Chapter 20
Allorecognition and Lysin Systems During Ascidian Fertilization

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Abstract Ascidians (primitive chordates) are hermaphroditic animals that release sperm and eggs almost simultaneously, but several species, including *Halocynthia roretzi* and *Ciona intestinalis*, show strict self-sterility. In *H. roretzi*, a 70-kDa vitelline coat (VC) protein consisting of 12 EGF-like repeats (HrVC70) appears to be a promising candidate for the self/non-self-recognition (or allorecognition) system during gamete interaction. After sperm recognizes the VC as non-self, the sperm extracellular ubiquitin-proteasome system appears to degrade HrVC70, allowing sperm to penetrate through the VC with the aid of sperm trypsin-like proteases.

In *C. intestinalis*, egg-side highly polymorphic fibrinogen-like ligands on the VC (v-Themis-A and v-Themis-B) and cognate sperm-side hypervariable region-containing polysystin-1-like receptors (s-Themis-A and s-Themis-B) seem to be responsible for allorecognition in gamete interaction. Recently, we noticed that a novel pair of v-Themis-B2 and s-Themis-B2 and an acid-extractable VC protein called Ci-v-Themis-like may take part in gamete interaction or allorecognition. When sperm recognizes the VC as self, the sperm undergoes a drastic Ca^{2+} influx, which is one of the major intracellular self-recognition responses within sperm, resulting in sperm detachment from the VC or in sperm becoming quiescent. These allorecognition systems and self-recognition responses within sperm are very similar to the self-incompatibility system in flowering plants.

Keywords Allorecognition • Ascidian • Lysin • Protease • Proteasome • Self-incompatibility • Sperm

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20.1 Introduction

Sexual reproduction is an excellent reproductive strategy to elicit genetic diversity in the next generation, but most flowering plants and several animals, including ascidians, are hermaphrodites. Therefore, it seems beneficial for these hermaphroditic organisms to acquire a self-sterility or self-incompatibility system to avoid self-fertilization.

Ascidians (tunicates) are hermaphroditic marine invertebrates (primitive chordates), but several species, including *Halocynthia roretzi* and *Ciona intestinalis*, that release sperm and eggs almost simultaneously show strict self-sterility. In animal fertilization, it is indispensable for sperm to penetrate through the proteinaceous egg investment called the vitelline coat (VC) in marine invertebrates and zona pellucida (ZP) in mammals (McRorie and Williams 1974; Morton 1977; Sawada 2002). Because VC-free eggs are self-fertile in ascidians, it is thought that a self/non-self-recognition system, which is also referred to as an allorecognition system, is involved in the interaction between sperm and the VC of eggs. Therefore, sperm lysin, a lytic agent that makes a small hole in the VC for sperm passage, must be activated or exposed to the sperm surface after sperm recognizes the VC as non-self (Sawada 2002).

To investigate the allorecognition and lysin systems during ascidian fertilization, we have been mainly using two solitary ascidian species, *H. roretzi* and *C. intestinalis*, because readily fertilizable sperm and eggs are easily obtained. *H. roretzi* is particularly useful for biochemical studies because this species is cultured in Japan for human consumption and also large quantities of gametes can be easily collected (Sawada 2002). On the other hand, *C. intestinalis* is very useful for genetic analysis and molecular biological approaches because a genome database is available and also genetic analysis can be easily carried out using adults that are sexually matured within 3 months. In this chapter, we summarize the sperm proteases, including the ubiquitin-proteasome system (UPS), that are involved in ascidian fertilization as lysins and also the allorecognition or self-incompatibility system functioning in fertilization of *H. roretzi* and *C. intestinalis*.

20.2 Allorecognition and Lysin Systems in *H. roretzi*

20.2.1 Allorecognition in H. roretzi

It is known that self-sterile *H. roretzi* eggs become self-fertile when the eggs are treated with acid (pH 2–3) for 1 min and also that immature and VC-free eggs are self-fertile (Fuke 1983; Fuke and Numakunai 1996). We therefore speculated that a certain allorecognition factor is attached to the VC during oocyte maturation and that the putative factor may be detached from the VC or denatured by a weak acid. To test this possibility, VCs were isolated from immature and mature eggs and

