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Sperm penetration through cumulus mass and zona pellucida

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ABSTRACT Mammalian fertilization requires sperm to penetrate the cumulus mass and egg zona pellucida prior to fusion with the egg. Although sperm penetration through these physical barriers is essential, the molecular mechanism has not yet been completely elucidated. In addition to sperm motility, hyaluronan-hydrolyzing and proteolytic enzymes of sperm have been suggested to participate in the penetration events. Here we focus on the functional roles of hyaluronidase and protease in sperm passage through the cumulus mass and zona pellucida.

KEY WORDS: *egg, zona pellucida, acrosome reaction, penetration, hyaluronidase, proteolysis*

Introduction

Mammalian fertilization involves a precisely ordered set of molecular and cellular events, including sperm penetration through the cumulus mass, sperm adhesion and binding to the zona pellucida (ZP), an extracellular glycoprotein coat surrounding the egg, acrosome exocytosis, sperm penetration through the ZP, and fusion between sperm and egg (Yanagimachi, 1994; Snell and White, 1996; Wassarman, 1999; Florman and Ducibella, 2006). Capacitation of sperm possessing the intact acrosome is required for sperm entry into the cumulus mass (Austin, 1960, Yanagimachi, 1994). Sperm penetration of the cumulus mass is important for fertilization, because the fertilization rate is greatly reduced by removal of the cumulus *in vitro* (Yanagimachi, 1994). As shown in Fig. 1, a large number of experimental results reported so far raise the possibility that the cumulus may function in stimulating the sperm motility and in promoting the acrosome reaction, or may control the sperm access to the egg ZP immobilized by the fibrous network of the cumulus matrix (Yanagimachi, 1994; Florman and Ducibella, 2006). However, the role of the cumulus matrix still remains uncertain. Since cumulus cells are embedded in the extracellular matrix abundant in hyaluronan (hyaluronic acid), sperm hyaluronidase identified approximately 60 years ago has long been believed to catalyze hyaluronan degradation to enable acrosome-intact sperm to penetrate the cumulus matrix and then reach the ZP (Yanagimachi, 1994; Florman and Ducibella, 2006). Although sperm hyaluronidase has not been well characterized, the functional roles of several hyaluronidase isoforms are becoming clear in the mouse in recent years.

Binding of acrosome-intact sperm to the egg ZP induces sperm to undergo fusion between the plasma and outer acrosomal membranes at the anterior region of sperm head, acrosome reaction. As a consequence of the exocytotic event, the acrosomal components are released and interact with the ZP. In general, both mechanical and enzymatic mechanisms have been postulated to explain sperm entry into and penetration through the ZP (Yanagimachi, 1994). Only sperm motility is required for sperm penetration through the ZP in the former mechanism, whereas in the latter, the acrosomal enzymes are important and sperm motility is of second importance. Although acrosomal and membranous trypsin-like serine proteases may play an important role(s) in sperm passage through the ZP (Stambaugh *et al.*, 1969; Miyamoto and Chang, 1973; Frazer, 1982; Yanagimachi, 1994), the true mechanism is still‘unclear. Nevertheless, the physiological significance of the acrosome reaction and subsequent penetration of sperm through the ZP is that the fertilizing sperm acquires the ability to fuse with egg plasma membrane, because the fusion event is achieved only by acrosome-reacted sperm (Yanagimachi, 1994; Florman and Ducibella, 2006).

Despite the importance of sperm penetration through the cumulus cell layer and egg coat in mammalian fertilization, the molecular mechanism is still controversial. Indeed, the general concept, which has been believed for a long time, is overthrown by the experimental results obtained by using gene-knockout

Abbreviations used in this paper: GPI, glycosylphosphatidylinositol; ZP, zona pellucida.

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This paper is dedicated to the late Dr. David L. Garbers.

