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[REVIEW]

Ascidian Sperm Lysin System

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ABSTRACT—Fertilization is a precisely controlled process involving many gamete molecules in sperm binding to and penetration through the extracellular matrix of the egg. After sperm bind to the extracellular matrix (vitelline coat), they undergo the acrosome reaction which exposes and partially releases a lytic agent called "lysin" to digest the vitelline coat for the sperm penetration. The vitelline coat sperm lysin is generally a protease in deuterostomes. The molecular mechanism of the actual degradation of the vitelline coat, however, remains poorly understood. In order to understand the lysin system, we have been studying the fertilization mechanism in ascidians (Urochordata) because we can obtain large quantities of gametes which are readily fertilized in the laboratory. Whereas ascidians are hermaphrodites, which release sperm and eggs simultaneously, many ascidians, including *Halocynthia roretzi*, are strictly self-sterile. Therefore, after sperm recognize the vitelline coat as nonself, the sperm lysin system is thought to be activated. We revealed that two sperm trypsin-like proteases, acrosin and spermosin, the latter of which is a novel sperm protease with thrombin-like substrate specificity, are essential for fertilization in *H. roretzi*. These molecules contain motifs involved in binding to the vitelline coat. We found that the proteasome rather than trypsin-like proteases has a direct lytic activity toward the vitelline coat. The target for the ascidian lysin was found to be a 70-kDa vitelline coat component called HrVC70, which is made up of 12 EGF-like repeats. In addition to the proteasome system, the ubiquitination system toward the HrVC70 was found to be necessary for ascidian fertilization. In this review, I describe recent progress on the structures and roles in fertilization of the two trypsin-like proteases, acrosin and spermosin, and also on the novel extracellular ubiquitin-proteasome system, which plays an essential role in the degradation of the ascidian vitelline coat.

Key words: fertilization, ascidian, sperm, egg, vitelline coat, lysin, acrosin, spermosin, proteasome, ubiquitin, trypsin-like enzyme

INTRODUCTION

Fertilization is achieved by the specific interaction between sperm and egg, which is mediated by many molecules of sperm and egg origin (McLeskey *et al*., 1998; Gilbert, 2000). The first step in fertilization is the species-specific binding of sperm to the extracellular matrix of the egg, called the vitelline coat in marine invertebrates and the zona pellucida in mammals. This allows sperm to undergo the acrosome reaction, an exocytosis of the acrosomal vesicle located on the tip of the sperm head (Dan, 1967; Tilney, 1985; Gilbert, 2000). A lytic agent called a sperm lysin is exposed at the surface of the sperm membrane and partially released from the sperm during the acrosome reaction (Hoshi, 1985). These events enable sperm to penetrate through the extracellular matrix of the egg and also enable membrane fusion between sperm and egg (Hoshi, 1985; Hoshi *et al*., 1994) (for the ascidian fertilization processes, see Fig. 1).

In mammals, it has long been believed that an acrosomal trypsin-like protease acrosin [EC 3.4.21.10] is a zonalysin, which allows sperm to penetrate through the zona apellucida (Morton, 1977; McRorie and Williams, 1974; Müller-Esterl and Fritz, 1981; Urch, 1986). However, molecular biological studies using the acrosin-knockout mice clearly showed that acrosin is not essential not only for sperm penetration of the zona pellucida but also for fertilization itself (Baba *et al*., 1994; Adham *et al*., 1997). Although acrosin is not essential for fertilization, there is a significant delay (about 30 min) in *in vitro* fertilization experiment (Baba *et al*., 1994). These results, together with the results of the

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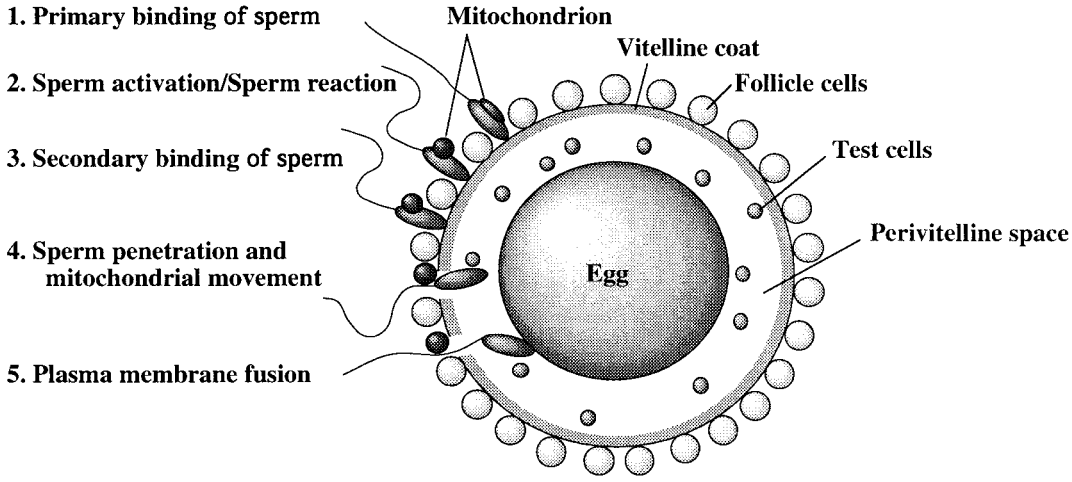


Fig. 1. Ascidian fertilization processes. 1, primary binding of sperm to the vitelline coat; 2, sperm activation (acrosome reaction); 3, secondary binding of sperm to the vitelline coat: 4, sperm penetration and mitochondrial movement; 5, plasma membrane fusion between sperm and egg. A Pro-rich region of the L1 light chain of spermosin and a putative HrVC70-ligand may play a key role in the primary binding of sperm to the vitelline coat. In addition, sperm glycosidases (α-L-fucosidase in the case of *H. roretzi*) appear to be involved in the primary binding of sperm to the carbohydrate moiety of the vitelline coat (Hoshi, 1986; Hoshi *et al*., 1994). After sperm recognizes the vitelline coat as nonself, sperm undergoes acrosome reaction (or sperm reaction), which exposes and partially releases the ubiquitin-proteasome system. The 70-kDa vitelline coat component (HrVC70), which is made up of 12 EGF-like repeats, is a candidate self-nonself recognition molecule in the interaction between gametes. HrVC70 is ubiquitinated by ubiquitin-conjugating enzymes in the sperm exudate (Sakai and Sawada, unpublished data), and then degraded by the activated 26S-like proteasome localized on the surface of the sperm head. Sperm exposes and partially secretes proacrosin upon acrosome reaction (or sperm reaction). Proacrosin seems to play a key role in the secondary binding of sperm to the vitelline coat via the N-terminal-side paired basic residues and C-terminal CUB domains. On the step of plasma membrane fusion, spermosin ECD motif may play an important role in the binding of sperm to the egg plasma membrane. Sperm metalloproteinase is reported to be involved in the membrane fusion between sperm and egg (De Santis *et al*., 1992).

effects of acrosin inhibitors on the acrosome reaction itself, lead to the current hypothesis that acrosin is involved in the dispersal of acrosomal proteins during acrosome reaction (Yamagata *et al*., 1998b). Since trypsin inhibitors are capable of inhibiting the *in vitro* fertilization in acrosin-knockout mice, it seems plausible that a sperm trypsin-like protease other than acrosin, which is sensitive to p-aminobenzamidine, functions as a zona-lysin in mammalian fertilization (Yamagata *et al*., 1998a).

Our studies on the role of lysin molecule(s) or the lysin-system, have benefited by using the ascidian (Urochordata) *Halocynthia roretzi* as an experimental animal, since a large number of sperm and eggs are obtainable from thousands of the *H. roretzi* Type C (Numakunai and Hoshino, 1980), one of the largest ascidians, cultivated in Onagawa Bay, Japan, for human consumption. In addition, these gametes are normally fertilized outside the parent making them very useful for studies on the functions of their sperm proteases.

