adham *et al.*, 1997, Yamagata *et al.*, 1998b). It is thought that the alkalization of the acrosomal contents is dependent on an influx of calcium into the acrosome since digitonin-permeabilized guinea pig spermatozoa undergo acrosomal matrix dispersion in response to 2.0 mM  $\text{CaCl}_2$  (Noland, 1990).

During acrosomal exocytosis, some components of the acrosomal matrix undergo post-translational modifications coincident with their release from the acrosome. These events are associated with the disassembly or breakdown of the acrosomal matrix, probably involving proteolytic cleavage by acrosin. The alkalization of the milieu surrounding the acrosomal matrix components leads to the proteolytic processing of proacrosin itself to active acrosin during acrosomal exocytosis in several species (Baba *et al.*, 1994b, Brown and Harrison, 1978, Green, 1978, Meizel, 1972, Nuzzo *et al.*, 1990, Tesarik *et al.*, 1990). In guinea pig sperm, proacrosin is detected in immunoblots of mature sperm, but is not detected in A23187-treated sperm, suggesting that the protein (or at least certain antigenic sites) had been modified during acrosomal exocytosis (Kim *et al.*, 2001b). Related to this conclusion, protease activity consistent with the activation of proacrosin to a lower molecular weight form of acrosin was detected in the soluble acrosomal components released from sperm during acrosomal exocytosis. Another guinea pig acrosomal matrix protein, AM50 (50,000 *M*) co-localizes with proacrosin in the M3 zone of the acrosomal matrix and is proteolytically processed to AM50<sub>AR</sub> (43,000 *M*) coincident with its release from the sperm during acrosomal exocytosis (Westbrook-Case *et al.*, 1994). The conditions under which this cleavage pattern occurs are consistent with proteolysis by acrosin. In addition, the AM50 to AM50<sub>AR</sub> cleavage occurs at acrosin-specific sites (Foster and Gerton, unpublished data), further evidence that this protease may function in the dispersion of the acrosomal matrix during acrosomal exocytosis. Thus, proteolytic activation of proacrosin and processing of AM50 corresponds with the disassembly of the acrosomal matrix during acrosomal exocyto-

sis. AM67, the guinea pig homologue of mouse ZP3R, does not appear to undergo processing during AM disassembly in the guinea pig (Kim *et al.*, 2001b).

In the mouse model, homologues of several guinea pig acrosomal matrix proteins (proacrosin, proacrosin-binding protein, and AM67/sp56/ZP3R) are present in the acrosome, but how these proteins are assembled into an acrosomal matrix is not currently understood. Resistance to extraction by Triton X-100 is a hallmark of AM proteins, and under these conditions it is possible to visualize a matrix-like material in the acrosome by indirect immunofluorescence (Kim *et al.*, 2001a) and scanning electron microscopy (Fig. 4). Mouse sp56/ZP3R is converted to a lower molecular weight form coincident with its release from the sperm into the supernatant during the course of spontaneous acrosomal exocytosis (Kim, Buffone, and Gerton, unpublished results). While a mouse homologue of AM50 would be a prime candidate substrate for acrosin, so far this protein has not been detected in the sperm acrosome.

Taken as a whole, the acrosomal matrix contains a set of proteins that interact with each other to form the particulate structure within the membrane boundaries of the acrosome. Furthermore, additional studies suggest that some matrix proteins may interact with either the outer or inner acrosomal membrane proteins. As previously mentioned, zonadhesin, which is considered an acrosomal matrix protein in mature boar and hamster sperm is initially synthesized as an acrosomal membrane protein that becomes proteolytically processed and incorporated into the matrix as the spermatid/sperm matures (Bi *et al.*, 2003, Olson *et al.*, 2004). In addition, inner acrosomal membrane proteins such as CD46 (membrane cofactor protein) may also influence the dispersion of acrosomal matrix components during the course of acrosomal exocytosis; males null for CD46 display a higher incidence of spontaneous acrosomal exocytosis and heightened fertilizing ability, suggesting that CD46 may have some role in regulating sperm acrosomal exocytosis (Inoue *et al.*, 2003).