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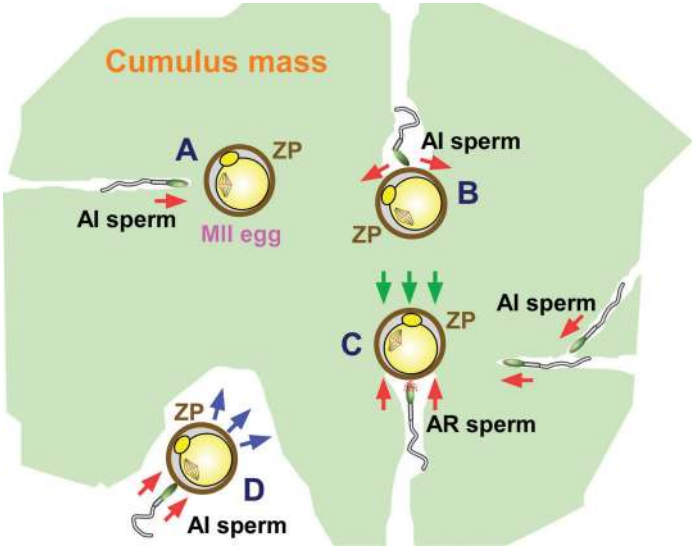

Fig. 1. Schematic model of sperm penetration through the cumulus mass. *Capacitated, acrosome-intact (AI) sperm penetrate the cumulus mass to reach the zona pellucida (ZP) of metaphase II (MII)-arrested egg* **(A)**. *Sperm hyaluronidase(s) may play an important role in sperm penetration through the cumulus and/or in local hyaluronan hydrolysis near or on the ZP surface to enable the proximal region of sperm tail to move freely* **(B)**. *The cumulus may assist acrosome-reacting or acrosome-reacted (AR) sperm in penetrating the ZP, because the egg movement is greatly blocked by the cumulus* **(C)**. *When the cumulus is absent, the eggs migrate due to the force of sperm motility, thus resulting in the block of sperm penetration into the ZP* **(D)**.

mice. Here we review our current knowledge of the mechanism of sperm penetration through the cumulus mass and egg ZP mainly in the mouse.

Sperm penetration through the cumulus mass

Hyaluronidase responsible for degradation of hyaluronan is distributed widely in mammals, insects, leeches, and bacteria (Kreil, 1995; Frost *et al.*, 1996). There are at least two isoforms of hyaluronidase in mouse epididymal sperm. A glycosylphosphatidylinositol (GPI)-anchored membranous protein, Ph-20, which was originally identified as a binding protein to the ZP in guinea pig sperm (Primakoff *et al.*, 1985), is structurally similar to bee venom hyaluronidase. Indeed, Ph-20 exhibits the hyaluronidase activity (Phelps *et al.*, 1988; Gmachl and Kreil, 1993; Gmachl *et al.*, 1993; Thaler and Cardullo, 1995; Cherr *et al.*, 2001). Ph-20 has long been thought to be the sole hyaluronidase involved in the sperm penetration through the cumulus mass, because other sperm hyaluronidase(s) were not characterized well. In 2002, mice carrying a targeted, disruptive mutation in *Ph-20* were produced by homologous recombination in embryonic stem cells (Baba *et al.*, 2002). When total hyaluronidase activity of *n*-octyl-β-D-glucopyranoside protein extracts from acrosome-intact sperm is measured, the Ph-20-deficient sperm extracts still contain approximately 40% of the enzyme activity in the wild-type sperm extracts. SDS-PAGE zymography in the presence of hyaluronan reveals that wild-type and Ph-20-deficient mouse sperm both contain another hyaluronan-hydrolyzing protein, Hyal5, with a molecular size of 55 kDa. Since an enzymatically active 52-kDa protein is completely absent only in Ph-20-deficient sperm, mouse epididymal sperm contain at least two isoforms of hyaluronidase, 52-kDa Ph-20 and 55-kDa Hyal5 (Baba *et al.*, 2002; Kim *et al.*, 2005).

The DNA-derived amino acid sequences indicate that mouse Ph-20 and Hyal5 are initially synthesized as a single-chain polypeptide of 512 and 526 amino acids with calculated molecular masses of 58,520 and 60,807 Da, respectively (Kim *et al.*, 2005). On the basis of the sequence similarity to guinea pig PH-20, the 36-residue sequences of mouse Ph-20 and Hyal5 at the N-terminus are predicted to function as the signal sequence for a nascent protein destined for initial transfer to the endoplasmic reticulum (Fig. 2A). The C-terminal 30- and 26-residue sequences of Ph-20 and Hyal5, respectively, may act as a signal for the GPI attachment, because these two proteins are both GPI-anchored on the sperm membrane. The catalytic (hyaluronidase) domain of Hyal5, which is assigned to the 306-residue sequence at positions 37-342, shares a noticeable degree of sequence identity (65%) with the corresponding region of Ph-20. The locations of 12 Cys residues in Hyal5 are completely conserved with those in Ph-20. Moreover, the entire sequences of Ph-20 and Hyal5 are relatively divergent to those of other mouse hyaluronidases, Hyal1, Hyal2, Hyal4, and Hyalp1 (less than 40% identity). Noteworthy is that Hyal5 is a single-chain molecule structurally distinguishable from Ph-20 consisting of two chains covalently linked by one of two pre-existing disulfide bridges (Hunnicutt *et al.*, 1996a and 1996b; Myles and Primakoff, 1997; Markovi-Housley *et al.*, 2000; Kim *et al.*, 2005). Indeed, the endoproteolytic cleavage-site sequences in guinea pig and mouse Ph-20, Arg^{346}-Ser^{347} and Arg^{347}-Ala^{348} are replaced by Thr^{347}-Met^{348} in mouse Hyal5, respectively (Kim *et al.*, 2005).