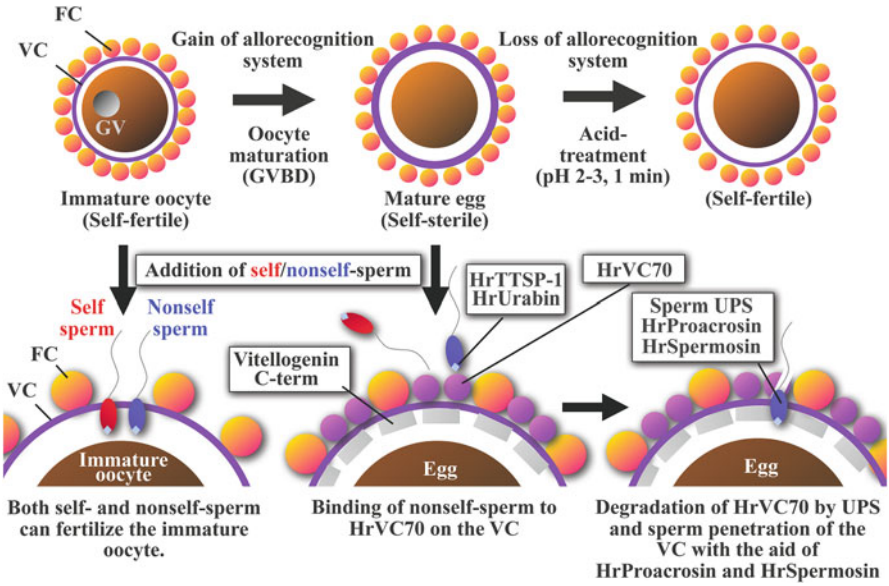

Fig. 20.1 Working hypothesis for the possible roles of sperm proteases as a lysin and allorecognition system in *Halocynthia roretzi*. In *H. roretzi,* HrVC70, consisting of 12 EGF-like repeats with a high degree of polymorphism among individuals, attaches to the vitelline coat (*VC*) during oocyte maturation, resulting in prevention of self-fertilization. The sperm-side binding partners of HrVC70 appear to be HrTTSP-1 and HrUrabin, and if the sperm recognizes the VC as non-self, sperm UPS may be activated, enabling sperm to penetrate through the VC. HrVC70 has a nature to be extracted by weak acid, which allows self-sperm to penetrate through the VC. During sperm passage through the VC, sperm trypsin-like proteases HrProacrosin and HrSpermosin also play some important roles by enabling movable binding to the VC components, which are C-terminal fragments of vitellogenin

subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We found that the amount of the 70-kDa main component, HrVC70, of mature eggs is markedly larger than that of immature oocytes, suggesting that HrVC70 is attached to the VC during oocyte maturation. In addition we noticed that HrVC70 is easily solubilized and extracted from the isolated VC by 1–10 mM HCl and that sperm are capable of binding to HrVC70-immobilized agarose beads. We also found that non-self sperm rather than self-sperm efficiently bound to HrVC70 and that HrVC70 isolated from non-self eggs more efficiently inhibited the fertilization than did that from self-eggs (Sawada et al. 2004). From these results, together with the fact that HrVC70 shows a high degree of polymorphism among individuals and that even a single amino-amino acid substitution in EGF-like repeat regions in Notch protein is sufficient to cause Notch-signaling diseases (Artavanis-Tsakonas et al. 1995), we concluded that HrVC70 is a promising candidate for allorecognition in fertilization of *H. roretzi*. Although it is still unclear whether the amino-acid substitution in HrVC70 is actually responsible for allorecognition during gamete interaction in *H. roretzi*, all the biochemical data so far obtained support the idea that HrVC70 is a key protein involved in allorecognition (Fig. 20.1).

As sperm-side binding partners of HrVC70, HrTTSP-1 (type II transmembrane serine protease) and *u*nique *RA*FT-derived *bin*ding (HrUrabin), partner for HrVC70 (a GPI-anchored CRISP family protein) have been identified by yeast two-hybrid screening (Harada and Sawada 2007) and far Western blot analysis, respectively (Urayama et al. 2008). HrTTSP1 has an estimated molecular mass of 337 kDa and contains 23 CCP/SCP/Sushi domains, 3 ricin B domains, and 1 CUB domain in its extracellular region. Although HrTTSP-1 contains several potentially interesting domains, its biological function has not been studied in detail. In contrast, HrUrabin appears to play a key role in fertilization because an anti-HrUrabin antibody can inhibit fertilization and also binding of allo-recognizable sperm to HrVC70 agarose beads. However, HrUrabin had little polymorphism among individuals and showed no difference in its binding ability to HrVC70 from self-eggs and non-self eggs. Therefore, it is thought that HrUrabin is unable to directly distinguish self- and non-self-HrVC70 but participates in the process of allorecognition in a broad sense, because the antibody against HrUrabin potently inhibited binding of allo-recognizable sperm to HrVC70 (Urayama et al. 2008) (Fig. 20.1).

