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Diversity in the fertilization envelopes of echinoderms

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Abstract

Cell surface changes in an egg at fertilization are essential to begin development and for protecting the zygote. Most fertilized eggs construct a barrier around themselves by modifying their original extracellular matrix. This construction usually results from calcium induced exocytosis of cortical granules, the contents of which in sea urchins function to form the fertilization envelope (FE), an extracellular matrix of cortical granule contents built upon a vitelline layer scaffold. Here we examined the molecular mechanism of this process in sea stars, a close relative of the sea urchins, and analyze the evolutionary changes that likely occurred in the functionality of this structure between these two organisms. We find that the FE of sea stars is more permeable than in sea urchins, allowing diffusion of molecules in excess of 2 megadaltons. Through a proteomic and transcriptomic approach, we find that most, but not all of the proteins present in the sea urchin envelope are present in sea stars, including SFE9, proteoliasin, rendezvin, and ovoperoxidase. The mRNAs encoding these FE proteins accumulated most densely in early oocytes, and then beginning with vitellogenesis, these mRNAs decreased in abundance to levels nearly undetectable in eggs. Antibodies to the SFE9 protein of sea stars showed that the cortical granules in sea star also accumulated most significantly in early oocytes, and different from sea urchins, they translocated to the cortex of the oocytes well before meiotic initiation. These results suggest that the preparation of the cell surface changes in sea urchins has been shifted to later in oogenesis and perhaps reflects the meiotic differences among the species—sea star oocytes are stored in prophase of meiosis and fertilized during the meiotic divisions, as in most animals, whereas sea urchins are one of the few taxa in which eggs have completed meiosis prior to fertilization.

Keywords

Fertilization envelope; SFE9; Rendezvin; Ovoperoxidase; Transglutaminase; p160; Cortical granules; Fertilization; Polyspermy

Introduction

Reproductive strategies differ amongst organisms based on their evolutionary history and the niche within which they compete. The reproductive strategy for most marine invertebrates includes broadcast spawning of their gametes, and if successful in fertilization, the embryos often utilize the water column as a food source for development before metamorphosing into an adult. Echinoderms are paradigmatic for this reproductive strategy, and have served as important research organisms for understanding mechanisms of sperm activation (Lee et al., 1983), chemoattraction of sperm to the egg (Ward et al., 1985), sperm-egg binding mechanisms (Vacquier and Moy, 1977), egg activation (Steinhardt et al., 1977), and the diverse evolutionary basis for sperm-egg interactions (Vacquier, 1998).

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Animal fertilization was first observed in sea urchins, where an envelope forms promptly after sperm fusion with the egg and thus provides a rapid metric for successful sperm-egg interaction (Derbes, 1847), (Briggs and Wessel, 2006). Fusion of the male and female pronuclei, when first seen in sea urchins by Hertwig (1886) and Fol (Fol, 1877), closed the chapter on the important role of sperm in the process of reproduction.

The extracellular matrix of the egg, while called many different names, e.g. vitelline layer, zona pellucida, serves two essential jobs. First, it interacts with sperm in a species-specific manner. While this function occurs in almost all animals, it is particularly striking in broadcast spawners, such as abalone and sea urchins, which can inhabit the same niches and often spawn in overlapping times. In such cases, species specificity in sperm-egg interactions relies heavily on the extracellular matrix. Following successful sperm-egg fusion, the egg's extracellular matrix quickly reveals its second job as it is transformed to minimize the chances of additional sperm from reaching the egg. This physical block to polyspermy is highly selected for because fusion of more than one sperm with an egg is lethal to the embryo. The block to polyspermy in some animals, such as sea urchins, is remarkable since sperm:egg ratios may reach the millions.

The fertilization envelope in sea urchins establishes a physical and biochemical barrier that protects the zygote from supernumerary sperm, as well as environmental and microbial agents (Wong and Wessel, 2006a). Cortical granules are the major source of proteins used to construct the fertilization envelope (Wessel et al., 2001), (Wong and Wessel, 2006a). These abundant organelles, ranging to 15,000 per egg in sea urchins, are synthesized during oogenesis and released following gamete fusion (Laidlaw and Wessel, 1994). In the sea urchin, contents of the cortical granules are secreted within 30 sec of insemination and mix with the egg's vitelline layer. Hydrostatic pressure and addition of glycoproteins from the cortical granules to the vitelline layer lift the nascent fertilization envelope off the egg surface, and associated enzymes transform the envelope into an effective barrier for early embryogenesis. Sea urchin cortical granules harbor the major structural proteins of the envelope as well as enzymes essential to stabilize the envelope until hatching (Wong and Wessel, 2008).