Ascidians occupy a phylogenetic position between invertebrates and segmented vertebrates (Swalla, 2001). While ascidians are hermaphrodites that release sperm and eggs simultaneously during the spawning season, several ascidians, including *Ciona intestinalis* (Morgan, 1938; Rosati and De Santis, 1978) and most pyurids including *Halocynthia roretzi* (Fuke, 1983), strictly prohibit self-fertilization. As vitelline-coat-free naked eggs are self-fertile, the sperm lysin system is considered to be activated after the sperm recognize the vitelline coat as nonself. Since neither major histocompatibility genes nor immunoglobulin genes have been known in ascidians (Kasahara, 2000), the self-nonself recognition system in ascidian fertilization is also a very intriguing issue.

In the present review, I summarize our current understanding and perspectives of the ascidian sperm lysin-system. The physiological substrate of the lysin system was recently revealed to be a 70-kDa vitelline-coat component VC70, which has sperm receptor activity (Sawada *et al*., 2002a). I would like to describe the molecular features of this novel sperm receptor in ascidians.

IDENTIFICATION OF ASCIDIAN LYSINS

Generally, the vitelline coat of ascidian eggs, which was previously called the chorion, is made up of a filamentous network, too tough to be easily dissolved by pancreatic trypsin or chymotrypsin (Sawada, unpublished) except in *Phallusia mammillata* (Zalokar and Sardet, 1984). Their sperm possesses a very small acrosome (Fukumoto, 1996; Fukumoto and Numakunai, 1993; De Santis *et al*., 1980; Rosati, 1985), which was not clearly detected in earlier studies (Kubo *et al*., 1978). In addition, ultrastructural analysis revealed that the vitelline coat is likely to be chemically or enzymatically dissolved by the sperm lysin rather than that the vitelline coat is mechanically drilled by sperm movement (see Fig. 2). These features led us to investigate the structures and functions of ascidian sperm lysin system. If a cer-

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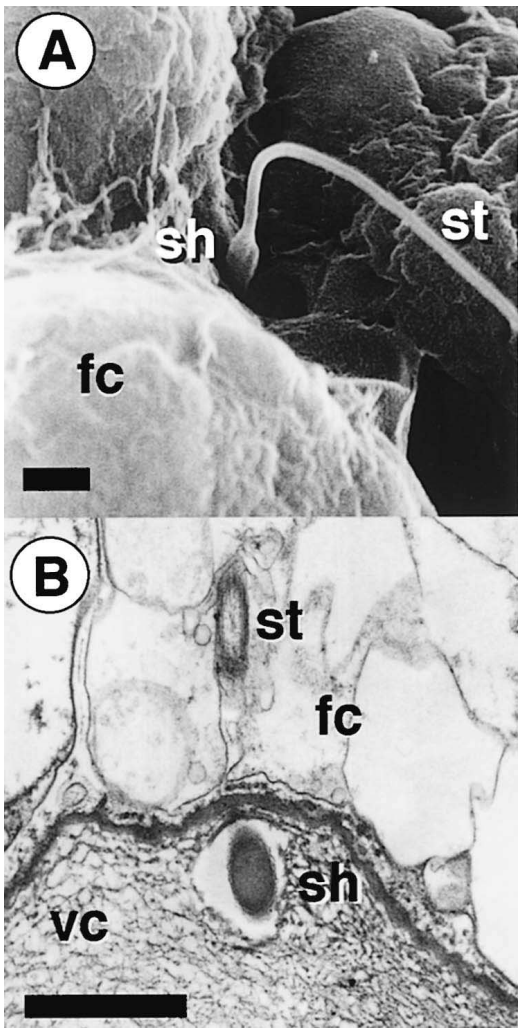


Fig. 2. Electron micrographs of ascidian spermatozoa penetrating through the egg investments. SEM (A) and TEM (B) images of *H. roretzi* oocytes 1 min after insemination. Scale bars indicate 1 μm. sh, sperm head; st, sperm tail; fc, follicle cell; vc, vitelline coat. Note that the vitelline coat around sperm head appears to be dissolved by the sperm lysin.

tain sperm protease is a lysin or one of the members of the lysin-system, the following three requisites must be fulfilled according to Hoshi (1985).

1. Specific inhibitors against the sperm protease(s) must block the fertilization of intact eggs but not of vitelline-coat-free naked eggs, since a target of the lysin has already been removed.
2. Lysin or at least one of the members of the lysin-system must degrade the vitelline coat, that is a physiological substrate of lysin.
3. The lysin molecule(s) must be localized on the sperm head surface during the sperm penetration process of the egg vitelline coat.

Using these criteria, Hoshi and his colleagues (Hoshi *et al*., 1981; see reviews Hoshi, 1985; Hoshi *et al*., 1994) first examined the effects of various protease inhibitors on the fertilization of intact eggs as well as of naked eggs in *H. roretzi*. They showed that of the trypsin inhibitors tested, two microbial inhibitors (leupeptin and antipain) and a proteinaceous trypsin inhibitor (soybean trypsin inhibitor) blocked the fertilization of intact eggs in a concentration-dependent manner (Hoshi *et al*., 1981). Also, chymotrypsin inhibitors including chymostatin and a proteinaceous inhibitor, potato proteinase inhibitor I, inhibited the fertilization of intact eggs. These results indicate that trypsin-like and chymotrypsin-like protease(s) are indispensable for fertilization of *H. roretzi* eggs, and also that the proteases in question function extracellularly. Since the inhibitory effects on fertilization of leupeptin and chymostatin, both of which were selected as representatives of trypsin- and chymotrypsin-inhibitors, respectively, were greatly reduced in the case of naked eggs, it was proposed that trypsin-like and chymotrypsin-like proteases play key roles in sperm penetration through the vitelline coat (Hoshi *et al*., 1981).

From these results, we attempted to purify the trypsin-like proteases from *H. roretzi* sperm using t-butyloxycarbonyl-Val-Pro-Arg-4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-MCA) as a substrate, which was the strongest inhibitor against the fertilization of *H. roretzi* among various trypsin substrates tested (Sawada *et al*., 1982). Two trypsin-like proteases (We designated as ascidian acrosin and spermosin.) were purified to homogeneity (Sawada *et al*., 1984a). The enzymatic properties of ascidian acrosin were similar to those of mammalian acrosin, whereas the enzymatic properties of ascidian spermosin were distinctively different from those of mammalian acrosin, especially in substrate specificity. It was revealed that ascidian spermosin specifically hydrolyzes Boc-Val-Pro-Arg-MCA among various fluorogenic trypsin substrates, suggesting that ascidian spermosin prefers P2 Pro and P1 Arg residues as a substrate (For the definition of P1 and P2 sub-sites, see Schechter and Berger, 1967). We showed that not only acrosin but also spermosin play essential roles in fertilization of *H. roretzi* eggs, by examining the effects on fertilization of leupeptin analogs including Val-Pro-Arg-H derivatives, which are potent spermosin inhibitors (Sawada *et al*., 1984b; Sawada and Someno, 1996). We also used an anti-ascidian spermosin antibody (Sawada *et al*., 1996), and an anti-ascidian acrosin antibody (Kodama, 2000; Kodama *et al*., unpublished data). These trypsin-like proteases, however, seem unlikely to directly degrade the vitelline coat because they have reduced or no degrading activity on isolated vitelline coats, although they contain several domains or motifs in their structures, which are involved in the interaction between sperm and the vitelline coat (see below and Table 1).