There is an orthologue of HrVC120, a precursor of HrVC70, in another ascidian species, *Halocynthia aurantium*, which is a close relative species of *H. roretzi* inhabiting the northern part of Japan. The mature protein HaVC80 consists of 13 epidermal growth factor (EGF)-like repeats, 1 repeat longer than HrVC70, and is derived from the precursor protein HaVC130 consisting of 14 EGF-like repeats and a C-terminal ZP domain (Ban et al. 2005). HrVC120 is very similar to HaVC130 (83.4 % identity based on their amino-acid sequences), and the 8th EGF domain of *HrVC120* gene appears to have been duplicated during evolution. HaVC80 is also highly polymorphic among individuals in restricted regions between the first and second Cys residues, the third and fourth Cys residues, and the EGF-domain-connected regions, where similar polymorphisms are observed in HrVC70 (Ban et al. 2005). Further studies are needed to elucidate the binding partners and roles of HaVC80 in fertilization.

20.2.2 Lysin in H. roretzi

Hoshi et al. (1981) first reported that trypsin-like protease(s) and chymotrypsin-like protease(s) are indispensable for sperm penetration through the VC of eggs in *H. roretzi* by examining the effects of various protease inhibitors on fertilization of intact and VC-free eggs (Hoshi et al. 1981; Hoshi 1985). Two trypsin-like proteases, HrAcrosin and HrSpermosin, were then purified from *H. roretzi* sperm using Boc-Val-Pro-Arg-MCA, the strongest inhibitor of fertilization among the substrates tested (Sawada et al. 1982, 1984a). Although it was suggested that both these proteases participate in fertilization by comparing the effects of various leupeptin analogues (peptidyl-rgininal) on fertilization and enzymatic activities (Sawada et al. 1984b; Sawada and Someno 1996) and also by examining the inhibitory ability of anti-spermosin antibody on fertilization (Sawada et al. 1996), the purified enzymes showed little degrading activity toward VC proteins (Sawada et al., unpublished data).

Both HrProacrosin (precursor of HrAcrosin) and HrSpermosin possess several candidate regions for protein–protein interaction, that is, two CUB domains in the C-terminus of HrProacrosin and a Pro-rich region in the N-terminus of the light chain of HrSpermosin (Kodama et al. 2001, 2002). Two VC proteins (25- and 30-kDa VC proteins) were identified as binding proteins to these proteases (Akasaka et al. 2010). By cDNA sequencing, it was revealed that the 25- and 30-kDa proteins correspond to the C-terminal region of high molecular mass vitellogenin, which belongs to a family of lipid transfer proteins (Akasaka et al. 2010, 2013). We propose that sperm binds to the C-terminal fragments of vitellogenin located on the VC, the binding being mediated by the sperm-side HrProacrosin CUB domain and HrSpermosin Pro-rich region, and then sperm proteases degrade these VC proteins or the precursor regions, enabling sperm to detach from and penetrate the VC. These sequential actions may explain the phenomena of sperm binding to and penetrating through the VC (Fig. 20.1) (working hypothesis: for details, see another chapter by Akasaka et al.).

Because the purified preparations of ascidian sperm trypsin-like proteases were unable to efficiently degrade the VC, we focused on a chymotrypsin-like protease as a potential VC lysin. As Suc-Leu-Leu-Val-Tyr-MCA was the strongest inhibitor of *H. roretzi* fertilization among the peptide substrates tested (Sawada et al. 1983), Suc-Leu-Leu-Val-Tyr-MCA-hydrolyzing protease was purified and identified as 20S and 26S (or 26S-like) proteasomes (Saito et al. 1993). The UPS is one of the most important intracellular protein-degradation systems in eukaryotic cells (Hershko and Ciechanover 1998; Finley 2009; Tanaka 2009). In this system, intracellular short-lived and aberrant proteins are tagged with ubiquitin by sequential actions of ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3 and then degraded by the 26S proteasome in an ATP-dependent manner. The 26S proteasome is made up of the 20S proteasome, a barrel-shaped protease complex consisting of four stacked heptameric rings $\alpha_7\beta_7\beta_7\alpha_7$, and the 19S regulatory particle (19S RP)/PA700, consisting of 19 subunits, including 6 ATPase subunits and a ubiquitin-recognizing subunit S5a. The 20S proteasome has three protease activities, that is, caspase-like (β_1), trypsin-like (β_2), and chymotrypsin-like (β_5) activities (Tanaka 2009).