The cortical granules contain several structural proteins and enzymes that give the fertilization envelope its distinct properties of stability yet permeability in the ocean environment. These proteins include the Soft Fertilization Envelope proteins SFE1 and SFE9, proteoliasin, and rendezvin; their cognate transcripts are specifically expressed in oocytes (Laidlaw and Wessel, 1994), (Wong and Wessel, 2004), (Wong and Wessel, 2006b). SFE1, SFE9 and proteoliasin are proteins rich in low-density lipoprotein receptor type A (LDLR_A) repeats involved in protein interaction (Wessel et al., 2000), (Wessel, 1995), (Wong and Wessel, 2004). Rendezvin (RDZ) is enriched in CUB domains, also involved in protein interaction. One RDZ gene is present in the sea urchin genome, but several transcripts are produced after alternative splicing. The full-length *rdz* transcript is alternatively spliced into at least three forms, encoding its majority proteins RDZ⁶⁰, RDZ⁹⁰, and RDZ⁴⁰. Two significantly less-abundant transcripts are also created, encoding RDZ¹²⁰ and RDZ⁷⁰. At the protein level, the different isoforms are differentially localized. RDZ⁶⁰, RDZ⁹⁰, RDZ⁴⁰, RDZ⁷⁰ only accumulate in the cortical granules, whereas RDV¹²⁰ is found in the vitelline layer (Wong and Wessel, 2006b). After fertilization, these segregated siblings reunite within the fertilization envelope, likely via heterologous CUB interactions.

Four major enzymatic activities are essential for the proper assembly of the sea urchin fertilization envelope: proteolysis, transamidation, hydrogen peroxide synthesis, and peroxidase-dependent dityrosine crosslinking. Serine protease activity from CGSP1 (cortical granule serine protease) is the only detectable class of protease activity of the cortical

granules necessary for the formation of the fertilization envelope (Vacquier et al., 1972); (Carroll and Epel, 1975); (Haley and Wessel, 1999). Full-length CGSP1 is enzymatically quiescent in the cortical granules, inactive at pH6.5 or below. Exposure of the protease to the pH of the seawater (pH8) at exocytosis immediately activates the protease through autocatalysis (Haley and Wessel, 2004b). CGSP1 cleaves a subpopulation of the granule content proteins, such as the enzyme ovoperoxidase to limit its activity and the β -1,3 glucanase to increase its activity. Another substrate targeted by CGSP1 is p160, a protein thought to link the vitelline layer to the plasma membrane (Haley and Wessel, 2004a). At fertilization, p160 cleavage allows for the separation of the fertilization envelope from the fertilized egg.

Transamidation is mediated by transglutaminases that crosslink glutamine and lysine residues to form N-epsilon (gamma glutamyl) lysyl isopeptide bonds (Greenberg et al., 1991). Two transglutaminases were found in the *Strongylocentrotus purpuratus* genome (Wong and Wessel, 2009). These two isoforms, derived from different genes, are differentially localized and were described as the extracellular transglutaminase (eTG), and the nuclear transglutaminase (nTG). Both transcripts are expressed in the oocyte. Whereas eTG mRNA persists in eggs, nTG mRNA is largely degraded during meiotic maturation (Wong and Wessel, 2009). These transglutaminases are activated by local acidification and act on fertilization envelope proteins such as SFE9, rendezvin, and ovoperoxidase.

Hydrogen peroxide is quickly synthesized at fertilization for ovoperoxidase cross-linking activity, and is synthesized by the dual oxidase homolog, Udx1 in the classically described respiratory burst (Warburg 1926). This calcium-dependent, pH sensitive enzyme is essential for completing the physical block to polyspermy (Wong et al., 2004). Unlike genes utilized exclusively for the formation of the fertilization envelope and expressed exclusively during oogenesis, such as the structural matrix proteins SFE1, SFE9, proteoliasin, rendezvin, and the enzyme ovoperoxidase, (Wessel et al., 2001) and (Wong et al., 2004), *Udx1* transcripts are present in eggs and later in development (Wong et al., 2004). Interestingly, Udx1 also plays a role in the early development as its specific inhibition induces a delay in cytokinesis (Wong and Wessel, 2005). In the egg, this hydrogen peroxide synthesis is necessary for the activity of the ovoperoxidase, a tyrosine crosslinking enzyme derived from the egg cortical granules (Foerder and Shapiro, 1977) and (LaFleur et al., 1998). In the sea urchin *S. purpuratus*, the ovoperoxidase mRNA is present exclusively in oocytes and is turned over rapidly following germinal vesicle breakdown (LaFleur et al., 1998). Under normal conditions, ovoperoxidase is specifically targeted to the FE via a calcium-dependent interaction with proteoliasin (Weidman et al., 1987). The ovoperoxidase activity is sensitive to transglutaminase (Wong and Wessel, 2009), CGSP1 (Haley and Wessel, 2004b), and Udx1 (Wong et al., 2004). Semi-*in vivo* crosslinking assay identifies four major targets of ovoperoxidase (Wong and Wessel, 2008): RDZ¹²⁰, proteoliasin, SFE1, and SFE9.

