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Nematode sperm maturation triggered by protease involves spermatid-secreted serine protease inhibitor (Serpin)

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Spermiogenesis is a series of poorly understood morphological, physiological and biochemical processes that occur during the transition of immotile spermatids into motile, fertilization-competent spermatozoa. Here, we identified a Serpin (serine protease inhibitor) family protein (As_SRP-1) that is secreted from spermatids during nematode Ascaris suum spermiogenesis (also called sperm activation) and we showed that As_SRP-1 has two major functions. First, As_SRP-1 functions in cis to support major sperm protein (MSP)-based cytoskeletal assembly in the spermatid that releases it, thereby facilitating sperm motility acquisition. Second, As_SRP-1 released from an activated sperm inhibits, in trans, the activation of surrounding spermatids by inhibiting vas deferens-derived As_TRY-5, a trypsin-like serine protease necessary for sperm activation. Because vesicular exocytosis is necessary to create fertilization-competent sperm in many animal species, components released during this process might be more important modulators of the physiology and behavior of surrounding sperm than was previously appreciated.

In most, if not all, animals, males produce sperm in their gonad that are fertilization-incompetent until they leave this organ and undergo further maturation. For instance, whereas mammalian spermatozoa form in the testes, they must undergo a maturation process called capacitation in the female reproductive tract before they become fertilization-competent (1). In nematodes, spermatids do not complete maturation into spermatozoa (spermiogenesis) until after they have left the testes. In the nematode Caenorhabditis elegans, sperm are made in both males and self-fertile hermaphrodites (there are no conventional females). Hermaphrodites have a testis that proliferates sperm and then it switches into an ovary and produces oocytes. The first ovulated oocyte pushes the stored spermatids from the gonad into the spermatheca, where they become fertilization-competent (1). In vitro and in vivo studies have suggested that protease activity is involved in sperm maturation triggered by vas deferens-derived As_TRY-5 involving sperm-secreted As_SRP-1 and that secreted As_SRP-1 in the medium inhibits activation of surrounding spermatids. This dual function of sperm-secreted As_SRP-1 might play a significant role during postcopulatory sexual selection.

Results
As_SRP-1 (1CB4 antigen) Is Translocated During Ascaris Sperm Activation. We found that the 1CB4 monoclonal antibody that recognizes C. elegans MOs (11, 16-18) also recognized Ascaris sperm MOs (Fig. 1 A and B). Immunofluorescence staining of permeabilized Ascaris spermatids or spermatozoa with 1CB4 revealed...
punctuate, peripherally located structures, similar to what is seen in *C. elegans* (11, 17). Cryo immuno-EM with 1CB4 confirmed that immuno-gold labeled tightly-packed stacks of membranes inside sperm (Fig. 1B, Upper), characteristics of MOs in *C. elegans* (2). Different from previous immuno-fluorescence studies in *C. elegans*, 1CB4 also stained the leading edge PM of *Ascaris* spermatozoon (Fig. 1B, Lower). Cryo-immuno-EM with 1CB4 revealed the clear immunogold labeling along the outer PM of spermatozoon (Fig. 1B, Lower). Third, from an in vitro MSP motility assay (Fig. 1D), in which the leading edge PM-derived vesicles from spermatozoon extracts recruit cytosolic components to trigger MSP fiber assembly (13), we found that 1CB4 immunofluorescence could be detected only in permeabilized fiber-growing vesicles. Given that these vesicles acquire an inside-out configuration during cell lysis (13), this result is consistent with the outer PM-localization of the 1CB4 target in *Ascaris* spermatozoon. Moreover, immunofluorescence quanti-
fication of the 1CB4 staining in nonpermeabilized spermatozoa demonstrates that the 1CB4 on the outer PM of spermatozoa was distinctly asymmetrical, i.e., the fluorescence intensity along the leading edge PM was 5.3-fold higher than that in the rear edge PM (Fig. 1C), in agreement with previous observations by quantitative immuno-EM in *C. elegans* spermatozoa (11).