The 26S proteasome-containing fraction partially purified from activated sperm showed a weak VC-degrading activity in *H. roretzi* (Saitoh et al. 1993). In addition, HrVC70 was ubiquitinated and degraded by the purified sperm 26S proteasome in the presence of ATP and ubiquitin (Sawada et al. 2002a). The extracellular UPS appears to play a key role in ascidian fertilization for the following main reasons. First, *H. roretzi* fertilization was inhibited by proteasome inhibitors, including MG115 and MG132, and also by anti-proteasome antibody and anti-multi-ubuiquitin chain-specific monoclonal antibody FK2 (Sawada et al. 2002a, b). Second, Suc-Leu-Leu-Val-Tyr-MCA-hydrolyzing proteasome activity, which was specifically inhibited by MG115, was detected in the sperm head region under an epifluorescence microscope when activated by alkaline seawater (Sawada et al. 2002b). Third, sperm proteasomes, as well as HrVC70-ubiquitinating enzyme, ATP, and ubiquitin, were partially released from sperm when activated by alkaline seawater (Sakai et al. 2003; Sawada et al., unpublished data). Fourth, HrVC70 on

the VC appears to be ubiquitinated upon insemination on the basis of results of Western blotting and immunocytochemistry using the monoclonal antibody FK2 (Sawada et al. 2002a; Sakai et al. 2003).

HrVC70-ubiquitinating enzyme has been purified from sperm exudate, a fraction released from activated sperm, by DEAE-cellulose chromatography, ubiquitin-agarose chromatography, and 10–40 % glycerol density gradient centrifugation (Sakai et al. 2003). The molecular size of the enzyme was estimated to be approximately 700 kDa by glycerol density gradient centrifugation (Sakai et al. 2003). The purified enzyme exhibited activity in artificial seawater and required a high concentration (~10 mM) of Ca^{2+} for its activity. These enzymatic features also support our idea that the purified enzyme functions extracellularly in seawater. Furthermore, apyrase, which depletes ATP and inhibits HrVC70-ubiquitinating activity, inhibited fertilization when added to the surrounding seawater. These results indicate that a novel extracellular 700-kDa HrVC70-ubiquitinating enzyme complex plays a pivotal role in ubiquitination of HrVC70. Although there are two Lys residues in HrVC70, Lys234 and Lys636, only Lys234 was identified to be ubiquitinated by a ubiquitin-conjugation assay using several site-directed Lys-to-Arg mutant recombinant proteins of HrVC70 (Sawada et al. 2005). Because it is widely believed that only one molecular species of E1 is committed to every ubiquitination reaction, our findings imply the existence of a novel E1-containing complex, functioning extracellularly during fertilization.

20.3 Allorecognition and Lysin Systems in *C. intestinalis*

20.3.1 Proposed Hypotheses of Allorecognition in C. intestinalis

Several candidate molecules involved in the self-incompatibility (SI) system in *C. intestinalis* have been proposed. Kawamura and colleagues found that an acid extract of the VC has the ability to inhibit the binding of non-self sperm, but not self-sperm, to the VC (Kawamura et al. 1991). They partially purified several factors responsible for this activity and revealed that there are a non-allo-recognizable glucose-enriched inhibitor of gamete-binding and Glu/Gln-enriched peptide modulators, which serve as acceptors of non-self sperm, and that certain combinations of these factors show specific inhibitory ability toward the binding of non-self sperm (Kawamura et al. 1991; Harada and Sawada 2008). However, further detailed studies have not been carried out. De Santis and colleagues showed that SI becomes effective several hours after germinal vesicle breakdown (De Santis and Pinto 1991). Because removal of follicle cells prevents the onset of self-sterility, they proposed that follicle cells release a certain self-sterility factor(s) that binds to the VC (De Santis and Pinto 1991). By analogy to the mammalian cellular immune system, they proposed that peptides produced by proteasome-mediated proteolysis are loaded onto Cihsp70,