Then, we attempted to purify the sperm chymotrypsin-like protease using succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) as a substrate, since this substrate most strongly inhibited the fertilization among the fluorogenic chymotrypsin substrates tested (Sawada *et al*., 1983). The Suc-Leu-Leu-Val-Tyr-MCA-hydrolyzing protease was purified from *H. roretzi* sperm and

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Table 1. Properties of *H. roretzi* sperm proteases

	Acrosin	Spermosin	Proteasome
Molecular mass	55 kDa (Preproenzyme) 35 kDa (Active acrosin)	42 kDa (Preproenzyme) 40 kDa (Type 1) 33 kDa (Type 2	620 kDa (20S proteasome) 930 kDa (26S-like proteasome)
Subunits	1.8-kDa L chain, 34-kDa H chain	Type 1 (12-kDa L1 chain + 28-kDa H chain) Type 2 (5-kDa L1 chain + 28-kDa H chain)	20-35 kDa subunits (20S) 20-110 kDa subunits (26S)
Isoelectric point	5.5 (active form)	6.5 (active form)	6.2 (20S)
Substrate specificity	Broad (Boc-Phe-Ser-Arg-MCA and other trypsin substrates)	Narrow (Boc-Val-Pro-Arg-MCA)	Broad (Suc-Leu-Leu-Val-Tyr-MCA, Z-Leu-Leu-Glu-MCA, Boc-Phe-Ser-Arg-MCA etc.)
Inhibitor	Leupeptin, STI, Dansyl-Leu-Arg-H	Z-Val-Pro-Arg-H, Dansyl-Val-Pro-Arg-H	Z-Leu-Leu-Nva-H (MG115), Z-Leu-Leu-Leu-H (MG132) chymostatin (20S proteasome) Propioxatin A (26S-like proteasome)
Localization	Tip of sperm head, Mitochondrial region (Partially released by sperm activation)	Sperm head (Partially released by sperm activation)	Sperm head Activated by alkaline seawater (Partially released by sperm activation)
Physiological functions	Binding to the 25-, 28-, 30-, 85-, 90-kDa VC components (CUB domain in proacrosin) Binding to VC (Lys56-His57)	Binding to the 28-kDa VC component (Pro-rich region in L1 chain)	Degradation of the vitelline coat (VC70 is degraded by the extracellular ubiquitin-proteasome system)

the enzyme was identified as a high-molecular-weight protease complex designated as a proteasome (Saitoh *et al*., 1993). Two species of the proteasome were isolated: the 620-kDa “20S proteasome” and the 930-kDa “26S-like proteasome” (for the definition, molecular structures and intracellular functions of the mammalian 20S and 26S proteasomes, see review Tanaka, 1998). The ascidian sperm 20S proteasome was potently inhibited by chymostatin, while the ascidian sperm 26S-like proteasome was inhibited by propioxatin A (Saitoh *et al*., 1993). It was also found that the proteasomes are secreted to the surrounding seawater upon sperm activation or sperm reaction that is accompanied by vigorous sperm movement and mitochondrial translocation mediated by Ca^{2+} influx (Lambert and Epel, 1979; Lambert and Koch, 1988). This sperm reaction appears to correspond to the acrosome reaction in sea urchins or mammals. We showed that a 1000-kDa fraction in the sperm exudate, which is a supernatant after centrifugation of the reacted sperm suspension, contained the 26S-like proteasome on the basis of dot blot analysis and that this proteasome-containing fraction showed a strong vitelline coat degrading activity (Saitoh *et al*., 1993). In addition, preliminary results showed that the vitelline-coat-degrading activity of the sperm exudate was inhibited by propioxatin A rather than chymostatin and that propioxatin A is a potent inhibitor against fertilization (Saitoh *et al*., 1993). These results indicate that acrosin, spermosin, and proteasomes are essential for the fertilization of *H. roretzi* eggs, most probably functioning as a member of the lysin-system, and also that the 26S-like proteasome is capable of degrading the vitelline coat component.

Although it has not been well established that the lysin system in *H. roretzi* is similar to the lysin systems of other ascidians, Hoshi (1985) has proposed that both trypsin-like and chymotrypsin-like proteases may be involved in the fertilization of Stolidobranch ascidians including *Halocynthia*, but that only chymotrypsin-like protease(s) including the proteasome is involved in Phlebobranch ascidians including *Ciona* and Ascidia (Hoshi, 1985; Sawada *et al*., 1998; Koch *et al*., 1994). In *Ciona intestinalis*, it is reported that the sperm 24-kDa chymotrypsin-like protease is a vitelline coat lysin, which is able to affect the electron-dense outer layer within the vitelline coat (Marino *et al*., 1992). In addition, we recently showed that the proteasome plays an important role in the fertilization of *C. intestinalis* (Sawada *et al*., 1998). More recent detailed studies by Lambert *et al*. (2002) using several ascidian species revealed that three sperm proteases (acrosin, spermosin, and the proteasome) appear to be present not only in Stolidobranch ascidians but also in Phlebobranch ascidians: the chymotrypsin-like activity is essential for penetration of the vitelline coat, but spermosin and acrosin both function to increase the rate of fertilization (Lambert *et al*., 2002). These results led me to assume that acrosin, spermosin, and chymotrypsin-like proteases including proteasomes play key roles in fertilization of all ascidians, although the extent of their participation in the fertiliza-

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tion process is different in each species. Since the molecular structures and functions of the acrosin and spermosin were recently elucidated, I would like to now review the structures and novel functions of the three sperm proteases from *H. roretzi* in the following sections (see also Table 1).

ASCIDIAN SPERM ACROSIN

A cDNA clone of ascidian acrosin was isolated from a *H. roretzi* gonad cDNA library, and the amino acid sequence was deduced from its DNA sequence (Kodama *et al*., 2001). The ascidian preproacrosin consists of 505 amino acid residues, and the molecular mass was estimated to be 55,003 Da (Figs. 3, 4). The His-76, Asp-132, and Ser-227 residues make up a catalytic triad. By analyses of the N-terminal amino acid sequence and of the amino acid composition of the purified acrosin heavy chain, together with analogy to mammalian acrosin, it is thought that ascidian acrosin is made up of a light chain (residues 20–35) and a heavy chain (residues 36-505). Taking into account the conserved positions of the Cys residues in mammalian acrosin, it is inferred that Cys-21 and Cys-152 are disulfide-bonded.

By motif search analysis, it was revealed that ascidian proacrosin has three potential N-glycosylation sites, paired basic residues (Lys-His (56–57)) in the N-terminal portion of the heavy chain, which are reported to be important for the binding of mammalian acrosin to the zona pellucida (Richardson and O'Rand, 1996; Jones, 1991; Jansen *et al*., 1998; Howes *et al*., 2001; Howes and Jones, 2002), and two CUB domains in the C-terminal portion. Homology in the amino acid sequence between mammalian acrosin and ascidian acrosin is 33–35%. Northern blot analysis showed that a 4.7