1CB4 is a monoclonal antibody generated using homogenates of whole *C. elegans* (16). Although it has been extensively used for labeling MOs in *C. elegans* (11, 17), the molecular identity of the antigen recognized by 1CB4 has not been determined. By using Western blotting, we found that a single polypeptide (~46 kDa) is recognized by 1CB4 in *Ascaris* sperm extract, and it was mostly in a soluble, cytosolic fraction (Fig. S1A). Isolation and purification of the 46 kDa protein were achieved by following the 1CB4 signal in Western blots from different cellular fractions (Fig. S1B). Initial MS analysis of the purified protein using a conventional database search strategy was ineffective because this protein was not in the database. We resorted to de novo sequencing analysis using the pNovo program (19) and extracted sequences directly from the tandem mass spectra of peptides derived from this protein (Fig. 1E and Fig. S2). We synthesized two peptides according to the pNovo result and found that the identification of these two sequences was fully supported by the parent masses and high-resolution MS/MS spectra of the synthetic peptides (Fig. S2). BLAST searches of these peptides against predicted *Ascaris* protein sequences in NEMBASE3 (20) revealed that the most abundant protein in the sample was a Serpin (Fig. 1E), belonging to the Serpin superfamily (we named it *As_SRP-1*). Using rapid amplification of cDNA ends by PCR (RACE PCR), we cloned the full-length cDNA of *As_srp-1* and deduced its amino acid sequence (Fig. 1F). When the original MS data were searched against a database containing the newly cloned *As_SRP-1* sequence using either Mascot or pFind, *As_SRP-1* was identified as the top hit, and the overlap between the database search result and the de novo sequencing result was extensive (Figs. S3 and S4). Amino acid alignment showed that *As_SRP-1* shares strong sequence homology with members of the clade B Serpin family. A highly conserved reactive site loop (RSL) containing a putative scissile bond (21) was detected in the sequence of *As_SRP-1* (Fig. 1F). When expressed in *E. coli*, the recombinant *As_SRP-1* displayed the same molecular mass as that of native *As_SRP-1* and was recognized by both 1CB4 and the polyclonal antibody we raised against purified native *As_SRP-1* (Fig. S1C). These data demonstrate that the target of the 1CB4 monoclonal antibody in *Ascaris* is *As_SRP-1*.

**As_SRP-1 Is Essential for MSP-Based Sperm Motility in Ascaris.** The localization of *As_SRP-1* on the outer PM of spermatozoon and its asymmetrical distribution at the leading edge (Fig. 1A-D) suggest that this protein probably plays a role in MSP cytoskeleton dynamics and sperm motility. To examine this possibility, we performed both ex vivo and in vitro experiments. When spermatozoa were perfused with the *As_SRP-1* antiserum (1:100 or 1:50 dilution), spermatozoa stopped crawling, their MSP cytoskeleton disappeared (66% or 98%, respectively), and cells rounded up (Fig. 2A and C). These defects in cytoskeleton dynamics and sperm morphology were almost completely reversed when the antiserum was first neutralized by adding purified native *As_SRP-1*, with <5% of spermatozoa exhibiting defects (Fig. 2A and C). Furthermore, *As_SRP-1* localization on the inner leaflet of the vesicle membrane (equivalent to outer PM) (Fig. 1D) is important for MSP fiber assembly in vitro.
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Density of MSP
at the N terminus of As_SRP-1 (Fig. 1F). Because the fusion of MOs with the PM during sperm activation is known to be
because extracts from As_SRP-1 antiserum-treated spermatozoa resulted in a significant decrease in both the growth rate and area
density of MSP fibers. Such motility defects disappeared in the extracts from spermatozoa that were perfused with the As_SRP-1
antiserum and the neutralizing As_SRP-1 (Fig. 2B, D, and E). Together, these data indicate that As_SRP-1 at the outer PM of
spERM pseudopod leading edge plays an essential role in regulating both MSP cytoskeleton assembly and cell motility. Our immuno-
data suggest that As_SRP-1 regulates Ascaris sperm mo-
tility probably through protein tyrosine phosphorylation (Fig. S5).