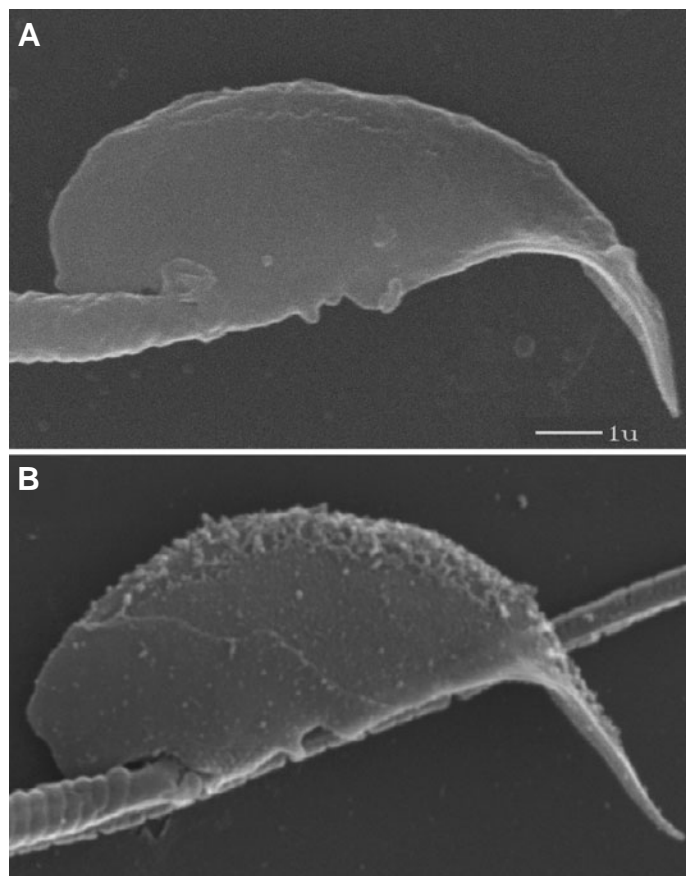
The microenvironment existing around the acrosomal matrix also influences its properties. Besides pH and proteolysis, other aspects of the milieu around the sperm undergoing exocytosis may also influence the state of hydration of the matrix, causing it to function more like a gel and impacting the rate of diffusion of substances trapped in the matrix. In the case of various osmoticants, it is possible to obtain fusion of the outer acrosomal membrane with the plasma membrane without the loss of the acrosomal matrix; under these circumstances, the sperm must be examined by electron microscopy to demonstrate that membrane fusion occurred (Green, 1991).

### Zona adhesion

The interaction between sperm and the ZP leads to the activation of sperm signaling events and consequently stimulates acrosomal exocytosis, which is marked by the fusion of the plasma membrane with the outer acrosomal membrane and the eventual release of the acrosomal contents (Yanagimachi, 1994). We refer the reader to other studies for a discussion of the communication between the sperm plasma membrane and the zona pellucida as well as the signaling pathways that are activated following these interactions (see for example the review by Kopf (2002)). For the purposes of this discussion we note that it

is often presumed that the outer acrosomal and plasma membranes must be intact for sperm to be capable of recognizing and binding to the ZP. However, there are several reports in the literature that sperm at various stages of acrosomal exocytosis may be competent to bind to the ZP (Huang *et al.*, 1981, Morales *et al.*, 1989, Valdivia *et al.*, 1999), suggesting that components from the interior of the acrosome, such as acrosomal matrix proteins or proteins on the inner acrosomal membrane, are also involved in the attachment of the sperm from some species to the zona. Indeed, some of the technical difficulty of establishing whether “acrosome-intact” or “acrosome-reacted” sperm bind to the ZP could have arisen due to the lack of appreciation of the acrosomal matrix and its exposure during intermediate stages of acrosomal exocytosis (see comments below concerning assays of acrosomal status).

A large number of sperm molecules have been demonstrated to have affinity for the ZP and to be involved in sperm-ZP interactions, these include attachment, binding, and penetration of the ZP. The initial attachment is apparently not particularly species-specific (Bedford, 1977, Hartmann *et al.*, 1972). This is followed by a tight binding between sperm and the ZP, which is species specific. Tight binding is differentiated from attachment



**Fig. 4. Scanning electron micrographs of mouse sperm.** (A) An acrosome-intact sperm shows some rippling of the membranes over the acrosome – perhaps in an early stage of acrosome reaction. (B) A demembrated sperm that was treated with 1% Triton X-100 and protease inhibitors shows acrosomal matrix material along the anterior edge of the acrosome.

by the resistance of sperm to being removed from the ZP by a physical force such as repeated pipetting or centrifugation (Bleil and Wassarman, 1980a, Saling and Storey, 1979).