In the human and mouse, at least six hyaluronidase-like genes (seven genes for mouse) are clustered as two tightly linked triplets on two chromosomes (Csóka *et al.*, 1999 and 2001; Kim *et al.*, 2005): *HYAL1*, *HYAL2*, and *HYAL3* on human chromosome 3p21 (*Hyal1*, *Hyal2*, and *Hyal3* on mouse chromosome 9F1-F2), and *HYAL4*, *PH-20/SPAM1*, and *HYALP1* on human chromosome 7q31 (*Hyal4*, *Ph-20*, *Hyalp1*, and *Hyal5* on mouse chromosome 6A2). Both *Ph-20* and *Hyal5* are a single-copy gene on the mouse genome, and contain four exons interrupted by three introns, and the translation initiator codon, ATG, is encoded within the second exons of *Ph-20* and *Hyal5*. A computer-aided search on the NCBI Genomic Biology database (http://www.ncbi.nlm.nih.gov/Genomes/) reveals that *Hyal5* is localized 57 kbps away from *Ph-20* on mouse chromosome 6A2 (Fig. 2B). Importantly, the genomic region carrying *Hyal5* is inserted onto the mouse, rat, and possibly hamster chromosomes (Kim *et al.*, 2005). No *Hyal5* orthologous gene is present at least in the corresponding region of the human, pig, cow, and chimpanzee chromosomes. Mouse *Hyal1*, *Hyal2*, and *Hyal3* are ubiquitously expressed in all tissues, whereas expression of *Hyalp1* and *Hyal5* is exclusive in the testis. *Ph-20* and *Hyal4* are expressed specifically in the testis and epididymis (Zhang and Martin-DeLeon, 2001; Kim *et al.*, 2005), and in the placenta and skeletal muscle (Csóka *et al.*, 1999), respectively. *Ph-20* and *Hyal5* mRNAs are first transcribed in the testis of 20-day-old mice, and the mRNA levels progressively increase during testicular development. Moreover, hyaluronan zymography indicates that mouse epididymal sperm may contain only Ph-20 and

Hyal5 (Kim *et al.*, 2005). It should be noted that a research group has reported that Hyalp1, in addition to Ph-20 and Hyal5, is enzymatically functional in sperm penetration of the cumulus in the mouse (Zhang *et al.*, 2005; Miller *et al.*, 2007), although other workers (Reitinger *et al.*, 2007) provide evidence that recombinant Hyalp1 is incapable of hydrolyzing hyaluronan.

Although Ph-20 and Hyal5 are both GPI-anchored on the plasma or acrosomal membranes of mouse epididymal sperm, these two hyaluronidases differ from each other in the subcellular localization (Baba *et al.*, 2002; Kim *et al.*, 2005). Ph-20 is all present on the plasma membrane of acrosome-intact sperm, and the localization appears unchanged after the acrosome reaction. On the other hand, Hyal5 is localized both on the plasma (approximately 60% of total) and acrosomal membranes (~40%). Most or all Hyal5 is released from the sperm membranes during the acrosome reaction. Thus, Hyal5 may function in sperm penetration through the cumulus mass, and in the local hyaluronan hydrolysis near or on the surface of the egg ZP to enable the proximal region of sperm tail to move freely (Kim *et al.*, 2005). It is also suggested that Ph-20 may compensate in part for the functional roles of Hyal5. In guinea pig and cynomolgus monkey, PH-20 is distributed uniformly on the plasma membrane of sperm head, including the acrosome and equatorial segment (Phelps *et al.*, 1990; Overstreet *et al.*, 1995). Following the acrosome reaction PH-20 is present on the inner acrosomal membrane and on the plasma membrane overlying the equatorial segment, indicating that PH-20 may migrate from the plasma membrane of the posterior sperm head to the inner acrosomal membrane after acrosomal exocytosis. A soluble form of monkey PH-20 is also released during the acrosome reaction (Cherr *et al.*, 1996). It is thus conceivable that the localization of mouse Ph-20 and Hyal5 is essentially similar to that of guinea pig and monkey PH-20 on the acrosome-intact and acrosome-reacted sperm, although we need to ascertain the PH-20 migration during the acrosome reaction.