**Secreted As_SRP-1 Blocks Sperm Activation in Surrounding Sper-
matids.** As shown in Fig. 1, the As_SRP-1 localization in Ascaris spermatids (in MOs) is different from that in spermatozoa (on the outer PM). A secretory signal peptide sequence is present at the N terminus of As_SRP-1 (Fig. 1F). Because the fusion of MOs with the PM during sperm activation is known to be
a regulated exocytosis process in C. elegans (11), exocytosed As_SRP-1 could be translocated from MOs to the outer PM
during Ascaris sperm activation. Indeed, when Ascaris spermatids were activated by sperm-activating substance (SAS) (the extract from vas deferens) (12), the amount of As_SRP-1 in the medium increased dramatically as shown by Western blotting analysis using anti-As_SRP-1 antibody (Fig. 3A and B). In contrast, only a weak signal of As_SRP-1, most likely from rare, spontaneous activation, was detected in the medium of sperm that were either not subjected to SAS or subjected to heat-inactivated SAS. The se-
cretion of a Serpin (As_SRP-1) during sperm activation (Fig. 3A) and the ability of proteases (6, 7, 12) to activate nematode sperm led us to test whether As_SRP-1 can inhibit SAS-induced sperm activation and whether the activity of a serine protease(s) in SAS is essential for sperm activation. Not surprisingly, we found that purified As_SRP-1 was able to inhibit SAS-induced sperm activation (Fig. 3B). Further experiments showed the inhibitory
target of As_SRP-1 was present in SAS but not on sperm itself because when sperm were treated with the mixture of As_SRP-1 and 0.5 or 5 μg/mL of SAS, the sperm activation rate was lower than that from sperm treated with As_SRP-1 first, then followed by SAS addition (Fig. S6). The importance of a serine protease activity in SAS-induced sperm activation is also supported by our pharmacological studies in which the effect of various specific protease inhibitors on SAS was tested. These results demonstrate that only serine protease inhibitors, and not other inhibitors, prevent SAS-induced sperm activation (Fig. S7). Furthermore, we found that As_SRP-1 could interact with the predicted serine protease(s) in SAS using immunodepletion assays (Fig. 3C). We immobilized As_SRP-1 to protein A beads through As_SRP-1 antibody, incubated the As_SRP-1 beads with SAS and separated the SAS supernatant from the beads. If physical interaction occurs, the protease should be depleted from SAS by As_SRP-1 beads, causing the supernatant to lose its activity. This was indeed what we observed (Fig. 3C, Top). Meanwhile in the control experiments, Protein A beads with mock immobilization of As_SRP-1 through control IgG (Fig. 3C, Middle) or beads preloaded with As_SRP-1 antibody alone (Fig. 3C, Bottom) failed to deplete the activity from the SAS supernatant. Therefore, As_SRP-1 can physically bind to the serine protease(s) in SAS. Collectively, these data suggest that the activity of a serine protease(s) in SAS is critical for Ascaris sperm activation and the secreted As_SRP-1 likely inhibits sperm activation through its physical interaction with this protease(s).

Although our data (Fig. 3 B and C and Fig. S7) and accumulating evidence (6, 7, 12) suggest that the activation of nematode sperm involves a serine protease(s), the identity of this protease(s) has been unknown. To identify the protease, we used conventional biochemical purification strategies to enrich the target protein by following its sperm activating activity (Fig. S8A). The Con A eluate showed strong activity in inducing sperm activation, and this activity could be inhibited by the serine protease inhibitor PMSF (Fig. S8J), suggesting that the fraction from Con A contains our target serine protease(s). This proteolytic fraction was shown to interact physically with As_SRP-1 (Fig. 3C), and the interaction was predicted to produce a large covalent protein complex containing the cleaved As_SRP-1 and target protease(s), according to the well-characterized Serpin–proteinase interaction mechanism. Indeed, as one of the two nonpolar bands of SDS/PAGE and Western blot, an ~9-kDa band (Fig. 3D, Left) appeared after As_SRP-1 (~46 kDa) was incubated with the Con A eluate. This 90-kDa band was then subjected to MS analysis and de novo peptide sequencing to identify the protease because its sequence was not present in existing databases. Using the pNovo algorithm (19), we obtained over a dozen high-quality peptide sequences that did not belong to any previously characterized protein (Fig. 3D, Right, and Fig. S9). A synthetic peptide was obtained for one of them, and its fragmentation spectra were found to be identical to those of the endogenous peptide (Fig. S9), thus validating the de novo sequencing results. We assembled these sequences into longer segments and found by BLAST search that they share homology with a trypsin-like serine protease (Fig. 3D, Right). Based on the peptides identified from de novo sequencing, we designed degenerative primers for RACE PCR and cloned the full-length cDNA (Fig. 3E). Sequence comparisons indicate that the protein encoded by this cDNA shares a high degree of homology, including a conserved catalytic triad, with other known serine proteases (Fig. 3E). We named this protein AsTRY-5 after its closest homolog, TRY-5, in C. elegans. Again, analysis of the original MS data against full-length TRY-5 using Mascot and pFind further confirmed the accuracy of sequence identifications made by pNovo (Figs. S10 and S11).

We further tested whether the inhibitory effect of the secreted As_SRP-1 on sperm activation could be rescued by the addition of specific antiserum of As_SRP-1. We activated the first batch of sperm with the Con A eluate, then collected the supernatant. When the supernatant was added to the second batch of sperm, no sperm activation was observed (Fig. 3F, Upper and Lower Left), probably because AsTRY-5 in the Con A eluate was inhibited by As_SRP-1 secreted from the first batch of sperm. As expected, when As_SRP-1 antiserum was added to neutralize As_SRP-1 secreted by the first batch of sperm, the resulting supernatant was able to activate the second batch of sperm (Fig. 3F, Center, Upper and Lower). As a control, the preimmune serum had no such effect (Fig. 3F, Upper and Lower Right). Thus, sperm-secreted As_SRP-1 during sperm activation blocks the activation of other sperm by inhibiting the glandular vas deferens-derived serine protease AsTRY-5.