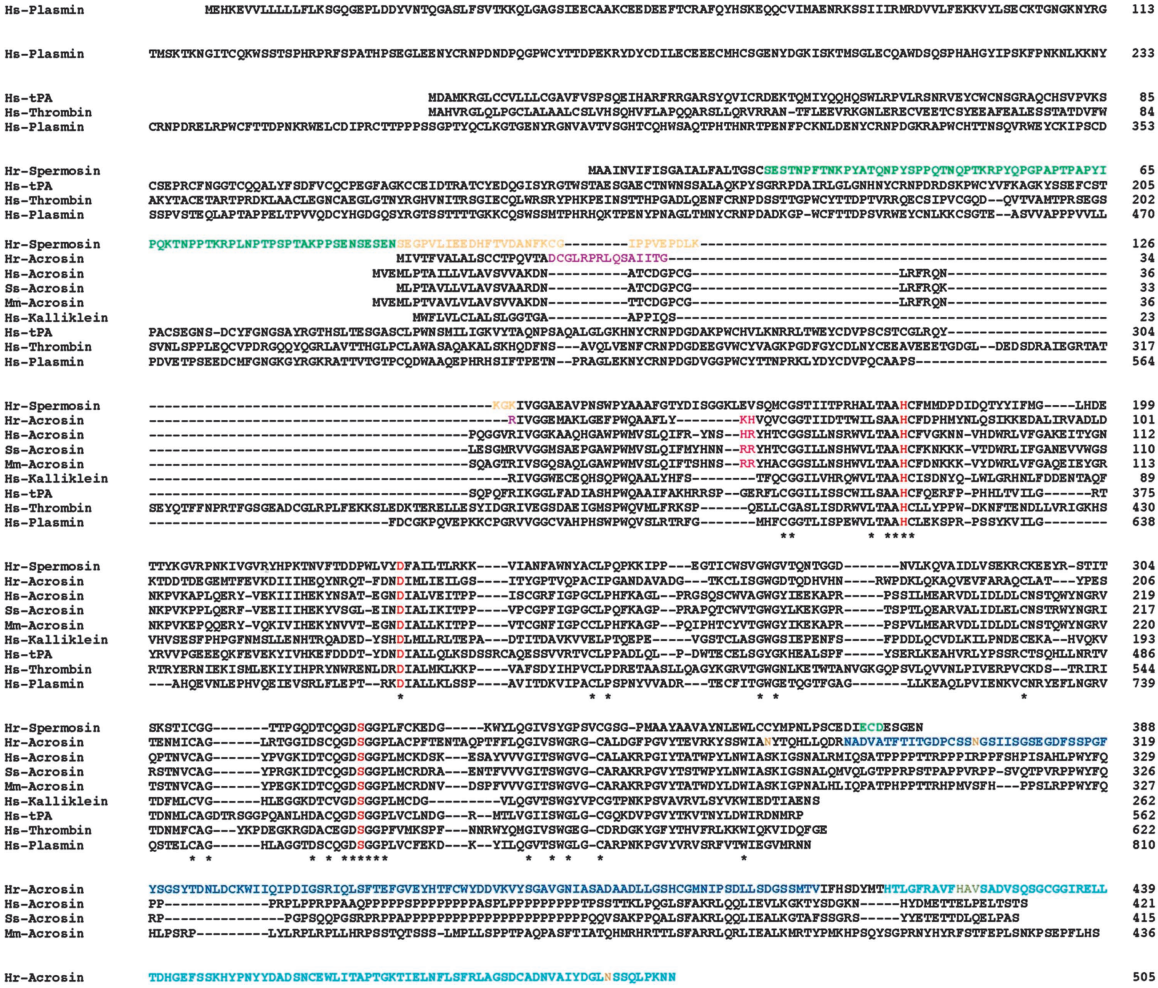


Fig. 3. Sequence alignment of *H. roretzi* spermosin and acrosin with those of trypsin-family proteases by ClustalW program. Identical residues in the sequences among serine protease are indicated by asterisks. Three conserved active-site residues in the S1 subfamily (Barrett *et al*., 1998) of trypsin-like protease are indicated by red. The locations of paired basic residues in the N-terminal regions of ascidian- and mammalian-acrosins are indicated by magenta. L1(ΔL2) region, L2 regions, and ECD motif of spermosin are indicated by green, orange, and pale green, respectively. Light chain, CUB domain 1, CUB domain 2, and HAV motif of ascidian acrosin were indicated by violet, blue, light blue, and olive drab, respectively. Potential *N*-glycosylation sites were indicated by brown. DDBJ/GenBank/EMBL accession numbers are as follows: Hr-Spermosin, *Halocynthia roretzi* spermosin, AB052776; Hr-Acrosin, *Halocynthia roretzi* acrosin, AB052635; Hs-Acrosin (human), Y00970; Ss-Acrosin (pig), J04950; Mm-Acrosin (mouse), D00754; Hs-Kallikrein (human), M25629; Hs-tPA (human), A01465; Hs-Thrombin (human), J00307; Hs-Plasmin (human), A22096.

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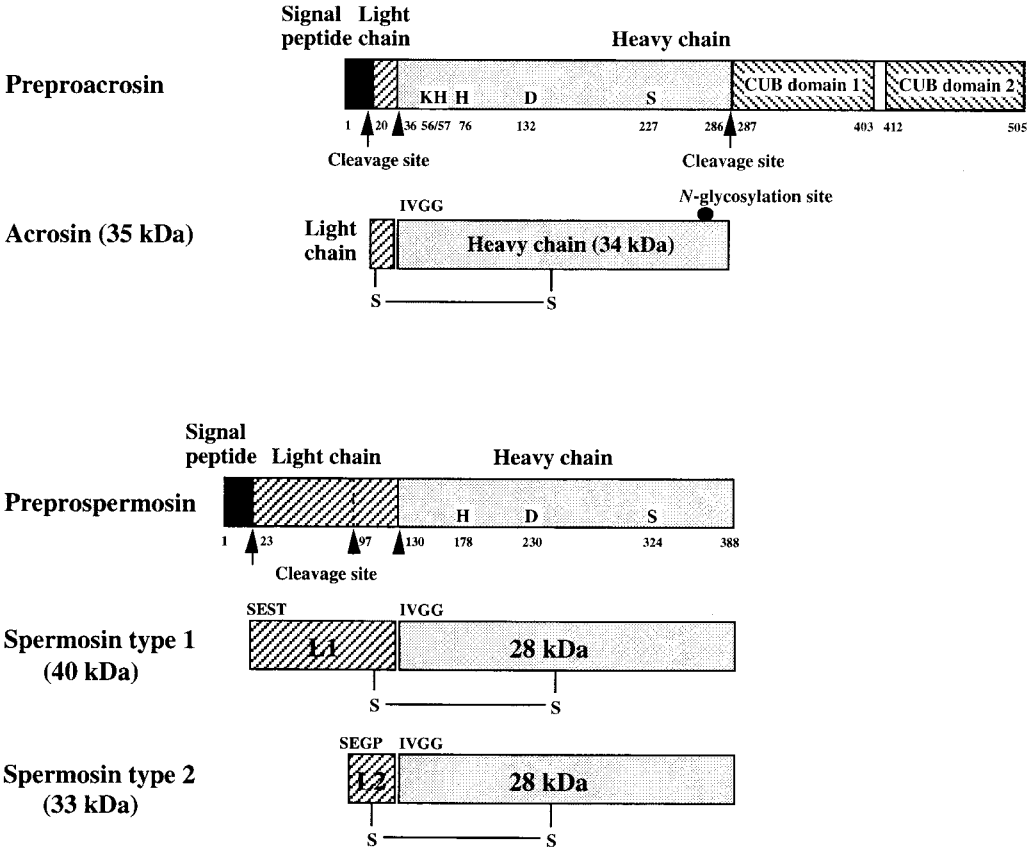


Fig. 4. Schematic representation of the structures of ascidian acrosin and spermosin. Ascidian proacrosin (53 kDa) is thought to be processed to the 35-kDa active acrosin. The active acrosin is made up of a light chain and a heavy chain, both of which are linked by a single disulfide bond. Two types (type 1 and 2) of active ascidian spermosin are made by limited proteolysis from prospermosin. Type 1 spermosin consists of L1 light chain and 28-kDa heavy chain, while Type 2 spermosin consists of an L2 light chain and the same heavy chain.