The vast majority of what is known about the fertilization envelope is from the study of a few sea urchin species, yet similar fertilization envelopes are utilized by other echinoderms. Here we explore the proteome of the fertilization envelope in sea stars, and compare its sequences to those in the pencil urchin, thought to be reflective of the ancient sea urchins within the fossil record, and to the well-known sea urchins *S. purpuratus* and *Lytechinus variegatus*, for which most work on the cortical granules and fertilization envelopes have been accomplished. The sea star family, the Asteroidea, contains an estimated 1,600 species worldwide (Blake, 1989). Their eggs are generally stored in prophase of meiosis I, and spawning activates release of the inducer for meiotic progression, 1-methyl adenine. Upon germinal vesicle breakdown, the oocyte becomes fertilization-competent, and following sperm-egg fusion, a robust fertilization envelope forms. Many sea stars rely on the fertilization envelope to limit exposure to harmful elements in the marine environment;

some species also rely on the envelope to constrain the blastomeres (Dan-Sohkawa, 1976), (Matsunaga et al., 2002). Removal of the fertilization envelope in many sea star species leads to blastomeres dissociating from each other and subsequent death, likely because of the absence of a distinct hyaline layer, an embryonic extracellular matrix found in sea urchins. Here we determine the genes responsible for formation of the fertilization envelope in the sea star *Patiria miniata* (the common batstar) by proteomic, genomic, and functional criteria.

Materials and methods

Animals

P. miniata were housed in aquaria with artificial seawater (ASW) at 16°C (Coral Life Scientific Grade Marine Salt; Carson, CA). Gametes were acquired by opening up the animals. Immature and full-grown oocytes were collected in filtered seawater and sperm was collected dry. Oocytes were separated by size using Nytex filters, and size separation was improved by manual sorting under the microscope. To obtain mature oocytes, the full-grown, immature oocytes were incubated for an hour in filtered sea water containing 2 μ M 1-methyladenine. After addition of sperm, fertilized eggs were cultured in filtered seawater at 16°C (Wessel et al., 2010).

S. purpuratus were housed in aquaria with artificial seawater (ASW) at 16°C (Coral Life Scientific Grade Marine Salt; Carson, CA). Gametes were acquired by either 0.5M KCl injection or by shaking. Eggs were collected in filtered seawater and sperm was collected dry. To obtain embryos, fertilized eggs were cultured in filtered seawater at 16°C.

Permeability assays

Fertilization envelope permeability was tested by measuring the diffusion of fluorophore-conjugated dextrans into the perivitelline space (Wong and Wessel, 2008). As appropriate, eggs were fertilized in filtered seawater or in filtered seawater containing 1 mM 3-aminotriazole (3-AT) and dejellied by acidic treatment (Foltz et al, 2004). Twenty minutes after fertilization, zygotes were incubated with 5 μ M fluorescein dextran 10,000 Daltons, (10-kDa-dex) and 50 nM Rhodamine dextran 2,000,000 Daltons, (2,000-kDa-dex) diluted in filtered sea water with or without 1 mM 3-AT. Ten minutes after exposure, zygotes were imaged for both fluorescein and rhodamine using a LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc.; Thornwood, NY). Average fluorescence intensity was measured using regions within the perivitelline space or the surrounding media using Metamorph software (Universal Imaging Corporation, Downingtown, PA). For each condition, measurements were made on 10 embryos.

Mass spectrometry analysis

Fertilization envelopes were separated from the cells by manual sorting under a dissecting microscope. Fifteen hundred fertilization envelopes were purified and loaded on a SDS PAGE gel for Coomassie staining. The proteins obtained were processed for *in gel* digestion using the *In gel* tryptic digestion kit (Pierce, Rockford, IL). Three hundred additional fertilization envelopes were purified for *in solution* digestion. Briefly, envelopes were resuspended in 100 mM NH_4HCO_3 , pH 8, and denatured for 5 minutes at 95°C. After addition of 20 mM DTT, the solution was incubated at 56°C for 45 minutes. The sample was alkylated during a 30-minute incubation at room temperature with 55 mM iodoacetamide. Proteins were digested overnight at 37°C in the presence of 10 ng/ μ l trypsin. Samples were identified using a Thermo-Finnigan LTQ linear ion trap mass spectrometer.

Phylogenetic analysis

Phylogenetic trees were made using the program PhyML available on the website phylogeny.fr (Dereeper et al., 2008).

Whole mount RNA *in situ* hybridization (WMISH)

Sequences used to make antisense WMISH probes for *Pm-rendezvin*, *Pm-SFE9*, and *Pm-proteoliasin* were amplified from *Pm* ovary cDNA and cloned into pGEM T-Easy (Promega). The corresponding primers are presented in supplemental data 1A. The pGEM T-Easy plasmids were linearized using either SalI (T7 transcription) or ApaI (SP6 transcription) (Promega; Madison, WI). Antisense, DIG-labeled RNA probes were constructed using a DIG RNA labeling kit (Roche; Indianapolis, IN).

WMISH experiments were performed as described previously (Minokawa et al., 2004), and the alkaline phosphatase reaction was carried out for 1h. A non-specific DIG-labeled RNA probe complementary to *neomycin*, obtained from the pSport 18 (Roche; Indianapolis, IN) was used as a negative control. Samples were imaged on a Zeiss Axiovert 200M microscope equipped with a Zeiss color AxioCam MRc5 camera (Carl Zeiss, Inc.; Thornwood, NY).