PH-20 from mouse and other animal origins has been reported to exhibit dual functions: the hualuronan-hydrolyzing and ZP-binding activities (Hunnicutt *et al.*, 1996a and 1996b; Myles and Primakoff, 1997; Cherr *et al.*, 2001). Particularly, PH-20 on the plasma membrane of acrosome-intact sperm and on the inner acrosomal membrane of acrosome-reacted sperm is thought to exhibit the hyaluronidase activity required for sperm penetration through the cumulus mass, and to participate in sperm-ZP binding known as "secondary sperm-ZP binding", respectively (Yanagimachi, 1994; Florman and Ducibella, 2006). Since PH-20 is enzymatically active at acid and neutral pH, the neutral enzyme activity is postulated to function in hyaluronan degradation of the cumulus cell layer during sperm penetration. It is also proposed that the acid-active enzyme may be important for digestion of hyaluronan near or on the ZP surface for sperm binding to and penetration into the ZP (Drobnis *et al.*, 1988; Cherr *et al.*, 1996). However, recent works regarding mouse hyaluronidase (Baba *et al.*, 2002; Kim *et al.*, 2005) have weakened the above two possibilities. Hyaluronan zymography reveals that Hyal5 is enzymatically active in the pH range 5-7, and inactive at pH 3 and 4 (Kim *et al.*, 2005). The hyaluronidase activity of Ph-20 is also detectable only at pH 6 and 7. Thus, the pH dependencies of these two mouse hyaluronidases are similar to each other, but distinguished from that of monkey soluble PH-20. Moreover, no significant difference of the ability to bind cumulus-free, ZP-intact eggs is observed between wild-type and Ph-20-deficient acrosome-intact sperm (Baba *et al.*, 2002). Indeed, ZP-binding assay *in vitro* demonstrates that both Ph-20 and Hyal5 barely exhibit the binding activity, while Adam3 (Shamsadin *et al.*, 1999; Nishimura *et al.*, 2001 and 2004) is capable of binding to the ZP tightly (Kim *et al.*, 2005). Neither Ph-20 nor Hyal5 may function in "secondary sperm-ZP binding."