which is a molecular chaperone assumed to be an ancestor protein of major histocompatibility complex (MHC) class I and II molecules in lower vertebrates or invertebrates, and delivered to the surface of the VC (Marino et al. 1998, 1999). They also showed that the proteasome inhibitor clasto-lactacystin beta-lacton and anti-HSP70 antibodies prevented the onset of self-sterility (Marino et al. 1999). From these results, they speculated that Cihsp70 and a self-peptide produced by proteasomal degradation might be involved in the SI system of *C. intestinalis*, which might share the origin of the vertebrate immune system. However, the antigenic peptide fragments on Cihsp70 on the VC have not yet been identified. On the other hand, Khalturin, Bosch, and colleagues performed PCR-based subtraction experiments and compared gonad cDNAs between genetically unrelated individuals. They identified several candidate genes that are expressed in developing oocytes or/and follicle cells and are polymorphic among individuals, including CiS7 (EGF-like repeat-containing gene), vCRL1 [Sushi (or SCR)-domain-containing gene], and multiple homologues of HrVC70 (EGF-like repeat- and ZP-domain-containing genes) (Khalturin et al. 2005; Kürn et al. 2007a, b). However, they recently reported that vCRL1 genes are not related to the SI system in fertilization of *C. intestinalis,* but the s- and v-Themis system, which we reported previously (Harada et al. 2008; described below), plays a key role in SI (Sommer et al. 2012). In addition, several molecular interactions appear to be involved in gamete interaction, although the involvement in allorecognition is not known. For example, it has been reported that the terminal fucose residue on VC glycoproteins (Rosati and De Santis 1980) and sperm-side fucosidase may make an enzyme–substrate complex, allowing interaction between sperm and the VC of eggs (Hoshi 1986; Matsumoto et al. 2002). On the other hand, we reported that sperm-side CiUrabin, a GPI-anchored CRISP family protein located at the sperm head and tail, is capable of binding to CiVC57, an EGF-like-repeat-containing major glycoprotein on the VC, which can support the interaction between sperm and the VC of eggs (Yamaguchi et al. 2011; Yamada et al. 2009).

20.3.2 Two or Three Pairs of s-Themis and v-Themis, the Key Molecules in Allorecognition in C. intestinalis

In the early part of the twentieth century, Morgan published several papers on the SI system of an ascidian, *C. intestinalis* (Morgan 1911, 1939, 1942, 1944). He reported that the VC is a barrier against self-fertilization and that the SI system is abolished by treatment of eggs with acidic seawater or protease (Morgan 1939). By acid-induced self-fertilization, he raised many selfed F_1 siblings and examined cross-fertility and cross-sterility among them (Morgan 1942, 1944). Cross-sterility is rarely observed in wild populations, but cross-sterile combinations are sometimes observed in selfed or experimentally cross-fertilized siblings, suggesting that self-sterility is genetically governed. The self-fertilized individuals gave a considerable number of cross-sterile combinations, among which he recognized two types of cross-sterility: bi-directional and one-way (Morgan 1942, 1944). Morgan proposed

a “haploid sperm hypothesis” to explain the occurrence of one-way cross-sterility (Morgan 1942, 1944), where SI specificity is determined by haploid expression in sperm and diploid expression in eggs. According to his hypothesis, a parent heterozygous at the SI locus (A/a) produces two populations of sperm (A-expressing and a-expressing sperm), either of which can fertilize both types of homozygous eggs (A/A and a/a eggs). In contrast, sperm (A-expressing and a-expressing sperm) from two types of homozygotes (A/A and a/a individuals) are sterile to heterozygous eggs (A/a eggs), because heterozygous eggs (or VC) express both types of female SI gene products, either of which must be recognized by sperm as self. Thus, once a one-way cross-sterile pair of individuals was found, an egg-donating individual should be a heterozygote at a SI-responsible locus, whereas a sperm-donating individual should be a homozygote.