Real-time quantitative PCR (QPCR)

RNA was extracted from young (100 μ m diameter), full-grown immature, and mature oocytes, or 30 min after fertilization using the RNeasy Micro Kit (Qiagen; Valencia, CA). cDNA was prepared using the TaqMan® Reverse Transcription Reagents kit (Applied Biosystems; Foster City, CA). QPCR was performed on a 7300 Real-Time PCR system (Applied Biosystems; Foster City, CA) with the SYBR Green PCR Master Mix Kit (Applied Biosystems; Foster City, CA). Experiments were run in triplicate, and the data were normalized to 18S RNA levels. The primers used to amplify *Pm-rendezvin*, *Pm-SFE9*, *Pm-proteoliasin* and *18S* are indicated in supplemental data 1B.

Antibody production

A region of *Pm-SFE9* was cloned using the primers F (5'-CCCAGACCTTGATGCAATG-3') and R (5'-CCCAGTCGAGCAATCTCTGTAC-3'). This sequence was inserted in the pNO-TAT vector in frame with a 6xHis tag (Nagahara et al., 1998). Recombinant protein was expressed in BL21 bacteria, purified on a ProBond nickel column (Invitrogen; Carlsbad, CA), and used to raise antiserum in rabbit as previously described (Wong and Wessel, 2004).

Western blot

Western blot analyses were performed following electrophoretic transfer of proteins from SDS-PAGE onto 0.22- μ m nitrocellulose membranes (Towbin et al., 1979). Membranes were incubated with antibodies directed against *Pm-SFE9* (1:1000) in 20 mM Tris-HCl (pH 7.6), 1% BSA, and 0.1% Tween-20, overnight at 4°C. The antigen-antibody complex was measured by chemiluminescence using horseradish peroxidase-coupled secondary antibodies according to the manufacturer's instructions (ECL; Amersham Pharmacia Biotech). Preimmune serum from the same rabbit was used as a control. Three hundred purified fertilization envelopes were loaded per lane.

Immunofluorescence

Oocytes and embryos were cultured as described above, and samples were collected at indicated stages for whole-mount antibody labeling. Cells were fixed overnight in 4% paraformaldehyde in ASW, washed 3 times with PBS-Tween, and stored at 4°C. Oocytes and embryos were blocked for an hour at room temperature in 4% sheep serum (Sigma; St.

Louis, MO) /PBS-Tween (blocking buffer). For labeling, the cells were incubated overnight at 4°C with the anti *Pm*-SFE9 serum diluted 1:1000 in blocking buffer. The preimmune serum, also diluted by 1:1000, was used as a control. The cells were washed 3 times with PBS-Tween, and then incubated with anti-rabbit Alexa Fluor 488 conjugated antibody (Invitrogen), diluted 1:500 in blocking buffer, for two hours at room temperature. Oocytes and embryos were then washed 3 times with PBS-Tween. Pictures were taken on a LSM 510 laser scanning confocal microscope (Carl Zeiss, Inc.; Thornwood, NY). These pictures were used to define the number of cortical granules in young oocytes. Five young oocytes, with an average diameter of 72.1µm, were used for the quantification. The number of cortical granules was manually counted in the optical slice obtained using a pinhole of 0.99. The volume of each optical slice was defined by the formula $V = \pi r^2 h$. An approximate number of cortical granules per µm³ per oocyte analyzed was calculated, which was then multiplied by the volume of the corresponding oocyte to obtain the number of cortical granules per oocyte.

Results

Sea star and sea urchin fertilization envelopes show differential permeability

Fluorophore-conjugated dextrans were used to compare the permeability of the fertilization envelope in the sea urchin *S. purpuratus* (*Sp*) and the sea star *P. miniata* (*Pm*; Fig 1). Twenty minutes after fertilization, dejellied zygotes were incubated with two different sized compounds, fluorescein-conjugated 10-kDa-dex and rhodamine-conjugated 2000-kDa-dex. The permeability of the fertilization envelope in sea urchins is known to be sensitive to 3-aminotriazole (3-AT), which is an inhibitor of ovoperoxidase activity (Showman and Foerder, 1979). We used this reagent to compare the dityrosine crosslinking in both species. Only 52% of the fluorescein 10-kDa-dex diffused through the fertilization envelope in sea urchin zygotes (Fig 1Aa), whereas this diffusion increases to 92% in the presence of 3-AT (Fig 1Ac). In sea stars, the perivitelline level increased to 66% for the 10 kD-dex (Fig 1Ae), and addition of the 3-AT increased this diffusion to 81%. (Fig 1Ag). Sibling zygotes were simultaneously exposed to the 2,000-kDa-dex. Sea urchin zygotes show a low permeability for this reagent: 1% of the fluorescence was found in the perivitelline space (Fig 1Ab), whereas the diffusion through the fertilization envelope increased to 51% in presence of 3-AT (Fig 1Ad). In sea stars, 30% of the rhodamine present in the media was found in the perivitelline space in normal conditions (Fig 1Af), while a 3-AT pre-treatment increased the diffusion to 56% (Fig 1Ah). Interestingly, the 3-AT increased the permeability of the sea urchin fertilization envelope by 1.8 times for the 10-kDa-dex and by 50 times for the 2000-kDa-dex, whereas 3-AT increased the diffusion of the 10-kDa-dex by 3.1 times, and only increased the diffusion of the 2000-kDa-dex by 1.8 in the sea star. Altogether, these results suggest that the fertilization envelope is more permeable in sea stars than in sea urchins, and that the sea star has dityrosine activity, which influences the functionality of the envelope. Due to its more porous nature, however, this barrier is only evident with the larger diffusion reagents. The observation that 3-AT significantly increased the permeability of the sea star envelope also demonstrates that the perivitelline space is not in itself restrictive to the diffusion of dyes.