Male mice lacking Ph-20 are still fertile (Baba *et al.*, 2002),

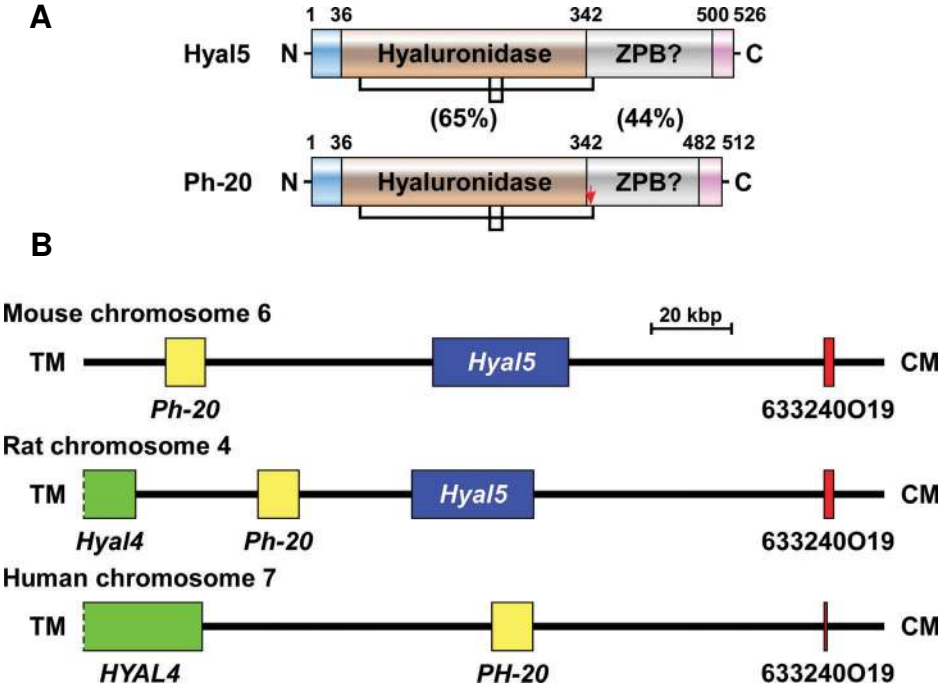

Fig. 2. Mouse sperm contain at least two isoforms of hyaluronidase. (A) *Schematic representation of mouse Hyal5 and Ph-20. These two proteins contain three putative domains: the N-terminal signal peptide domain, catalytic domain as hyaluronidase, and C-terminal recognition domain for attachment to GPI. Although the presence of ZP-binding domain (ZPB) has been postulated, both Hyal5 and Ph-20 barely exhibit the binding activity. Two disulfide-bond arrangements are assigned by the sequence similarities of Ph-20 and Hyal5 with guinea pig PH-20. The sequence identities of the catalytic and possible ZP-binding domains between Hyal5 and Ph-20 are also indicated (percentages in parentheses). An arrow represents the position of the endoproteolytic cleavage site determined in Ph-20.* **(B)** *Insertion of* Hyal5 *onto mouse and rat chromosomes. Locations of* Hyal4 *(*HYAL4*),* Ph-20 *(*PH-20*),* Hyal5*, and RIKEN cDNA 6332401O19 on mouse, rat, and human chromosomes are indicated. The genomic region carrying* Hyal5 *(approximately 60 to 90 kbp) is inserted only onto the mouse and rat chromosomes. TM, telomere; CM, centromere.*

providing evidence that Ph-20 is not essential for fertilization in the mouse. Although wild-type and Ph-20-deficient mouse sperm are capable of fertilizing the cumulus-intact eggs equally 3 h after insemination *in vitro*, the fertilization rate in Ph-20-deficient sperm is significantly lower than that in wild-type sperm only at the early stages (1 and 2 h) after insemination. When the eggs are inseminated with an equally mixed suspension of wild-type and Ph-20-deficient sperm, the mutant sperm are approximately three times slower to fertilize the eggs than wild-type sperm. Moreover, no significant difference in sperm binding to the ZP is found between the wild-type and Ph-20-deficient mice, as described above. Thus, the reduced fertilization rate in Ph-20-deficient sperm is probably due to the delay of sperm penetration through the cumulus mass. It is further suggested that Hyal5 may play the crucial role in sperm penetration through the cumulus mass, possibly in cooperation with Ph-20, because a Hyal5-enriched protein fraction prepared from Ph-20-deficient mouse sperm is capable of dispersing cumulus cells from the cumulus mass (Kim *et al.*, 2005).

Sperm penetration through the egg zona pellucida

Following passage through the cumulus cell layer, sperm gain access to the egg ZP, adhere and bind onto the ZP surface, undergo acrosome exocytosis induced by ZP3 known as one of three ZP glycoproteins in mouse and human, and then penetrate the ZP (Yanagimachi, 1994; Florman and Ducibella, 2006). Thus, the egg coat is the physical barrier for fertilizing sperm before the fusion event between sperm and egg takes place (the structural features of egg ZP and the mechanism of sperm-ZP interactions should be referred to excellent reviews described by P. Wassarman and B. Shur in this issue). Although the true mechanism is still unclear, it has long been hypothesized that sperm penetration through the ZP is dependent entirely on the mechanical force provided by sperm flagellar motility, or partly or mainly dependent on proteolytic hydrolysis of ZP assisted by acrosomal and/or membrane proteases of sperm (Yanagimachi, 1994; Florman and Ducibella, 2006). There are three candidate enzymes involved in sperm penetration through the ZP: acrosomal serine protease acrosin (Baba *et al.*, 1989), serine protease TESP5 GPI-anchored on the sperm membranes (Honda *et al.*, 2002b), and multi-subunit proteolytic holoenzyme proteasome in the acrosome (Sawada *et al.*, 2002; Sutovsky *et al.*, 2004) (Fig. 3). The physiological role of sperm entry into and penetration through the ZP is most likely to remove the acrosome from the fertilizing sperm. The reason is because only acrosome-reacted sperm are capable of fusing with the egg plasma membrane (Yanagimachi, 1994). Also, we must keep in mind that all sperm bound onto the egg ZP does not acrosome-react, and all acrosome-reacting or acrosome-reacted sperm on the ZP surface does not penetrate the ZP.