kb mRNA of *H. roretzi* acrosin is specifically expressed in the gonad but not in other tissues or organs, including hepatopancreas, branchial basket, or intestine (Kodama *et al.*, 2001). Western blot analysis of the sperm extract revealed the existence of 35-, 40-, and 50-kDa proteins in *H. roretzi* sperm, which were recognized by anti-acrosin antibody. Since proacrosin has a molecular mass of 53,052 Da, the 50-kDa protein seems to be proacrosin. Ascidian proacrosin with a molecular mass of 53 kDa appears to be processed to the 35-kDa acrosin via the 40-kDa intermediate protein. Since acrosin was detected by Western blotting in the sperm exudate, ascidian acrosin seems to be secreted or exposed to the sperm cell surface during fertilization (Kodama *et al.*, 2001). The paired basic residues located on the N-terminal side of the heavy chain must play a key role in the binding of ascidian (pro)acrosin to the egg vitelline coat, since the binding ability of a synthetic peptide A1 (AAFLY**KH**VQVCG (residues 51–62 in *H. roretzi* proacrosin)) to the vitelline coat was significantly higher than that of a peptide A1(KH/AA) (AAFLY**AA**VQVCG) to the vitelline coat. These results accorded well with the results obtained with mammalian proacrosin, which showed the importance of the paired basic residues in the interaction between proacrosin and the zona pellucida by using the synthetic peptide (Moreno and Barros, 2000) or by site-directed mutagenesis (Richardson and O'Rand, 1996; Jansen *et al.*, 1998). The paired basic residues in ascidian acrosin may interact with the sulfated *O*-linked glycans of the vitelline coat, which is reported to be involved in gamete interaction (Baginski *et al.*, 1999). Recently, three-dimensional structures of ram and boar acrosin were reported (Tranter *et al.*, 2000). According to their studies, not only the N-terminal paired basic residues but also the other clusters of the basic amino acid residues seem to be crucial in the interaction between (pro)acrosin and the sulfated polysaccharide portion of the zona pellucida (Tranter *et al.*, 2000; Jansen *et al.*, 1998). However, since basic residues other than the N-terminal-side paired basic residues are not observed in the ascidian (pro)acrosin, the above-mentioned basic clusters in mammalian acrosin are unlikely to be essential for the binding of ascidian (pro)acrosin to the vitelline coat.

Several mammalian proacrosins, including the human and porcine proacrosin, but not the rodent proacrosin, contain a Pro-rich region in the C-terminal portion. This region may participate, at least in part, in the binding of proacrosin to the zona pellucida (Urch and Patel, 1991), although there is an objection for the participation of the Pro-rich region in

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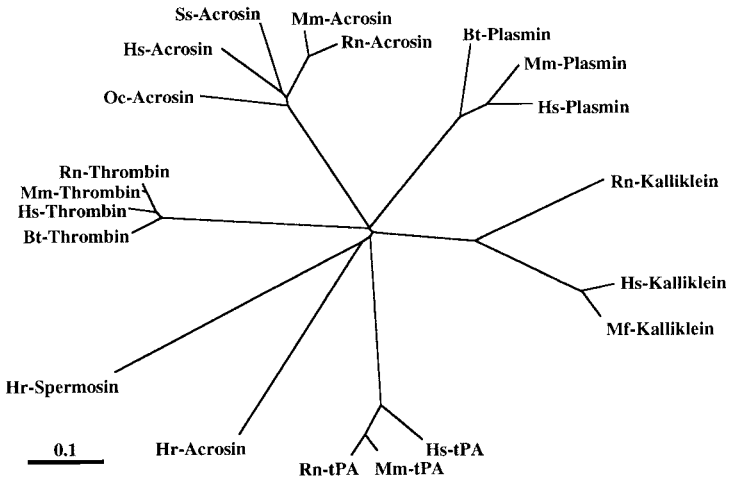


Fig. 5. Dendrogram depicting the relationship between ascidian acrosin, ascidian spermosin and mammalian trypsin family. Clustral W was used to generate alignments of the amino acid sequences of the preproenzyme, which were then displayed with Tree-View. A scale bar indicates the 0.1 amino acid substitutions per residue. The DDBJ/GenBank/EMBL accession numbers of respective proteases are described below. Hr-Acrosin, *Halocynthia roretzi* (ascidian) acrosin, AB052635; Hr-Spermosin, *Halocynthia roretzi* (ascidian) spermosin, AB052776; Ss-Acrosin (pig), J04950; Hs-Acrosin (human), Y00970; Mm-Acrosin (mouse), D00754; Oc-Acrosin (rabbit), U05204; Rn-Acrosin (rat), X59254; Hs-Kallikrein (human), M25629; Mf-Kallikrein (monkey), L10039; Rn-Kallikrein (rat), M19648; Bt-Plasmin (bovine), X79402; Hs-Plasmin (human), A22096; Mm-Plasmin (mouse), J04766; Hs-tPA (human), A01465; Mm-tPA (mouse), J03520; Rn-tPA (rat), A19618; Bt-Thrombin (bovine), J100041; Hs-Thrombin (human), J00307; Mm-Thrombin (mouse), M81394; Rn-Thrombin (rat), M81397.

the interaction between proacrosin and the zona pellucida (Jones, 1991; Howes and Jones, 2002). Whereas the existence of a C-terminal extension in the pro-enzyme is one of the characteristic features of proacrosin in a trypsin family (see Barrett *et al*., 1998), the physiological functions of these extensions of mammalian proacrosin remain unclear. Then, we attempted to elucidate the biological function of the two CUB domains located in the C-terminal portion of ascidian proacrosin, one (CUB domain 1) being complete and the other (CUB domain 2) being incomplete. Two pentadeca peptides derived from CUB domains 1 and 2 of ascidian proacrosin were synthesized and their effects on fertilization were examined (Kodama *et al*, 2001). Very interestingly, we found that the CUB1 peptide is able to strongly inhibit ascidian fertilization in a concentration-dependent manner. We also found that five vitelline-coat components with molecular masses of 25-kDa (N-terminal amino acid sequence: TATARNNNFFVVISTTTYEQMWQ), 28-kDa (not detected), 30-kDa (KGVPAL**HAV**LLEAYEAYESVATNTEEAXIYRNM), 85-kDa (EVTYRKLPESEHAF), and 90-kDa (QLPG) were able to bind to CUB1 peptide-immobilized agarose beads (Kodama *et al*., 2001). Therefore, it is inferred that the CUB1 peptide binds to the vitelline coat, resulting in inhibition of fertilization. It is also interesting to note that the 30-kDa component of the vitelline coat, which is able to associate with the CUB1 peptide, contains a His-Ala-Val sequence (HAV motif), which is an important element in homophilic cadherin interactions (Blaschuk *et al*., 1990). Since the CUB domain 2 also contains a HAV motif, CUB domains 1 and 2 both may be essential in the binding of ascidian proacrosin to the vitelline coat (see Figs. 3 and 4).

Since the occurrence of acrosin homologs in animals other than mammals has not been reported, ascidian acrosin is the first example of a non-mammalian acrosin. Although ascidian acrosin and mammalian acrosin are not classified in the same clade among mammalian trypsin family members by dendrogram analysis, ascidian acrosin may be classified in the clade of acrosin subfamily after elucidation of the sequences of acrosins originated from non-mammalian vertebrates (see Fig. 5). Localization of acrosin in *H. roretzi* sperm was examined by using a fluorescent acrosin inhibitor Dansyl-Leu-Arg-H (unpublished data). The results showed that acrosin is localized on the tip of the sperm head and mitochondrial region. This suggests that acrosin is localized not only in the tip (probably in an acrosome) of the sperm head but also around the mitochondrial region.