Only three of the five proteins found in the sea urchin fertilization envelope are present in the sea star

Purification of fertilization envelope proteins from the sea urchin *S. purpuratus* resulted in the identification of SFE9, rendezvin, ovoperoxidase, SFE1 and proteoliasin (Wong and Wessel, 2006b). To identify the components of the fertilization envelope in the sea star *P. miniata*, fertilization envelopes were purified and subjected to SDS-PAGE electrophoresis. After Coomassie blue staining, eight main protein bands were visualized and cut out of the

gel for mass spectrometry analysis (Fig 2). Three main proteins were identified: SFE9, rendezvin, and proteoliasin. Except for bands 4 and 8, which were identified as SFE9 and rendezvin, respectively, the other bands contained either SFE9 and proteoliasin or SFE9 and rendezvin, or all three proteins together. The combinatorial results suggest that these proteins might be crosslinked. According to the transcriptome data, the molecular weight of SFE9 and rendezvin were predicted to be 101-kDa and 201-kDa respectively. Proteoliasin was expected at a molecular weight higher than 79-kDa. The identification of these proteins in bands with a higher molecular weight than expected supports the hypothesis of crosslinking activity. To address the possibility that some fertilization envelope components might be in low abundance and not visualized by Coomassie blue staining, another purification of fertilization envelope was performed and subjected to direct *in solution* trypsin digestion before mass spectrometry analysis. The same proteins, previously obtained after *in gel* trypsin digestion, were identified (data not shown). These results indicate that in the sea star *P. miniata*, the fertilization envelope is primarily composed of SFE9, rendezvin, and proteoliasin.

The proteins involved in fertilization envelope formation differs among Echinoderms

The fertilization envelope formation is well described in the sea urchin *S. purpuratus*. To address the evolution of the fertilization envelope within Echinoderms, we considered another sea urchin species, *L. variegatus* (*Lv*), the pencil urchin *Eucidaris tribuloides* (*Et*), and two sea stars: *P. miniata* (*Pm*), and *Asterias forbesi* (*Af*). *S. purpuratus* diverged from *L. variegatus* between 30 and 50 million years ago (Smith et al., 2006). Sea urchins and pencil urchins diverged around 250 million years ago (Smith et al., 2006). Sea urchins and sea stars diverged approximately 500 million years ago (Hinman et al., 2003). The transcriptomes of *Lv*, *Et*, *Pm*, and *Af*, were obtained from ovary (Adrian Reich, unpublished data). We first looked for the transcripts encoding the three proteins found in both *Sp* and *Pm* fertilization envelopes. SFE9, proteoliasin, and rendezvin were present in all five species (Fig 3 and Supplemental data 2). We found that, in sea star, the permeability of the fertilization envelope for the high molecular weight is sensitive to 3-AT (Fig 1), but an ovoperoxidase ortholog was not detected (Fig 2). Interestingly, the transcript encoding an ovoperoxidase was found in both sea stars, as well as in the sea urchin *Lv* and in the pencil urchin transcriptomes (Fig 3). Altogether, these results indicate that some proteins involved in the formation of the fertilization — SFE9, proteoliasin, and rendezvin — are conserved among sea urchins, pencil urchin, and sea stars.

Rendezvin, SFE9, and proteoliasin transcripts are specifically expressed during the early oogenesis

To determine when the genes that encode the major fertilization envelope proteins are active in sea stars, *rendezvin*, *SFE9*, and *proteoliasin* mRNA probes were synthesized for *in situ* hybridization (Fig 4). A probe against *neomycin* was used as a negative control. The overall results show similar mRNA accumulation profiles for *rendezvin*, *SFE9*, and *proteoliasin*. The mRNAs accumulate uniformly throughout the oocyte, and at highest levels in young oocytes. Interestingly, the transcript levels are barely detectable in the full-grown, immature oocytes and in embryonic stages. Quantitative PCR was used to measure the relative RNA levels of *Pm-SFE9*, *proteoliasin*, and *rendezvin* in young oocytes (100- μ m diameter), full-grown immature oocytes, mature oocytes, and fertilized eggs (Fig 5). All values were normalized against 18S RNA and the corresponding Ct values are presented in supplemental data 3. These qPCR data confirm the RNA expression results obtained by *in situ* hybridization. For the three transcripts, the level of mRNA decreases during later oogenesis, reaching its lowest level in the full-grown immature oocytes, mature oocytes, and fertilized eggs. These results indicate that the transcript level of the proteins found in the sea star fertilization envelope uniformly accumulate in the early oocytes.