Based on these criteria, Harada et al. (2008) searched for a candidate SI locus by determining the DNA sequence at about 70 genetic markers in 14 chromosomes, and they noticed that two loci, loci A and B, located in chromosomes 2q and 7q, respectively, are involved in SI in this species (Harada et al. 2008) (Figs. 20.2, 20.3). Among the proteins encoded in locus A, a fibrinogen-C-terminus-like protein, referred to as v-Themis-A, with a high degree of polymorphism among individuals was detected on the VC by proteome analysis. On the other hand, of genes encoded in locus A, four genes were found to be expressed in the testis, among which a PKD-1-like protein, referred to as s-Themis-A, was identified as a candidate sperm-side receptor protein with a hypervariable region in its N-terminal region (Themis is a Greek goddess who is the embodiment of divine order, law, and custom and prohibits incest.). Although there is no overall synteny between loci A and B, a similar gene pair of proteins (v-Themis-B and s-Themis-B) was identified in locus B (Harada et al. 2008). Interestingly, *v-Themis-A*/*B* genes were located in the first intron of *s-Themis-A*/*B* genes, respectively, in the opposite direction in both cases (Fig. 20.2a). These features indicate a tight linkage between *s-Themis* and *v-Themis* genes, not allowing the segregation of putative binding partners. Based on results of genetic analysis, it has been proposed that when sperm-side s-Themis-A and s-Themis-B interact with the same allelic v-Themis-A and v-Themis-B, respectively, on the VC, sperm must recognize the VC as self, resulting in blocking fertilization.

Polymorphisms among individuals of *s*/*v-Themis-A* and *s*/*v-Themis-B* genes were investigated: molecular phylogenetic analysis indicated coevolution between *s-Themis-A*/*B* and *v-Themis-A*/*B* (unpublished data).

Within locus B, the genome sequence data showed one additional pair of *s-Themis* and *v-Themis* genes. We first thought that this gene model is caused by a possible miss-assembly. To clarify this point, we determined the DNA sequence in this region after cloning from the genome derived from one individual. The data confirmed the occurrence of a novel pair of *s*/*v-Themis* genes, which we tentatively call *s-Themis-B2* and *v-Themis-B2*. The sequence of this gene pair is almost identical to that of the pair of *s*/*v-Themis-B* except for the *v-Themis-B2* region and a hypervariable region of *s-Themis-B* (Fig. 20.2c). In addition, *v-Themis-B2* was detected in the VC by proteomic analysis (Yamada et al., unpublished data). Furthermore, cross-fertility/sterility experiments among individuals, whose *s*/*v-Themis-A*/*B*/*B2*