ASCIDIAN SPERM SPERMOSIN

An open reading frame of ascidian spermosin cDNA encoded 388 amino acids (Figs. 3, 4), and the molecular mass of preprospermosin was estimated to be 41,896 Da. By analogy, it is thought that the His-178, Asp-230 and Ser-324 residues consist of a catalytic triad of serine protease: ascidian spermosin is classified in a family S1 (trypsin family) of clan SA serine protease. This protease is thought to be a novel trypsin-like protease, rather than an ascidian homolog of mammalian acrosin, since there are no paired basic residues on the N-terminal portion and also there is no C-terminal extension in the pro-enzyme (Fig. 3). Dendrogram analysis also supported this conclusion (Fig. 5). The amino acid sequence of preprospermosin shows 32% homology to that of mouse plasma kallikrein and 27%

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homology to those of human and ascidian acrosins. A single 1.9 kb transcript of spermosin was detected in the gonads only of *H. roretzi*. SDS-PAGE of the purified spermosin afforded a single band with a molecular mass of 28 kDa under reducing conditions, whereas it gave two bands with molecular masses of 33 and 40 kDa under nonreducing conditions. The N-terminal amino acid sequence analyses of these bands revealed that the 28-kDa protein is a heavy (H) chain of spermosin, while the 30-kDa spermosin consists of the same H chain (residues 130–388) and an L1 light chain (residues 97–129), and the 40-kDa spermosin consists of the same H chain (residue 130–388) and an L2 light chain (residues 23–129). These results indicate that there are two forms of ascidian spermosin with different light chains in *H. roretzi* sperm. The light and heavy chains in both forms appear to be linked through a single disulfide bond via the Cys-116 and Cys-251.

The L1 chain, but not the L2 chain, contains a Pro-rich region, although it is not so abundant compared with the number of Pro residues in the C-terminal portion of non-rodent proacrosin. Then, we tested whether the Pro-rich region of spermosin is involved in the binding of spermosin to the vitelline coat. Three GST fusion proteins (GST-L1, GST-L2, and GST-L1(ΔL2)) were incubated with the Triton X-100-solubilized vitelline coats and adsorbed to glutathione-Sepharose beads. After washing, the vitelline coat components, which are capable of associating with the GST-L1 or GST-L1(ΔL2) fusion protein, were analyzed by SDS-PAGE. From these experiments, we found that a 28-kDa component (SAXARNQNFG) of the vitelline coat is capable of binding to the Pro-rich region in the light chain of spermosin, although it is presently unclear whether the CUB1 peptide-interacting 28-kDa component of the vitelline coat is identical to this protein or not.

With respect to the putative functional domain in the C-terminal region of ascidian spermosin, it is interesting to note that ascidian spermosin contains the ECD (Glu-Cys-Asp) motif in the C-terminal portion. Mammalian sperm ADAM (a disintegrin and metalloproteinase) family protein fertilin β contains an ECD motif, instead of a typical RGD (Arg-Gly-Asp) motif, in the disintegrin domain (Zhu *et al*., 2000). Recent study revealed that this motif is involved in the binding of fertilin β molecule to the integrin molecule located at the egg plasma membrane (Zhu *et al*., 2000). In the case of fertilin β, the ECD motif is enough for its binding to the egg plasma membrane (Zhu *et al*., 2000). It is reported that fusion proteins consisting of maltose-binding-protein and ECD-containing peptides are capable of binding to the egg plasma membrane (Zhu *et al*., 2000). Taking into account these results, it is very likely that spermosin may function as a multi-functional protein, which is responsible for the sperm binding to the egg plasma membrane, as well as its binding to the vitelline coat.

The presence or absence of spermosin homologs in mammalian sperm is an intriguing issue. In connection with this, it is interesting to note that mouse epididymis sperm extract gave a 27-kDa single band by Western blotting with the anti-spermosin antibody (Kodama *et al*., 2002). This implies the occurrence of a spermosin-like protein in mouse sperm.

ASCIDIAN SPERM UBIQUITIN-PROTEASOME SYSTEM

As described above, the ascidian sperm proteasome seems to play a pivotal role in the lysin-system in *H. roretzi*. In order to prove that the sperm proteasome functions as a lysin, elucidation of the localization of the proteasome in the sperm cell surface during fertilization, particularly in the sperm penetration of the vitelline coat, appears to be essential. It is widely believed that proteasomes are mainly localized within the cytoplasm and are involved in the degradation of intracellular short-lived proteins including cell-cycle regulators (cyclins, p53,Cdk-inhibitors, etc.) and transcription factors and their regulators (NF-κB, IκB, Myc, Mos, etc.) (see reviews Hershko and Ciechanover, 1998; Peters *et al*., 1998). Because of the widespread evidence that proteasomes function within cells we had to very carefully investigate from several different angles whether they actually function extracellularly in ascidian fertilization. First, when the cell surface proteins in intact and reacted sperm were labeled with cell-impermeable NHS-LC-biotin and were then extracted with 20 mM CHAPS, 30–40% of the proteasomal activity was adsorbed to avidin-immobilized agarose regardless of the occurrence of the sperm reaction, indicating that a substantial amount of the proteasome is localized on the cell surface of both intact and reacted sperm (Sawada *et al*., 2002b). Second, the proteasome activity was partitioned into both the detergent phase and the aqueous phase when treated with Triton X-114, suggesting the existence of a membrane-associated form of the sperm proteasome (Sawada *et al*., 2002b). Third, the 26S-like proteasome was purified from the membrane fraction of ascidian sperm by affinity chromatography on an anti-proteasome antibody column, suggesting the existence of the 26S-like proteasome in the sperm membrane fraction on the basis of subunit composition (Sawada *et al*., 2002b). Fourth, the Suc-Leu-Leu-Val-Tyr-MCA-hydrolyzing proteasome activity was detected on the sperm head region only after sperm activation, which is strongly inhibited by the proteasome inhibitor MG115 (Sawada *et al*., 2000b). Fifth, ascidian fertilization was strongly inhibited by the anti-proteasome antibody (Sawada *et al*., 2002b). From these results, we concluded that the sperm proteasome is localized on the sperm head surface and functions extracellularly in the ascidian fertilization. In human sperm, it is reported that the proteasome is localized on the sperm head surface by immunocytochemistry (Wójcik *et al*., 2000). In addition, antibodies obtained from male infertility patients were found to recognize sperm surface proteins, which were identified as proteasome subunits (Rümke, 1954; Bohring *et al*., 2001). These results led me to speculate that proteasomes are localized extracellularly on the sperm cell surface of the head region.

We then investigated the physiological substrate of the

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proteasome. Vitelline coats labeled with ^{125}I were digested with crude sperm proteasome extract. A 70-kDa major component of the vitelline coat (HrVC70) was found to be degraded (Fig. 6A). Since the 26S-like proteasome purified from *H. roretzi* sperm did not efficiently degrade the HrVC70 molecules, we examined the likelihood that ubiquitination might be necessary for degradation of HrVC70 by the purified proteasome. To assess this possibility, HrVC70 was ubiquitinated by reticulocyte ubiquitin-conjugating enzymes E1/E2/E3, and then digested with the purified sperm 26S-like proteasome in the presence of ATP. The degradation of HrVC70 molecules was specifically monitored by Western blotting using the anti-HrVC70 antibody. The ubiquitinated HrVC70 molecules were detected as high-molecular-weight bands on SDS-PAGE, and a 45-kDa band, which was recognized by FK2 antibody, a multi-ubiquitin chain-specific monoclonal antibody, was newly formed. Since the amino acid sequence of this 45-kDa band was identical to the sequence of ubiquitin, the 45-kDa band is thought to be a degradation intermediate of the ubiquitinated HrVC70.