Cortical granules translocate to the cell periphery during early oogenesis

A polyclonal antibody was generated against *Pm*-SFE9, and was used to determine the pattern of synthesis, location and fate of the major fertilization envelope proteins after fertilization. The transcript found in the *Pm* transcriptome contains 2775 nucleotides, leading to a protein sequence of 924 amino acids, with an expected size of 101-kDa. The antibody was first tested by immunoblot on purified fertilization envelopes (Fig 6). One protein was detected at the estimated molecular weight of 365-kDa (arrow), at higher relative size than predicted by primary sequence alone. This difference in molecular weight could be explained by the crosslinking of SFE9 to other proteins present in the fertilization envelope, as also found in the sea urchin (Wong and Wessel, 2008) and/or by post-translational modifications such as glycosylation. The preimmune serum did not detect this band, and demonstrates the specificity of the antiserum.

By immunofluorescence, *Pm*-SFE9 was detected in the cells from early oocytes to fertilized eggs. During oogenesis, especially in 110- μ m oocytes (Fig 7B) to mature oocytes (Fig 7E), SFE9 is highly enriched at the periphery of the cytoplasm. Consistent with other cortical granule content proteins, SFE9 was exocytosed at fertilization and it incorporated into the fertilization envelope (Figure 7 and Supplemental data 5). The preimmune serum did not detect any fluorescence using the same conditions (Supplemental data 4). Moreover, *Pm*-SFE9 antibody didn't detect any signal in embryos post-hatching, indicating its specificity for construction of the fertilization envelope (supplemental data 6). The cortical granules of young oocytes smaller than 100 μ m (Fig 7A and Fig 8) were distributed throughout the entire cytoplasm, and this signal became restricted to the cortex in oocytes larger than 100 μ m. These results suggest that the major period of cortical granule protein synthesis and cortical granule construction occurs early in oogenesis. After image quantification, we found that young oocytes, smaller than 100 μ m, contain approximately $11,400 \pm 2519$ ($n=5$) cortical granules per oocyte.

Discussion

In the sea urchin *S. purpuratus*, cortical granules accumulate throughout the cytoplasm until germinal vesicle breakdown, and then translocate to the cell periphery (Wessel, 1995) and (Laidlaw and Wessel, 1994). Our results suggest that, in contrast to the sea urchin, sea star cortical granules translocate to the cortex as they are synthesized. This early translocation seems more similar to the mechanism described in mice, in which the density of cortical granules present in the cortex increases continually during oogenesis (Ducibella et al., 1994). The production and migration of sea star cortical granules are continuous processes. Since the cortical granules are already at the oocyte surface prior to meiosis, what happens to them during meiosis, especially during the formation of the polar bodies and meiotic spindles? Does the meiotic spindle displace the cortical granules prior to polar body formation, or do they exocytose prematurely, as in mice? We found no evidence of precocious fertilization envelope formation in the sea star in the area of the meiotic spindle and polar body so we anticipate a cortical granule displacement is made at meiosis.

Cortical granules were previously analyzed in the sea star *Pisaster ochraceus* (Reimer and Crawford, 1995). Using a monoclonal antibody against a 120-kDa protein, it was shown that in immature oocytes, cortical granules were concentrated in the periphery of the cytoplasm, but were also found throughout the cytoplasm. In mature oocytes, a larger number of granules were located at the periphery of the cytoplasm, but some granules were still present throughout the cytoplasm. After fertilization, the staining was predominantly found in the perivitelline space, although several brightly stained granules remained in the cell cytoplasm. Later in development at the blastula, the fertilization envelope was not stained by this monoclonal antibody, but labeled granules were present in blastomeres (Reimer and

Crawford, 1995). Thus, it is not clear how selectively this antibody identifies cortical granules, or whether it includes recognition of other secretory organelles. To follow the cortical granule biogenesis in the sea star *P. miniata*, we used an antibody against SFE9 to learn that in this species they move to the cell periphery during early oogenesis. The contrasting results between these two species might be explained by the different target proteins studied as well as biological trafficking of different proteins. This may also be simply a matter of species difference in the cortical granule strategy. The granules found at the oocyte periphery might contain both SFE9 and the 120-kDa protein, whereas the granules persisting in the *P. ochraceus* embryos might contain only the 120-kDa protein and/or could play a different role in the development, such as the deposition of a more general extracellular matrix protein (Wong and Wessel, 2006a), e.g. decapod oocyte granules.