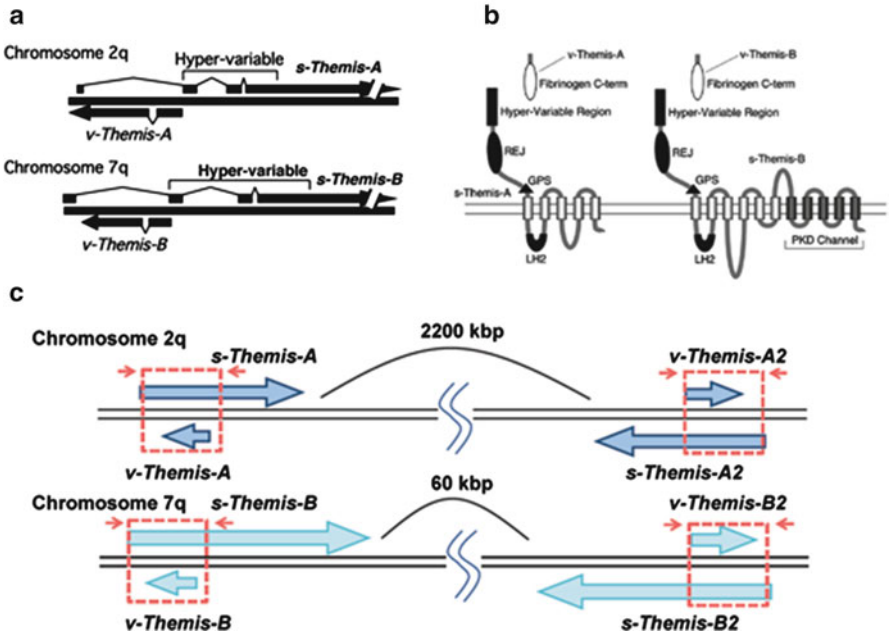

Fig. 20.2 Genes and proteins involved in the self-incompatibility (SI) system in *Ciona intestinalis*. **a** The pair of *s-Themis-A* and *v-Themis-A* located in chromosome 2q and the pair of *s-Themis-B* and *v-Themis-B* in chromosome 7q play a pivotal role in SI of *C. intestinalis*. **b** s-Themis-A and s-Themis-B possess a hypervariable region in their N-termini and have 5 and 11 transmembrane domains, respectively, in their C-termini. Five C-terminal transmembrane domains of s-Themis-B (and s-Theims-B2) showed homology to a cation channel. **c** A novel pair called *s-Themis-B2* and *v-Themis-B2*, which resides 60 kbp apart from s/v-*Themis-B* loci, may be involved in this SI system. A newly found pair of *v-Themis-A2* and *s-Themis-A2*, which resides 2.2 Mbp apart from *s/v-Themis-A* loci, appears to be a pseudo-gene pair. *Arrows* indicate the amplified regions to identify the allelic variety (haplotypes). Note that s-Themis-B and s-Themis-B2 contain a cation-channel domain in their C-terminal regions, which may be responsible for ionic flow during SI response. It is also known that sperm undergoes drastic Ca^{2+} influx when attached to the self-VC, a self-recognition signal in *C. intestinalis* (see also Fig. 20.3)

genes were checked by direct sequencing, showed that not only *s/v-Themis-A* and *s/v-Themis-B* but also *s/v-Themis-B2* plays a key role in the SI system (Yamada et al., unpublished data). By close inspection of the DNA sequence around the *s/v-Themis-A* region, we noticed one additional gene pair similar to *s/v-Themis-A*, called *s/v-Themis-A2*. However, our genetic analysis revealed that this gene pair appears to be a pseudo-gene pair (Yamamoto et al., unpublished data).

Sperm behavior and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to self/non-self recognition were also investigated. We found that sperm motility markedly decreased within 5 min after attachment to the VC of self-eggs but not after attachment to the VC of non-self eggs and that the apparent decrease in sperm motility was suppressed in low-Ca^{2+} seawater (Saito et al. 2012). It was also revealed that sperm detach from the self-VC or stop motility within 5 min after binding to the self-VC. As s-Themis-B contains a cation-channel domain in its C terminus, we monitored

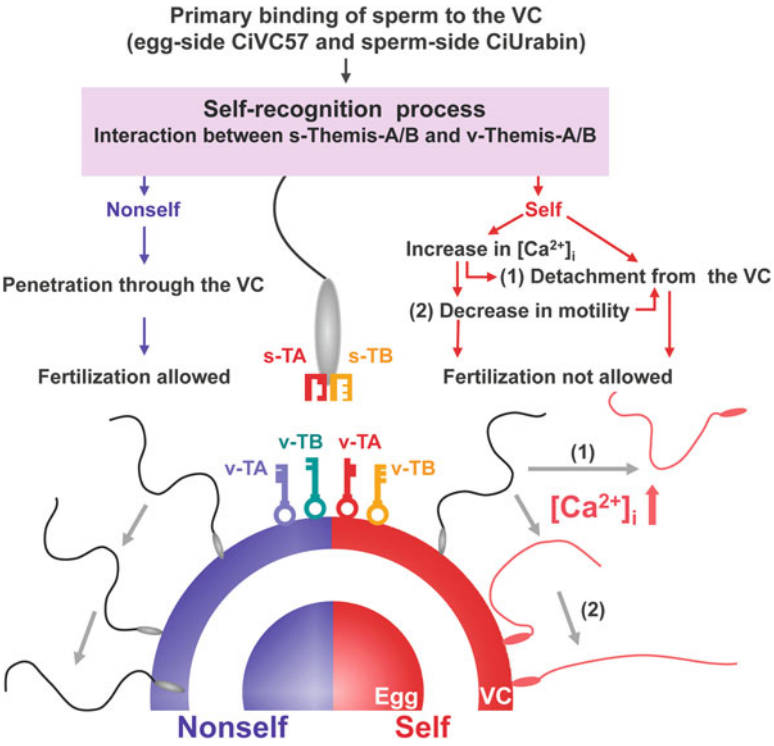

Fig. 20.3 Working hypothesis of self-incompatibility in *Ciona intestinalis*. We propose that sperm increase $[Ca^{2+}]_i$ and detach from the VC when both s-Themis-A/B (s-TA and S-TB; "keyholes") on the sperm surface recognize respective v-Themis-A/B (v-TA and v-TB; "keys") on the VC as self. Sperm remaining on the self-VC change their waveform and motility. Non-self sperm remain on the VC and penetrate through the VC to fertilize the egg. Although only s/v-Themis-A and s/v-Themis-B are depicted in this figure, s/v-Themis-B2 may also participate in this SI system