Discussion

After the meiosis, spermatids are transcriptionally and translationally silent and, thus, sperm activation, motility acquisition, sperm competition, and fertilization are performed without new gene expression (22). Our ex vivo data provide evidence that motile spermatozoa are biochemically active in contributing a protein (AsTRY-1) to the seminal fluid and that this protein might coordinate both spermatozoon motility and sperm competition in vivo. On the one hand, for activated sperm in the uterus, AsTRY-1 is necessary for MSP cytoskeleton assembly and sperm motility acquisition (Fig. 2), thus improving the competitiveness of spermatozoa. Although the mechanism by which AsTRY-1 modulates cytoskeleton dynamics remains unclear, our data suggest that AsTRY-1 might act through protein tyrosine phosphorylation (Fig. S5), which has been known as a molecular switch in the regulation of MSP-based cell motility (14). On the other hand, for nonactivated sperm in the uterus from other males, AsTRY-1 irreversibly terminates the activity of a vas deferens-derived serine protease, AsTRY-5 (Fig. 3), thus inhibiting the activation of other spermatids. The spatially and temporally controlled encounter of nonmotile spermatids with the activating protease AsTRY-5 and the regulated release of the dual-function serine protease inhibitor AsTRY-1 during sperm activation constitute an elaborate opposing but complementary mechanism to coordinate sperm maturation and likely sperm competition in vivo.

Sperm competition in polyandrous species has been widely recognized as one of the most potent driving forces in the evolution (23). Studies on sperm-competition mechanisms have focused on the physical traits of sperm [reviewed in (23, 24)], such as number of sperm inseminated, cell size, swimming velocity, and on seminal fluid produced by several accessory glands in the male body [reviewed in (25, 26)]. Several seminal fluid proteins in insects were involved in sperm competition by sperm displacement, sperm incapacitation or sperm ejection by females (27–30). Real-time live cell-imaging studies of sperm competition in transgenic flies with different fluorescent protein-labeled sperm support the sperm displacement mechanism, but not sperm incapacitation mechanism (31). Interestingly, the presence of sperm in addition to seminal fluid from second males would significantly enhance the magnitude of sperm displacement compared with that caused only by seminal fluid from spermless males (32). Seminal fluid is produced principally by accessory glands in the male body (25, 26), but our data show that sperm can secrete a component of this fluid. An interesting avenue for future investigation may be to determine whether other animal species besides nematodes also use sperm-secreted components mechanism to modulate sperm competition.

Some of our data from Ascaris not only agree with those from C. elegans but also further the understanding of C. elegans sperm activation. For example, it has been known for over three decades that nematode sperm can be activated in vitro by proteases, but the physiological relevance of this in vitro phenomenon is uncertain. A recent genetic study suggests that C. elegans sperm...
Materials and Methods

Ascaris sperm were obtained by dissecting males to recover seminal vesicles, which were processed to release seminal fluid into HKB buffer [50 mM Hepes, 70 mM KCl, 10 mM NaHCO₃ (pH 7.1)]. Spermatozoa were obtained using activating spermatids with the addition of SAS (vas deferens extract) (12). We observed sperm after various treatments as described in SI Materials and Methods using a DIC microscope (Axio Imager M2, Carl Zeiss) and MSF fibers assembled in vitro (13) using a phase-contrast microscopy (Axio Observer, Carl Zeiss). All images were processed using MetaMorph (Universal Imaging). For additional details on fiber assembly in vitro, native protein purification, MS analysis, recombinant protein expression, gene cloning, antibody preparation, immunodepletion, immunofluorescence, and cryo-immuno-EM assays, see SI Materials and Methods.

Note Added in Proof

While this paper was under review at PNAS, Smith and Stanfield (39) reported that C. elegans TRY-S, found in the male seminal fluid, is required for male-mediated sperm activation, consistent with our data described here.

Acknowledgments. We thank Dr. Li-Lin Du for valuable ideas regarding proteolysis-mediated sperm activation might have broad phylogenetic conservation and the proteolytic activity outside of sperm is essential for male reproductive success. As SRP-1 has dual functions in the modulation of nematode sperm maturation, providing insights for the fine-tuning of sperm function and male fertility before and postsemination. In taxa outside Nematoda that produce flagellated sperm, regulated exocytosis is also required to create fertilization-competent sperm and to achieve reproductive success (38). Thus, sperm from different taxa might use this active secretion mechanism to alter their immediate environment to enhance their own competitiveness.