It is not our purpose to compare and contrast the various zona pellucida-binding proteins and attempt to rank them in terms of relative importance. Of the many proteins that have been characterized as having some role in sperm-zona interactions; a few of these are truly associated with the surface of acrosome-intact sperm (such as  $\beta$ -1,4-galactosyltransferase (Shur and Hall, 1982) and SED-1 (Ensslin and Shur, 2003)) while others have been conclusively demonstrated to be components of the acrosomal matrix (see discussion above concerning specific acrosomal matrix components). The observations that so many acrosomal matrix proteins are involved in zona pellucida binding suggests that the interactions between the acrosomal matrix and the zona pellucida may be more akin to the interface between extracellular matrices of two interacting cell types rather than a lock and key mechanism displaying species-specific binding. The one exception may be zonadhesin which exhibits species-specific ZP-binding (Hardy and Garbers, 1995). In any event, sperm interaction with the ZP seems to be a very redundant process since the targeted deletion of SED-1,  $\beta$ -1,4-galactosyltransferase, ZPBP1, ZPBP2, or proacrosin does not necessarily result in a complete block to sperm-zona pellucida binding (Baba *et al.*, 1994a, Ensslin *et al.*, 2007, Lin *et al.*, 2007, Lu and Shur, 1997). Rather, the subfertility or infertility seen with these gene targeted null animals results from defects in acrosome biogenesis, sperm morphogenesis, or dysregulation of acrosomal exocytosis. Finally, although a protein like proacrosin may not be absolutely essential for fertilization, its presence may provide an advantage when competing with sperm lacking that protein (Adham *et al.*, 1997).

### Zona penetration

For many years, it was presumed that the process was enzymatic and dependent upon acrosin. However, in the mouse, this enzyme was eliminated as a possible zona lysin by the gene knockout studies from the Baba and Engel laboratories demonstrating that acrosin-null male mice are perfectly fertile (although fertilization is slower due to delays in the dispersal of the acrosomal matrix) (Adham *et al.*, 1997, Baba *et al.*, 1994a, Yamagata *et al.*, 1998b). It has been proposed that other, membrane-bound proteases may be responsible for the penetration of the mouse zona pellucida (Honda *et al.*, 2002, Yamagata *et al.*, 1998a). Alternatively, some investigators have proposed that a non-hydrolytic process may be involved in penetrating the zona pellucida. For example, in the case of the abalone, the vitelline lysin functions in a stoichiometric fashion to disassemble the vitelline coat in a non-enzymatic manner (Lewis *et al.*, 1982). Whether the mammalian sperm acrosome contains a protein with a similar activity has not been determined. The review by Bedford (1998) addresses inconsistencies in the literature that challenge the premise of zona enzymatic lysis. He discusses largely unnoted idiosyncratic features of the behavior of fertilizing spermatozoa in the eutherian mammals and analyzes the evidence for and the evidence against the enzymatic hydrolytic concept. The mechanism whereby a sperm penetrates the zona pellucida is surely in need of further study.

Another issue concerning the attachment and penetration of the zona pellucida by sperm is: once a sperm has bound to the ZP,

how does it release from its initial site and move forward without falling off? It does not matter whether the sperm uses an enzymatic mechanism or some other way to create a path through the ZP, it still must release from the original point of contact and move forward without losing its "grip". We conceptualize that the nature of the acrosomal matrix may assist in the following manner. Any adhesion mediated by the acrosomal matrix will occur at the outer surface. As the matrix is gradually dispersed, proteins will be shed from the outer surface of the matrix, exposing new acrosomal matrix proteins for renewed adhesion to the zona pellucida. In this manner, the sperm may ratchet its way through the ZP as it is cyclically gripping new zona molecules while leaving behind a trail of shed acrosomal matrix proteins (Gerton, 2002).