In order to elucidate the structure of HrVC70, a physiological substrate of the proteasome, we attempted to isolate a cDNA clone encoding HrVC70 from the *H. roretzi* gonad λgt11 cDNA library. A single open reading frame encoded the 120-kDa HrVC70-precursor (HrVC120) of 1162 amino acids (Fig. 6C). The deduced amino acid sequence contains a sequence (21–58) that corresponds to the N-terminal amino acid sequence of HrVC70. A homology search revealed that HrVC120 contains thirteen EGF-like repeats (*albeit* its incomplete homology in the 13th EGF module), a mammalian zona pellucida glycoprotein (ZP)-homologous domain, and a single transmembrane domain in the C-terminal region (Fig. 6C). As in the case of mammalian zona pellucida glycoproteins, HrVC120 contains furin cleavage site (Arg-Lys-Arg-Arg (1044-1047)). This protein also contains 4 potential *N*-glycosylation sites at the C-terminal side, and 5 potential *O*-fucosylation sites (Cys-X-X-Gly-Gly-Ser/Thr-Cys) located between the second and third Cys residues in EGF-domains 3, 5, 7, 9, and 11. By C-terminal sequence analysis the HrVC70 molecule (68,800 Da) appears to be generated by a trypsin-like proteolytic cleavage at the C-terminal side of the Arg-668 residue located between the 12th and 13th EGF-like repeats. These findings were further supported by MADLI-TOF mass spectrometric analysis, and amino acid composition analysis.

By Northern blot analysis, a high level of expression of HrVC120 mRNA was observed in the gonad but not other organs or tissues. Interestingly, even- and odd- numbered EGF-like repeats appear to be more evolutionarily related with one another: EGF-5, EGF-7, and EGF-9 are highly homologous, while EGF-6, EGF-8, and EGF-10 are highly related to one another (Fig. 6D). HrVC120 (residues 773-1032) shows a significant similarity to components of the mammalian zona pellucida (*i.e.*, 19% identity with human sperm receptor ZP3), although it is presently unknown whether the ZP-domain is excised and attached to the vitelline coat.

Transmembrane proteins with EGF-like repeats including Notch/Delta are involved in cell-cell interaction and recognition. Therefore, we examined the binding activity of HrVC70 toward *H. roretzi* sperm. First, we immobilized the HrVC70 molecule to a NHS-activated agarose beads (Affi-Gel 10), and the beads were tested for the ability to bind sperm. The number of DAPI-labeled sperm bound to each HrVC70-immobilized agarose bead was found to be significantly higher than those of control beads, indicating that HrVC70 has sperm receptor activity (Fig. 6B). In *Caenorhabditis elegans*, the sperm membrane protein Spe-9, which has 10 EGF-like repeats in its extracellular milieu, is reported to be essential for fertilization (Singson *et al.*, 1998), though its precise role is unknown. Several mutants with amino acid substitutions in EGF modules display a defect in sperm-egg interaction. By analogy with the Notch/

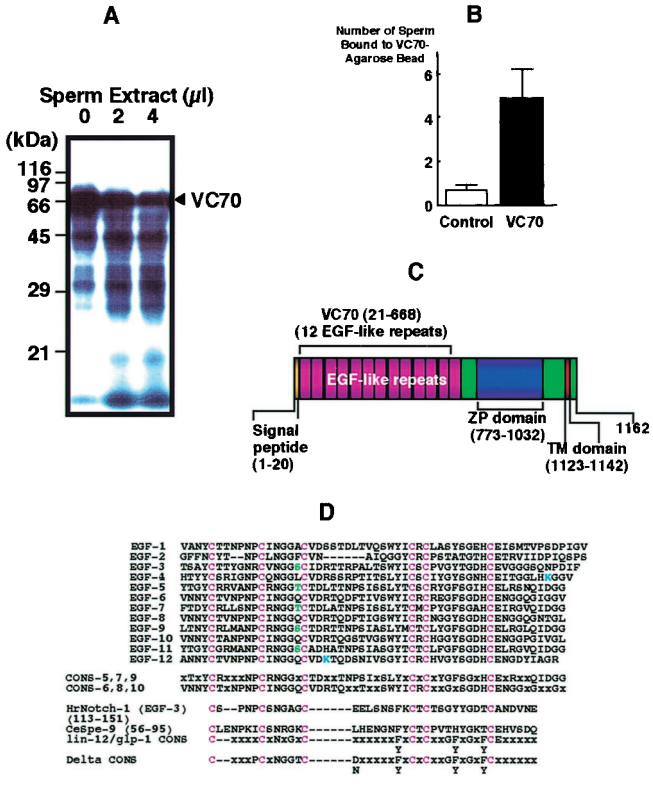


Fig. 6. Structure and function of HrVC70. (A) HrVC70 from ^{125}I-labeled vitelline coat was specifically degraded by the ascidian sperm extract as is revealed by SDS-PAGE and autoradiography. (B) HrVC70 has sperm receptor activity. (C) The domain structure of HrVC120. ZP domain, zona pellucida glycoprotein-homologous domain; TM domain, transmembrane domain. (D) Similarity in EGF-like repeats in HrVC70 and their comparison with *H. roretzi* Notch-1 (HrNotch-1) (3rd EGF-repeat: residues 113–151), *C. elegans* sperm membrane protein Spe-9 (CeSpe-9) (residues 56-95). Consensus sequences in the odd- (CONS-5, 7, 9) and even-numbered EGF-like repeats (CONS-6, 8, 10) in HrVC70 are aligned with the consensus sequences in lin-12/glp-1 (lin-12/glp-1 CONS) and Delta protein (Delta CONS). Two Lys residues in HrVC70 are shown by blue, and cysteine residues are indicated by magenta. Potential *O*-fucosylation sites are indicated by green.

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Delta interaction, we hypothesize that the physiological sperm ligand for HrVC70 is a Spe-9-like transmembrane protein possessing several EGF-like repeats. Alternatively, since the Pro-rich region of spermosin L1 chain is able to bind to the 70-kDa protein of the vitelline coat (Kodama and Sawada, unpublished results), one of the candidate ligands for HrVC70 may be spermosin located on the surface of the sperm head.

The HrVC70 has only two Lys residues (Lys-234 and Lys-636). One (Lys-234) is located in the linker region between EGF-4 and EGF-5 domains, while the other (Lys-636) is located in the region between the 3rd and 4th Cys residues in EGF-12 domain (see Fig. 6D). Two GST-fusion proteins carrying an HrVC70 fragment (residues 227-270), derived from EGF-4 and EGF-5 domains, and its lysine to arginine mutant were purified by glutathione-Sepharose chromatography, and were incubated in the presence of *H. roretzi* sperm exudate, ATP/Mg and ^{125}I-labeled ubiquitin. The wild-type GST-fusion protein (GST-VC70$_{227–270}$ WT) was efficiently multi-ubiquitinated, but the Lys-substituted mutant (GST-VC70$_{227–270}$ (K234R)) was not, indicating that the HrVC70, a main component of the vitelline coat, is multi-ubiquitinated *in vitro* at least via the lysine 234 by a putative ubiquitin-conjugating enzyme system in the sperm exudate (Sawada *et al*., 2002a).

In order to assess the issue whether HrVC70 is ubiquitinated *in vivo*, the vitelline coats isolated from the unfertilized and fertilized eggs (5 min after insemination) from the same individual, were subjected to SDS-PAGE and Western blotting using the anti-HrVC70 and FK2 antibodies. High-molecular-weight bands due to ubiquitinated HrVC70 molecules were detected only in the fertilized eggs under our experimental conditions (Fig. 7A). Furthermore, FK2 antibody recognized the high-molecular weight bands by Western blotting, the mobilities of which are identical to those of the high-molecular weight bands of HrVC70 detected by the anti-VC70 antibody on the basis of Western blotting. These results indicate that HrVC70 is ubiquitinated upon sperm-egg interaction under *in vivo* conditions. In addition, the HrVC70 is a preferred or sole substrate for ubiquitination after insemination among the various vitelline coat components under the *in vivo* conditions (see Fig. 7A).