Although an ovoperoxidase protein was not directly captured during proteomic analysis, we have lines of evidence to suggest that it is present. First, we found the sequence encoding the ovoperoxidase enzyme within each oocyte transcriptome from five echinoderm species analyzed, including two sea star species. Second, we indirectly observed its enzymatic activity: 3-AT is a specific inhibitor of ovoperoxidase, a myeloperoxidase-type enzyme (Daiyasu and Toh, 2000), and exposure to 3-AT resulted in significantly increased dextran diffusion in the sea star, similar to that documented in the sea urchin ((Wong and Wessel, 2008); Figure 1). Thus, we believe ovoperoxidase is one of the conserved fertilization envelope proteins of echinoderms, although unlike in sea urchins, in sea stars, its abundance may be limiting or it may diffuse away from the structure when its crosslinking activity is complete.

In *P. miniata*, the transcripts encoding the major cortical granule proteins (SFE9, proteoliasin and rendezvin) are synchronously regulated. Their RNA is highly expressed in early oocytes, and is rapidly lost in later oogenesis. The timing of this degradation coincides with the translocation of the majority of cortical granules to the cell periphery. In sea urchins, the RNA levels of most of fertilization envelope protein transcripts also decrease during oocyte maturation, particularly when the cortical granules move to the cell periphery (Laidlaw and Wessel, 1994). These two events occur at different phases of sea urchin and sea star oogenesis, but the parallels in relative timing suggest a common mechanism linking the reduction in RNA with the translocation of the cortical granules.

This observation opens two important considerations: Are mRNAs degraded by a shared mechanism, such as miRNAs or specific 3'UTR degradation elements or are the genes regulated by the same transcription factors to synchronize timing and protein stoichiometry? Further, cortical granule mRNA degradation begins as the oocytes rapidly increase in size, a phenomenon consistent with vitellogenesis. In echinoderms, the vitellogenin appears to be made in the digestive tract of the adult and is transported to the ovary where it is taken up into yolk granules (Brooks and Wessel, 2003). That uptake begins with a vitellogenic phase of oogenesis, a transitional period in development of this cell. Although we do not know how this transition is activated, this period may include a transition that involves reallocation of energy and resources, repressing cortical granule assembly, and the associated expression of genes that encode their content, in favor of processes that will enhance embryo viability.

Our results demonstrate that the fertilization envelope in sea urchins is a much more selective barrier than in the sea star. Three similar structural proteins rich in LDLrA repeats: proteoliasin, SFE9, SFE1 compose the fertilization envelope in sea urchin, but no SFE1 ortholog was identified in the sea star. This suggests that SFE1 is not required to form a fertilization envelope, but might be key to efficient packing of the envelope proteins to

reduce permeability. SFE1 may have appeared in sea urchins by duplication of SFE9 or proteoliasin to multiply the level of LDLrA rich proteins in the fertilization envelope and thus to form a structure more efficiently protective of the egg. One way to increase protein levels of an envelope protein may be to duplicate a gene and regulate its expression in a manner similar to other genes encoding envelope proteins. This may have been an evolutionary transition that occurred between sea stars and sea urchins. Yet, the diversification in sequence motifs between the two species was otherwise minimal. Perhaps this conservation is a result of compatibility within the complex—several proteins must rapidly and effectively self-assemble, and if they are delayed or compromised in their sperm-blocking, or pathogen blocking ability, the embryos may rapidly die. Even though separated from a common ancestor by approximately 0.5 billion years, the envelope proteins between sea urchins and sea stars remained largely similar in terms of composition, motif, and function.

The human renal glomerulus filters particles on the order of <50-kDa, somewhat smaller than serum albumin, and this extracellular matrix filter takes a few weeks to develop. In contrast, the *S. purpuratus* fertilization envelope filters materials of <~40-kDa (Wong and Wessel, 2008) and takes ~30 seconds to form. Based on the morphology of the fertilization envelope in sea stars, i.e. forms more slowly (several minutes) and is significantly thicker than the sea urchin fertilization envelope, we anticipated it would be relatively impermeant. Remarkably, it was far more permeable, allowing dextrans of 2000-kDa in size to diffuse through. Although still an effective barrier to sperm, it is clearly more passive to large molecules. This changes what we think about its role in the sea star environment – nutrients would be far more accessible to the developing embryo, and perhaps the embryo is able to endocytose larger nutrient particles for growth. On the other hand, it would be less capable of blocking toxins in the environment, perhaps even allowing small viral particles to diffuse through. These embryos may be more susceptible to environmental insults, especially in areas close to human effluents at the exact time in development that is most sensitive to the insulting agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SFE	soft fertilization envelope
FE	fertilization envelope

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Figure 1. The fertilization envelope is more permeable in sea star than in sea urchin

(A) After fertilization, dejellied zygotes from sea urchin *Sp* (a, b, c, d) and sea star *Pm* (e, f, g, h) were simultaneously exposed to a 10-kDa fluorescein dextran (a, c, e, g), and a 2000-kDa rhodamine dextran (b, d, f, h). In both species, the diffusion of the fluorophore dextran into the perivitelline space was analyzed in normal conditions (a, b, e, f) or in presence of 3-AT, (c, d, g, h). Perivitelline space is found between the zygote (zyg, white arrow), and the fertilization envelope (black arrow). (B) Determination of the dextran permeability index, corresponding to the ratio of fluorescence present in the perivitelline space to the media, results are shown as percentages. Ten individuals were measured in each condition.