### Role of ZP in acrosome interactions

Other than being a barrier to sperm penetration, the zona pellucida influences sperm in two other ways. It contains ligands for sperm binding and signaling molecules that affect acrosomal exocytosis (Dean, 2007, Wassarman, 2005). In addition, assays *in vitro* have implicated some of the carbohydrate residues in mediating sperm-zona interactions in mice (Bleil and Wassarman, 1988, Florman and Wassarman, 1985, Miller *et al.*, 1992) although recent studies with genetically manipulated mice call into question the roles of terminal galactose or N-acetylglucosamine residues on the zona pellucida glycans (Williams *et al.*, 2007). Following fertilization, egg cortical granules exocytose their contents, which modify the zona matrix so that additional sperm do not bind or penetrate (Ducibella *et al.*, 1993, Miller *et al.*, 1993a).

A recent paper published by Baibakov *et al.* (2007) challenges some aspects of the current model for events at the interface of the sperm and the zona pellucida. They showed that transgenic mouse sperm adhering to the zona of mouse eggs retained green fluorescent protein (GFP) for an extended period of time and proposed that sperm binding is not sufficient to induce acrosome exocytosis. The authors articulated a model of sperm-egg recognition in which the cleavage status of ZP2 determines whether the three-dimensional zona matrix will be permissive (ZP2 intact) or non-permissive (ZP2 cleaved) for sperm adherence, independent of fertilization and cortical granule exocytosis. The observed ability of sperm to remain acrosome-intact (as assayed by the presence of GFP) – despite hours-long adherence to ZP – is inconsistent with the ligand-receptor signal transduction model in acrosome exocytosis. To address this enigma, they proposed that a 'mechanosensory' signal generated by the penetration of the sperm through the zona pellucida matrix is sufficient for inducing acrosomal exocytosis. Unreconciled with this paradigm are the many, rather unambiguous observations that acid-solubilized ZP or purified ZP3 can stimulate acrosomal exocytosis (Bleil and Wassarman, 1980b, Bleil and Wassarman, 1983, Kopf, 2002). From our perspective, any model for acrosomal exocytosis must take into account the microenvironment at the site of sperm-zona pellucida interaction as well as the nature of the acrosomal matrix. For example, several studies have noted that GFP loss from the acrosomes of transgenic sperm on the zona is slow and interpret this to mean that acrosomal exocytosis has not occurred (Baibakov *et al.*, 2007, Nakanishi *et al.*, 1999). However, we have demonstrated an intermediate of mouse acrosomal exocytosis in which GFP-transgenic mouse sperm have exposed acrosomal matrix material (as demonstrated by the binding of beads coated

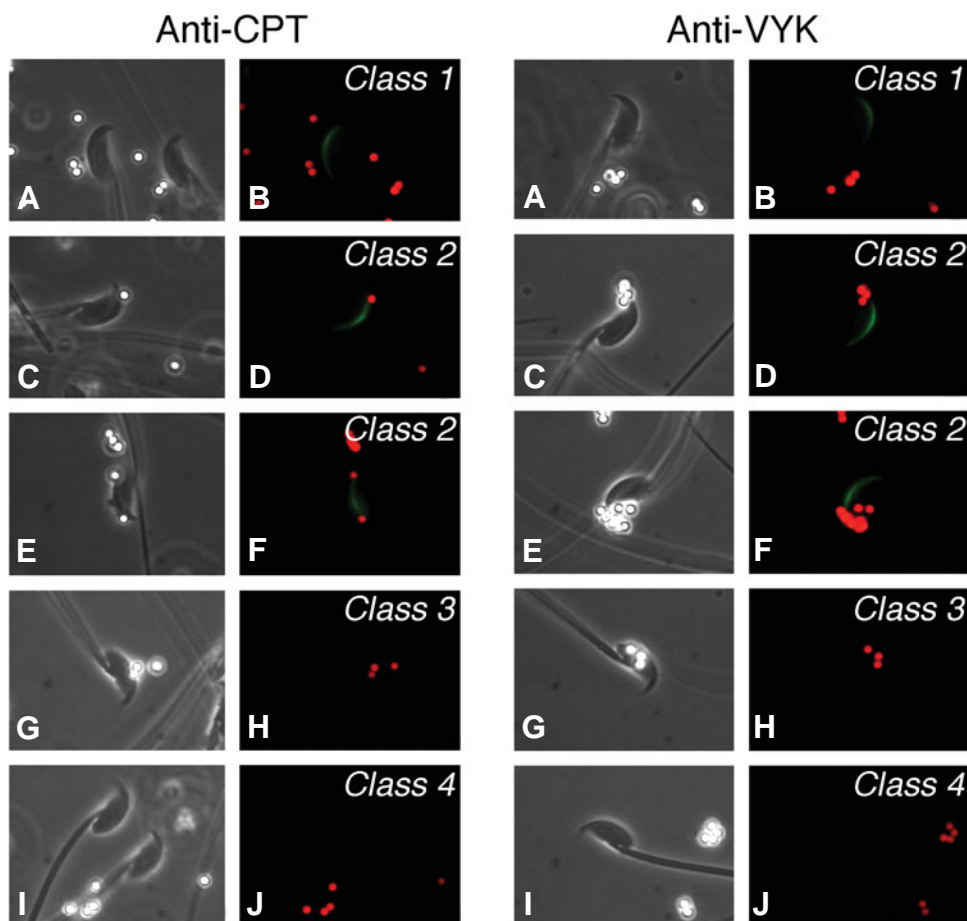
with anti-sp56 antibodies) but still retain GFP in their acrosomes (Kim and Gerton, 2003). To explain the conundrum of sperm bound to the ZP retaining GFP, we envision that the biophysical environment at this site is not fully appreciated in these studies and that the glycoprotein nature of the acrosomal matrix and ZP components may create a gel-like microenvironment. If so, the diffusion of GFP away from the acrosomes of sperm embedded in the surface of the ZP may be impeded relative to sperm swimming free in solution. These observations underscore the need to challenge and re-evaluate current paradigms of sperm-ZP interactions.