Immunocytochemistry by using FK2 monoclonal antibody showed that strong FK2 immunofluorescence due to the presence of ubiquitin-conjugates was observed on the vitelline coat after, but not before, insemination (Fig. 7B). These results also show that the vitelline coat proteins are ubiquitinated upon fertilization in *H. roretzi*. In order to examine whether this ubiquitination is essential for fertilization, we tested the effect of the FK2 antibody on fertilization and found that the FK2 antibody potently inhibited the fertilization in a concentration-dependent manner. These results suggest that the extracellular formation of multi-ubiquitin chains catalyzed by a ubiquitin-conjugating enzyme system, probably derived from the sperm, is essential for ascidian fertilization.

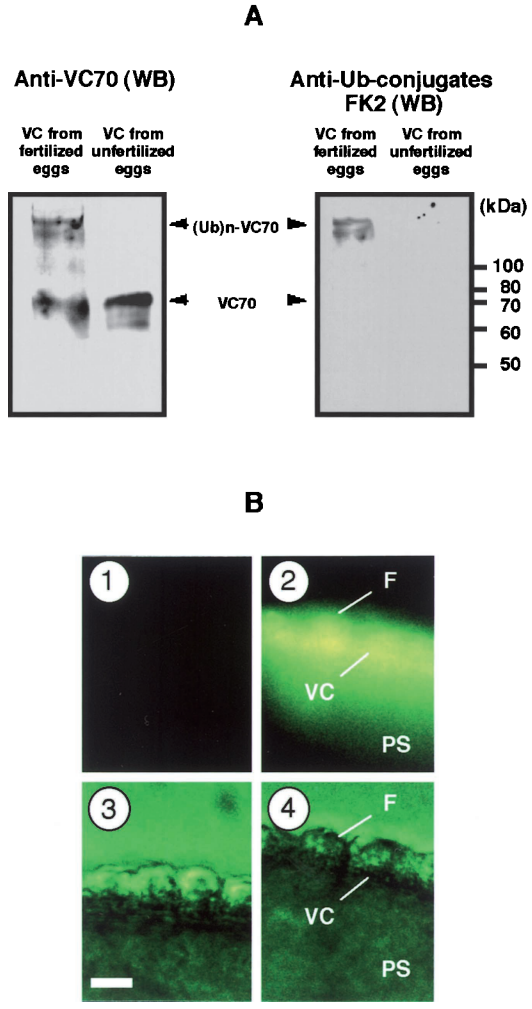


Fig. 7. *In vivo* ubiquitination of HrVC70 in the vitelline coat of *H. roretzi* eggs upon insemination with sperm. **(A)** *Left panel*. Vitelline coats (VCs) were isolated from unfertilized and fertilized (5 min after insemination) eggs, and then subjected to SDS-PAGE, followed by Western blotting (WB) using an anti-HrVC70-peptide antibody. *Right panel*. The same membrane in left panel was reprobed with FK2 antibody. (Ub)n-VC70, high-molecular-weight ubiquitinated HrVC70. **(B)** Immunocytochemistry of *H. roretzi* vitelline coats before (*1*, *3*) and after insemination (*2*, *4*) using the FK2 antibody as a primary antibody and the FITC-conjugated anti-mouse IgG goat antibody as a secondary antibody. The upper (*1*, *2*) and lower (*3*, *4*) panels represent the fluorescence and bright field respectively, under a fluorescence microscope.

In connection with the extracellular ubiquitin, there is an intriguing report showing that free ubiquitin is detected in human seminal plasma at a high concentration (2–19 μg/ml) (Lippert *et al*., 1993). Although the local concentration of ubiquitin at the site of sperm-egg interaction in *H. roretzi* remains to be determined, it appears likely that free ubiquitin is secreted from somatic cells such as follicle cells as well as from germ cells. These reports support our current conclusion that the extracellular ubiquitin-proteasome system plays a decisive role in ascidian fertilization. This may be first definitive demonstration that ubiquitin has any function outside the cell.

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HrVC70 IS A CANDIDATE SELF-NONSELF RECOGNITION MOLECULE

In *H. roretzi*, immature oocytes are self-fertile, but mature oocytes are self-sterile (Fuke and Numakunai, 1996). This suggests that a specific molecule, which is responsible for self-nonself recognition, may become associated with the vitelline coat during the oocyte maturation process. In addition, as suggested by Morgan (1938) and verified by Byrd and Lambert (2000) using *C. intestinalis*, acidic seawater-treated oocytes become self-fertile. This suggests that something is added to the vitelline coat during final maturation that can be removed. Since the vitelline coat is thought to be the place for self-nonself recognition in sperm-egg interaction, we investigated the components of the vitelline coat by SDS-PAGE. Very interestingly, we found that a large amount of HrVC70 was detected in the vitelline coats isolated from mature oocytes, but not from the vitelline coats of immature oocytes. In addition, we noticed that the HrVC70 was found to be detached and released to the surrounding seawater after treatment with acidic seawater (Sawada, unpublished data; Sawada and Yokosawa, 2001; Sawada *et al*., 2000). These results indicate that the HrVC70 is a candidate self-nonself recognition molecule in the interaction between sperm and the egg in addition to its role in sperm adhesion. Furthermore, we recently determined that the amino acid sequence of HrVC70 molecule appears to be different from each individual (Sawada and Yokosawa, 2001; Sawada *et al*., 2000). Since a single amino acid substitution in EGF-like domain is enough for changing the binding ability of EGF-like repeat-containing molecules in cell-cell interaction (Singson *et al*., 1998), it seems likely that a point mutation in the EGF-like repeats would cause alteration in the binding capacity between the HrVC70 molecule and its sperm ligand.

CONCLUDING REMARKS AND PERSPECTIVES

We revealed that a novel extracellular ubiquitin-proteasome system plays a key role in the fertilization of the ascidian *Halocynthia roretzi*. This indicates that the extracellular ubiquitin-proteasome system is an essential component of the sperm recognition, binding and penetration system in ascidian fertilization. If such is the case, several questions will arise. What is the mechanism of the secretion or exposure of the proteasome to the surface of the sperm head? What is the mechanism of the activation of the proteasome in response to the sperm reaction? What kinds of molecules or systems are involved in the extracellular ubiquitination to the HrVC70 molecules? Where are ubiquitin and ATP derived from? Although these problems remain to be solved in the future, the results that the proteasome is localized on the surface of the sperm head in human, together with the fact that ubiquitin content in seminal plasma is considerably high, led me to propose that an extracellular ubiquitin-proteasome system may function not only in ascidian- but also in mammalian-fertilization. It has been recently reported that ubiquitination of sperm mitochondria is responsible for the specific degradation of paternal mitochondria after sperm entry into the ovum (Sutovsky *et al*., 1999) and also that the abnormal sperm appears to be degraded by the extracellular ubiquitination system (Sutovsky *et al*., 2001). Although the ubiquitin-mediated degradation of sperm mitochondria is not necessary for ascidian fertilization because the sperm mitochondrion never enters the perivitelline space, the extracellular ubiquitination system toward abnormal sperm may be related to the ubiquitination system observed in ascidian fertilization.

Our preliminary data show that a HrVC70 homolog is present also in *Halocynthia aurantium*, though we have not yet succeeded to find a HrVC70 homolog in *Ciona intestinalis* (Ban, Yokosawa and Sawada, unpublished data). In *H. aurantium*, the HrVC70-homologous protein appears to contain 13 EGF-like repeats instead of 12 repeats as in the case of *H. roretzi*.

In mammals, it is known that acrosin is not essential for fertilization or sperm penetration of the zona pellucida, but is involved in the dispersal of acrosomal contents during acrosome reaction (Yamagata *et al*., 1998b). In ascidians also, we failed to detect classical lysin activity in sperm acrosin or spermosin preparations. Taking into account the binding ability of ascidian acrosin and spermosin to the vitelline coat, ascidian acrosin and spermosin may be important in sperm binding to the vitelline coat rather than in the degradation process of the vitelline coat. Cloning and characterization of the vitelline coat components, which are capable of interacting with sperm proteases, are interesting issues that remain to be solved.

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