sperm $[Ca^{2+}]_i$ by real-time $[Ca^{2+}]_i$ imaging using Fluo-8H-AM, a cell-permeable Ca^{2+} indicator. Interestingly, we found that sperm $[Ca^{2+}]_i$ rapidly and dramatically increased and was maintained at a high level in the head and flagellar regions when sperm interacted with the self-VC but not when the sperm interacted with the non-self VC (Saito et al. 2012). The increase in $[Ca^{2+}]_i$ was also suppressed by low-Ca^{2+} seawater (Saito et al. 2012). These results indicate that the sperm self-recognition signal triggers $[Ca^{2+}]_i$ increase or Ca^{2+} influx, which induces an SI response to reject self-fertilization.

As described in the preceding section, it has been reported that a non-self sperm-recognizing factor was identified in an acid extract of the VC in *C. intestinalis*. However, v-Themis-A and -B were hardly solubilized from the VC by acid treatment. In contrast, a novel factor, called Ci-v-Themis-like, which has the same molecular architecture, consisting of a coiled-coil domain and C-terminal fibrinogen-like domain, as that of v-Themis-A and -B except for having no apparent polymorphism, is a major acid-extractable VC protein. Ci-v-Themis-like appears to be an ancestral

protein of v-Themis-A/B based on results of molecular phylogenetic analysis (Otsuka et al. 2013). Although there is no direct evidence indicating the participation of this protein in SI, our preliminary data showed interaction with v-Themis-A proteins, suggesting that the interaction between Ci-v-Themis-like on the VC and its sperm-side binding partners participates in gamete interaction to support the Themis-mediated allorecognition system. Further studies on the role of Ci-v-Themis-like are now in progress in our laboratory.

20.3.3 Lysin in C. intestinalis

To elucidate the VC lysin in *C. intestinalis*, effects of protease inhibitors on fertilization were examined (Pinto et al. 1990; Hoshi 1985). In contrast to *H. roretzi*, chymostatin, but not leupeptin, showed a strong inhibitory effect on fertilization. Then, a chymotrypsin-like protease was purified from *C. intestinalis* sperm (Marino et al. 1992). The purified preparation of a 24-kDa protease had a weak activity to impair the VC on the basis of electron microscopic observation. Later, it was found that proteasome inhibitors, rather than chymostatin, potently inhibited the fertilization (Sawada et al. 1998). By analogy to the UPS, which functions as a lysin in *H. roretzi*, the UPS may play a key role in fertilization, probably as a lysin, also in *C. intestinalis* (Sawada et al. 1998).

20.4 Future Perspective

As discussed here, allorecognition or self-incompatibility systems in ascidians were seen to be very similar to the SI system in flowering plants. In flowering plants, SI-responsible proteins are different in different families (Table 20.1). In Brassicaceae, both genes of pollen-side SP11/SCP and pistil-side SRK (S-receptor kinase) are highly polymorphic and tightly linked (Takayama and Isogai 2005; Iwano and Takayama 2012). A drastic increase in intracellular Ca^{2+} within pollen is known in Papaveraceae, resulting in caspase-like protease-mediated cell death, which appears to be similar to the SI response in *C. intestinalis*. From this aspect, sexual reproductive strategies might be much more common between animals and plants than previously thought, although the SI-responsible proteins themselves are considerably diverged.

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Table 20.1 Self-incompatibility system in plants and animals

Family	Female determinant	Male determinant
Flowering plants		
Brassicaceae	SRK	SP11/SCR
Solanaceae, Rosaceae	S-RNase	SLF/SBP
Papaveraceae	PrsS	PrpS
Ascidians		
Cionidae		
(*Ciona intestinalis*)	s-Themis-A, -B, -B2	v-Themis-A, -B, -B2
(*Ciona intestinalis*)	Not identified	(Gln-enriched VC peptides, Cihsp70, vCRL1 etc.)
Pyuridae		
(*Halocynthia roretzi*)	HrVC70	HrTTSP-1[a], HrUrabin[a]
(*Halocynthia aurantium*)	HaVC80	Not identified

For details, see reviews (Takayama and Isogai 2005; Iwano and Takayama 2012; Sawada 2002; Harada and Sawada 2008)

[a]These proteins are potential candidates, but it is not known whether these proteins are directly involved in SI

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