#### Acrosome transitions and differential release of acrosomal proteins

We concur with other scientists that acrosomal exocytosis is not an all or none event where the acrosome is either “intact” or “reacted”. As determined by transmission electron microscopy of human sperm undergoing acrosomal exocytosis, six stages can be identified, with the intermediate ones involving loss of acrosomal matrix material while outer membranes appear to retain their integrity (Stock and Fraser, 1987, Yudin *et al.*, 1988). In our own laboratory, we demonstrated that there is a temporal relationship among four stages of acrosomal exocytosis in the mouse: plasma membrane intact over the acrosome (Class 1), plasma membrane initiating fusion with outer acrosomal membrane with soluble components retained within the acrosome (Class 2), soluble acrosomal proteins lost but acrosomal matrix materials still present (Class 3), and most acrosomal components lost (Class 4) (Fig. 5). We conclude that these patterns represent successive transitional stages leading to the complete release of acrosomal components (Kim and Gerton, 2003). To visualize the soluble compartment, we utilized sperm from transgenic mice that carry soluble GFP in their acrosomes and, as a means to assess the exposure of acrosomal matrix components, we tested the ability of these sperm to bind beads coated with antibodies against sp56/ZP3R. The loss of GFP from the acrosomes and the binding of the beads by the sperm undergoing capacitation serve as indicators of different stages of acrosomal exocytosis suggesting that the exposure and release of soluble and matrix acrosomal proteins during spontaneous acrosomal exocytosis is not synchronous but is regulated as the sperm are incubated under capacitating conditions.

Based on this evidence and expanding on the work of others (DiCarantonio and Talbot, 1988, Hardy *et al.*, 1991), we proposed an alternative model for acrosomal exocytosis in mouse that considers the role of these intermediates of exocytosis during capacitation and sperm-ZP interactions and the particulate nature of the acrosomal matrix. We initially tested this hypothesis by examining materials released from guinea pig sperm undergoing acrosomal exocytosis in response to ionophore A23187 and comparing these with the proteins that remained associated with the sperm (Kim *et al.*, 2001b). For instance, soluble compartment component CRISP2 (autoantigen1) was readily released from sperm during acrosomal exocytosis whereas acrosomal matrix protein AM50 remained with the sperm for a longer period of time. AM67 was also released more slowly than CRISP2 but was more readily solubilized than AM50 (in the supernatant fluid 10 minutes after AR induction).