Acrosin has long been believed to participate in limited proteolysis of the egg ZP to assist sperm in penetrating the egg coat. However, acrosin-deficient mice conclusively show that this serine protease is not essential both for sperm penetration through the ZP and for fertilization (Baba *et al.*, 1994). The loss of acrosin results in delayed sperm penetration of the ZP at the early stages of fertilization *in vitro*, probably owing to the delay in dispersal of acrosomal proteins during the acrosome reaction (Yamagata *et al.*, 1998b). Indeed, when cumulus-intact eggs are inseminated with an equally mixed suspension of wild-type and acrosin-deficient mouse sperm, only wild-type sperm fertilize the eggs, verifying a reduced ability of the mutant sperm to penetrate the ZP or fertilize the eggs (Adham *et al.*, 1997). Moreover, *p*-aminobenzamidine, a competitive inhibitor for trypsin-like serine proteases, blocks penetration of acrosin-deficient sperm through the ZP (Yamagata *et al.*, 1998a). Thus, other *p*-aminobenzamidine-sensitive protease(s) besides acrosin may function in the penetration event, possibly in cooperation with acrosin. This speculation appears reasonable because acrosin is present not on the sperm surface but in the acrosome.

In an attempt to characterize a gelatin-hydrolyzing serine protease with a molecular mass of 42 kDa present in the sperm extracts of wild-type and acrosin-deficient mice (Baba *et al.*, 1994; Yamagata *et al.*, 1998a and 1999), five different serine proteases, termed Tesp1 (testicular serine protease 1), Tesp2, Tesp3, Tesp4, and Tesp5, were identified as candidates for the 42-kDa serine protease (Kohno *et al.*, 1998; Ohmura *et al.*, 1999; Honda *et al.*, 2002b). Of these five serine proteases, only Tesp5, which is identical to esp-1 (Inoue *et al.*, 1998), testisin (Scarman *et al.*, 2001), and tryptase 4 (Wong *et al.*, 2001) belonging to a 21st member of serine protease family Prss21, corresponds to the 42-kDa sperm protein (Honda *et al.*, 2002b). Tesp5 is initially synthesized as a 43-kDa precursor in the testis, and the precursor is converted into the 42- and 41-kDa active enzymes during sperm transport in the epididymis. The enzymatic property of Tesp5 is similar to but distinguishable from those of acrosin and trypsin by the substrate specificity and inhibitory effects of serine protease inhibitors. Importantly, Tesp5 is localized on

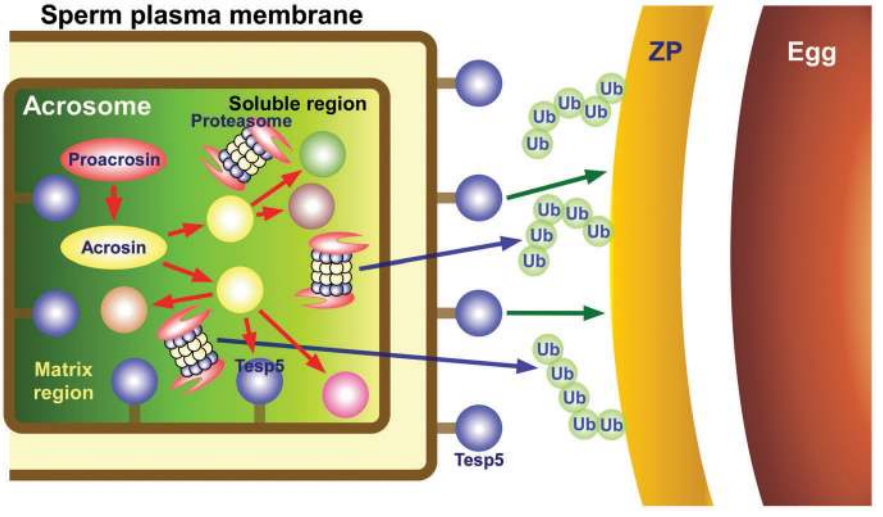

Fig. 3. Schematic representation of three candidate enzymes involved in sperm penetration through the egg zona pellucida in the mouse: *acrosomal serine protease acrosin, serine protease Tesp5 GPI-anchored on the sperm membranes, and multi-subunit proteolytic holoenzyme proteasome in the acrosome. For details see the text. Ub, ubiquitin; ZP, zona pellucida.*

the sperm plasma membrane and as a GPI-anchored protein (Honda *et al.*, 2002b; Kim *et al.*, 2005).