Significance was assessed for each condition between *Sp* and *Pm* using Student's *t*-test $P < 0.05$. Significant differences were obtained in normal condition between *Sp* and *Pm* for the diffusion of the 10-kDa (a) and 2000-kDa (b) dextran, and between *Sp* and *Pm* in the presence of 3-AT for the diffusion of the 10-kDa dextran (c). Significant differences were also obtained for *Sp* (d, e) and *Pm* (f, g) between normal condition without 3-AT and the addition of 3-AT for both conjugated dextrans.

Figure 2. In the sea star *Pm*, the fertilization envelope is composed of three major proteins: SFE9, rendezvin, and proteolaisin

(A) Fertilization envelopes were isolated from zygotes and loaded on a SDS-PAGE gel. After Coomassie blue staining, eight main bands were obtained (a). Each band, represented by a black box (b), was cut out of the gel for mass spectrometry analysis. (B) Results of the mass spectrometry. The purification was done twice using different *Pm* sea stars for the fertilization. The same bands were obtained in both experiments and analyzed by mass spectrometry. The letters a and b following the band numbers correspond to the duplicates for each band. The table lists the name of the protein identified in each band followed by the corresponding transcript number found in the *Pm* transcriptome.

Figure 3. Phylogenetic trees representing the proteins involved in the formation of the fertilization envelope

The amino acid sequences of SFE9, proteoliasin, rendezvin and ovoperoxidase, identified in the five species *S. purpuratus* (Sp), *L. variegatus* (Lv), *E. tribuloides* (Et), *P. miniata* (Pm), *A. forbesi* (Af), were used to design phylogenetic trees. (A) Tree for SFE9. Homo sapiens LDLr (NCBI: NP000518) was used as an outgroup. (B) Tree for proteoliasin. Homo sapiens LDLr (NCBI: NP000518) was used as an outgroup. (C) Tree for rendezvin. Homo sapiens Bmp1 (NCBI: AAI01764) was used as an outgroup. (D) Tree for ovoperoxidase. Homo sapiens proteins myeloperoxidase, MPO (NCBI: AAA59863) was used as an outgroup. For each tree, the scale bar indicates an evolutionary distance of amino acid substitutions per position.

Figure 4. *Pm rendezvin*, *SFE9*, and *proteolaisin* mRNAs are highly and uniformly expressed during early oogenesis

Whole mount *in situ* hybridization in the sea star *P. miniata*, using probes against *Pm-rendezvin*, *SFE9*, and *proteolaisin*, in young oocytes, immature full-grown oocytes, mature oocytes, 30 minutes after fertilization, at the two-cell stage, in blastula, and in gastrula. *Neomycin* is used as a negative control.

Figure 5. *Pm-SFE9*, *proteoliasin*, and *rendezvin* RNA levels decrease during oogenesis

QPCR was used to measure the RNA levels of *Pm-SFE9*, *proteoliasin*, and *rendezvin*, at the indicated developmental stages: young oocytes (100 μ m diameter), full-grown immature oocytes, mature oocytes, and 30 minutes after fertilization. All values were normalized against 18S RNA and represented as a fold-change relative the amount of RNA present in the young oocytes. Significance was assessed for each transcript between young oocytes and each other developmental stage using Student's *t*-test, $P < 0.05$. Significant differences were obtained between the young oocytes and all the other stages: immature oocytes, mature oocytes and fertilized eggs for each transcript: *SFE9* (a), *proteoliasin* (b), and *rendezvin* (c).

Figure 6. *Pm*-SFE9 antibody specifically recognizes one high molecular weight bands
 Western blot using the antiserum raised against *Pm*-SFE9 (B) or its preimmune serum (A) on proteins obtained from purified fertilization envelopes (A, B). 300 fertilization envelopes were loaded in each well.

Figure 7. The protein *Pm-SFE9* is present throughout oogenesis, maturation, and fertilization
Immunofluorescence using the antiserum against *Pm-SFE9* (A, B, C, D, E, F) on 85-μm diameter oocytes (A), 110-μm diameter oocytes (B), 130-μm diameter oocytes (C), full-grown oocytes (D), mature oocytes (E), and fertilized eggs (F). The corresponding differential interference contrast images are respectively shown in G to L. For each developmental stage, the overlay of the fluorescence and the DIC image is represented in M to R. Scale bar, 100μm. Pictures were taken using the same microscope settings (laser intensity, pin-hole opening) at 200× magnification.

Figure 8. Sea star cortical granules move to the periphery of the cell during early oogenesis
 Immunofluorescence using the antiserum against *Pm*-SFE9 (B, D) or its preimmune serum (A, C) on oocytes having a diameter smaller (A, B) or greater than 100 μm (C, D). The corresponding differential interference contrast images are respectively shown from E to H. For each developmental stage, the overlay of the fluorescence and the DIC image is represented in I to L. Scale bar = 100 μm . Pictures were taken using the same microscope settings (laser intensity, pin-hole opening) at 400 $\times$ magnification.