In this differential time-released hypothesis, the AM components of sperm undergoing acrosomal exocytosis remain asso-



**Fig. 5. Simultaneous detection of acrosomal GFP in transgenic mouse sperm and the binding of anti-sp56 antibody-coated FluoSpheres.** GFP expression, driven by the proacrosin promoter, was targeted to the mouse sperm acrosome using the signal sequence and part of the N-terminus of proacrosin. Some sperm bound antibody-coated FluoSpheres, demonstrating the exposure of the acrosomal matrix in those sperm. Four classes of sperm were identified by this assay. Anti-CPT and anti-VYK are two peptide-specific antibodies to mouse sp56. Left panel: Anti-CPT-coated FluoSpheres; Right panel: Anti-VYK-coated FluoSpheres. Paired phase-contrast (A,C,E,G,I) and fluorescence (B,D,F,H,J) (GFP, green; FluoSpheres, red) images of sperm. Reproduced from Kim and Gerton (2003).



ciated with sperm longer than soluble acrosomal proteins. The solubilization of the acrosomal matrix proteins may be coupled with post-translational modifications as they are released from the acrosome. As previously discussed in regard to the disassembly or breakdown of the acrosomal matrix, some proteins are proteolytically cleaved such as proacrosin, AM50 and ZP3R (Kim, Buffone, and Gerton, unpublished results) coincident with acrosomal exocytosis. Guinea pig AM67 is apparently not processed as a consequence of acrosomal exocytosis.

Based on work in the mouse, it is generally thought that the outer acrosomal and plasma membranes must be intact for sperm to be capable of binding to the ZP. Following what is called "primary binding", the OAM and PM fuse, releasing the contents of the acrosome (Yanagimachi, 1994). After the acrosomal contents are released, a second kind of interaction called "secondary binding" of the sperm to the ZP is mediated by proteins on the inner acrosomal membrane. The concept that the OAM and PM are intact when the sperm encounters the ZP arose over 25 years ago from studies in the mouse (Florman and Storey, 1982, Saling *et al.*, 1979, Saling and Storey, 1979). However, there is evidence that guinea pig and human sperm in the process of undergoing acrosomal exocytosis may be competent to bind to the ZP (Huang *et al.*, 1981, Morales *et al.*, 1989). Furthermore, rabbit sperm isolated from the perivitelline space of fertilized eggs and, therefore, presumed to have undergone acrosomal exocytosis, are capable of fertilization in a second insemination (Valdivia *et al.*, 1999). Thus, although the current perspective is that sperm must be "acrosome-intact" to bind to the ZP, one must consider these other findings and realize that the story may be more complex in other species.

One last point to emphasize is the need to consider the type of assay used in various investigations to assess the integrity of the acrosome with particular regard to scoring whether or not exocytosis has occurred. By standard light microscopy, the presence of an intact acrosomal matrix may make the acrosome appear to be intact even though fusion of the membranes has begun to occur. The same may be true with sperm that have been stained with Coomassie blue dye, antibodies against acrosomal matrix components, protease inhibitors, or lectins to detect acrosomal status (Foster *et al.*, 1997, Larson and Miller, 1999, Morales and Cross, 1989, Tollner *et al.*, 2000). Some reporter dyes detect ionic or pH gradients that exist between the external environment of the cell and the internal acrosomal compartment (Rockwell and Storey, 2000, Saling and Storey, 1979). Other methods utilize sperm from transgenic mice that express the green fluorescent protein in the acrosomes (Nakanishi *et al.*, 1999). These latter two assays have a greater likelihood of determining whether the membranes are intact or not. Normally, the gold standard for acrosomal integrity has been transmission electron microscopy, but even this procedure has its shortcomings since only a small section of each cell is normally examined. The use of acrosomally targeted GFP as a reporter of membrane integrity was a major advance but our results indicate that acrosomal matrix proteins can be exposed in individual sperm without the complete or immediate loss of the GFP (Kim and Gerton, 2003). More recently, immunodetection of the exposure of the inner acrosomal membrane protein IZUMO1 has been used as another approach to assess acrosomal status (Yamashita *et al.*, 2007). The main

point to keep in mind is that it is difficult to compare studies that have not used the same method for assessing acrosomal integrity.

## Conclusion

The complex organization of the acrosomal contents was beautifully demonstrated by electron microscopic images of the guinea pig acrosomal apical segment by Fawcett and Hollenberg (1963). Over the years many researchers have contributed to this field so that we now have a better understanding of the structure of the acrosomal matrix and the proteins that comprise it. However, even though there is much evidence to raise doubts about the old "bag of enzymes" paradigm of acrosomal function, this is the model that seems to prevail. In this review we have highlighted some of the work that contributes to a model of acrosomal function that suggests a more complex role for the acrosome in fertilization in which the acrosomal matrix is a scaffold for sperm-ZP interactions that self-regulates by a controlled disassembly mechanism. Future work will test this hypothesis.

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