To elucidate the functional role of Tesp5 in fertilization, we have produced mutant mice lacking Tesp5 (unpublished data). Our preliminary data indicate that Tesp5-deficient epididymal sperm are severely defective in the ability to undergo the ZP-induced acrosome reaction, to penetrate the ZP, and to fuse with the egg membrane *in vitro*. Thus, Tesp5 may play an important role in the acrosome reaction and subsequent sperm penetration through the ZP at least in the mouse. Combining the experimental results concerning the phenotypes of acrosin- and Tesp5-deficient mouse sperm, the normal presence of Tesp5 on acrosin-deficient sperm (unpublished data) may explain the ability of acrosin-deficient sperm to penetrate the ZP despite the time delay (Baba *et al.*, 1994). Intriguingly, Tesp5 is activated by addition of exogenous pancreatic trypsin to protein extracts of acrosin-deficient sperm *in vitro* (Yamagata *et al.*, 1998a and 1998b; Honda *et al.*, 2002a), thus implying that Tesp5 may be activated by acrosin released from the acrosome onto the ZP surface after acrosome exocytosis. We have currently produced mutant mice lacking both acrosin and Tesp5. Comparative analysis of acrosin-, Tesp5-, and both-deficient mouse sperm presumably provides a new insight into the mechanism of sperm penetration through the ZP.

In this review, we discuss the possible mechanism of sperm penetration through the egg ZP mainly in the mouse model, but there is a discrepancy in the serine protease systems between mouse and other animal sperm (Honda *et al.*, 2002a). On the basis of gelatin zymography, the level of total gelatin-hydrolyzing activity in mouse sperm is much lower than those in rat and hamster sperm (Yamagata *et al.*, 1999). Notably, rat and hamster sperm barely contain Tesp5, whereas a very small amount of acrosin is present in mouse sperm as compared with rat and hamster sperm. These data imply that the serine protease system in the mouse may differ from those in other animals, including the rat and hamster, and hence the mechanism of sperm penetration through the ZP may be essentially similar but different between the mouse and other mammals.

Two trypsin-like serine proteases, acrosin and spermosin, and ubiquitin-proteasome system in sea squirts and sea urchins are demonstrated to play key roles in sperm binding to and penetration through the egg vitelline envelope (Sawada *et al.*, 1984 and 2002; Sawada and Someno, 1996; Yokota and Sawada, 2007). Ascidian sperm contain 26S or 20S proteasome, ubiquitin, and ubiquitin-conjugating enzyme that are released from the acrosome to ubiquitinate and degrade a sperm receptor, HrVC70, as an analogue of mammalian ZP3 on the surface of the vitelline coat during fertilization (Sawada *et al.*, 2002). Acrosomal proteasomes of sea urchin sperm are responsible for the acrosome reaction and sperm penetration through the vitelline envelope (Yokota and Sawada, 2007). In mammals, proteasome subunits are also localized in the acrosome of human (Morales *et al.*, 2004; Pasten *et al.*, 2005), pig (Sutovsky *et al.*, 2004), and mouse sperm (unpublished data). Pig sperm proteasomes present both in the acrosomal matrix and on the inner acrosomal membrane are released by the acrosome reaction to degrade already ubiquitinated ZP proteins on the ZP surface (Sutovsky *et al.*, 2004). The proteosome inhibitors and antibodies against proteasome subunits are capable of blocking completely sperm penetration through the egg ZP in the mouse and pig, without affecting sperm capacitation, sperm motility, sperm-ZP binding, and acrosome reaction (Wang *et al.*, 2002; Sutovsky *et al.*, 2004). Thus, the ubiquitin-proteasome system is a reliable tool for penetration of a fertilizing sperm through the ZP. However, the functional correlations in limited hydrolysis of the ZP among acrosin, Tesp5, and proteasomes of mammalian sperm (acrosin, spermosin, and proteasomes for the ascidian model) still remain to be uncovered. At any rate, the physiological importance of acrosome exocytosis should be revisited to elucidate the mechanism of sperm passage through the ZP, because acrosin and 26S or 20S proteasome are localized in the sperm acrosome.

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