Major components of a sea urchin block to polyspermy are structurally and functionally conserved

Julian L. Wong and Gary M. Wessel*

Department of Molecular Biology, Cellular Biology, and Biochemistry, Box G-J4, Brown University, Providence, RI 02912, USA

*Author for correspondence (email: rhet@brown.edu)

SUMMARY One sperm fusing with one egg is requisite for successful fertilization; additional sperm fusions are lethal to the embryo. Because sperm usually outnumber eggs, evolution has selected for mechanisms that prevent this polyspermy by immediately modifying the egg extracellular matrix. We focus here on the contribution of cortical granule contents in the sea urchin block to polyspermy to begin to understand how well this process is conserved. We identified each of the major constituents of the fertilization envelope in two species of sea urchins, Strongylocentrotus purpuratus and Lytechinus variegatus, that diverged 30 to 50 million years ago. Our results show that the five major structural components of the fertilization envelope, derived from the egg cortical granules, are semi-conserved. Most of these orthologs share sequence identity and encode multiple low-density lipoprotein receptor type A repeats or CUB domains but at least two contain radically different carboxy-terminal repeats. Using a new association assay, we also show that these major structural components are functionally conserved during fertilization envelope construction. Thus, it seems that this population of female reproductive proteins has retained functional motifs while gaining significant sequence diversity—two opposing paths that may reflect cooperativity among the proteins that compose the fertilization envelope.

INTRODUCTION

If multiple sperm fuse with an egg at fertilization, the resultant zygote usually dies. This lethality is usually caused by the multiple paternal centrioles inherited by the embryo, which compete for the extra chromosomes and disrupt the establishment of the cleavage furrow. Thus, eggs have evolved barriers that are erected immediately after first insemination to block such polyspermy. Two mechanisms exist within the animal kingdom to achieve this block to polyspermy: a fast and a slow block. The fast block, first identified in echinoderms, is a rapid change in membrane potential that impairs additional sperm binding (Jaffe 1976; Schatten and Hulser 1983; Jaffe and Schlichter 1985; Glahn and Nuccitelli 2003). This event occurs within milliseconds of sperm-egg fusion and endures for about 60 sec. The slow block is a physical modification of the egg’s extracellular matrix that results in a new physical barrier between the fertilized egg and the unsuccessful sperm (Shapiro et al. 1989). This barrier persists through early development and protects the embryo against additional sperm penetration, invasive pathogens, and physical damage.

Two spatially distinct protein populations, both made during oogenesis, are needed to assemble the physical block to polyspermy. One population is an extracellular glycoprotein coat known as the zona pellucida in mammals and marsupials, the vitelline membrane in amphibians and avians, the chorion in teleosts, and the vitelline layer in echinoderms. This layer protects the oocyte, functions in sperm recognition and sperm activation, and scaffolds assembly of the slow block to polyspermy. The second population of proteins necessary for the physical block is derived from secretory vesicles found near the egg cortex, usually referred to as egg cortical granules.

The physical block to polyspermy in sea urchins involves the creation and elevation of a fertilization envelope, a shell that prevents extra sperm from reaching the new zygote. As with other egg-derived barriers, the sea urchin fertilization envelope is assembled from secreted cortical granule components laid onto a preexisting matrix, the carbohydrate-rich vitelline layer or glycocalyx. Little is known about the constituents of the vitelline layer, although one- and two-dimensional gel electrophoresis demonstrate that it consists of at least 25 different proteins (Gache et al. 1983; Niman et al. 1984).

The major constituents of the sea urchin fertilization envelope originate from the cortical granules. In ascending order by mass from Strongylocentrotus purpuratus, they are ovoperoxidase (Somers et al. 1989; LaFleur et al. 1998), the cortical granule component of rendezvin (rendezvin®; unpublished data), SFE9 (Wessel 1995), SFE1 (Wessel et al., 2000), and proteolaisin (Somers et al. 1989; Somers and...
After cortical granule secretion, these proteins self-assemble within the vitelline layer scaffold as it detaches and lifts from the plasma membrane. The completed fertilization envelope is approximately four times greater in surface area than the original vitelline layer (Fig. 1A). Such an expansion is accomplished via the hydration of glucosaminoglycans from the cortical granules and the activity of CGSP1, a serine protease that severs the proteinaceous contacts between the plasma membrane and the vitelline layer (Carroll and Epel 1975a,b; Shapiro et al. 1989; Haley and Wessel 1999). Final modifications to the fertilization envelope involve ovoperoxidase, an enzyme that generates di-tyrosine cross-links during the “hardening process” of fertilization envelope construction (Foerder and Shapiro 1977; Deits et al. 1984; Kay and Shapiro 1987; LaFleur et al. 1998). In the absence of ovoperoxidase activity, the fertilization envelope still forms, but it remains a pliable or “soft” fertilization envelope (SFE), thereby losing its effectiveness as a physical shield (Showman and Foerder 1979).

Differences in cortical granule morphology between species (Runnstrom 1966) (Fig. 1B) suggest that the fertilization envelope proteins have diverged widely. In *S. purpuratus*, the cortical granules are 1.2 μm in diameter and contain a characteristic lamellar substructure, as documented by transmission electron microscopy (Fig. 1B). In contrast, all granules of *Lytechinus variegatus* eggs contain a mosaic pattern of electron lucent and dense organization (Fig. 1B), whereas *Arbacia punctulata* granules contain electron-dense stellate structures (Anderson 1968) and those of *Paracentrotus lividus* possess a series of interconnected concentric lamella (Runnstrom 1966). Given that the biogenesis of regulated secretory vesicles in many cell types shares a common

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**Fig. 1.** Comparison of cortical granule morphology and fertilization envelopes from *Lytechinus variegatus* versus *Strongylocentrotus purpuratus*. (A) Light microscopy image of *L. variegatus* and *S. purpuratus* eggs (egg) and one-cell zygotides (zygote). Bar, 50 μm. (B) Transmission electron micrographs of cortical granules from *L. variegatus* (left) and *S. purpuratus* (right). Bar, 500 nm. (C) Transmission electron micrographs of cross-sections from fertilization envelopes of *L. variegatus* (left) and *S. purpuratus* (right). Fertilization envelopes are oriented with the zygotic-face directed to the left. Bar, 50 nm. (D) Coomassie staining of 5 μg of total soft fertilization envelope (SFE) isolated from *L. variegatus* or *S. purpuratus* and separated by SDS-PAGE.
pathway through the rough endoplasmic reticulum and Golgi apparatus, the striking morphological differences in cortical granules among sea urchins is predicted to be a consequence of alternative packaging and/or different content proteins (Tooze and Stinchcombe 1992; Arvan and Castle 1998; Tooze et al. 2001; Wessel et al. 2001). Because the biophysical properties of content proteins in mature secretory granules usually dictate the final condensation and aggregation state within the granule lumen (Arvan and Castle 1998; Thiele and Huttner 1998), one model would predict that different content proteins account for the variability in cortical granule morphology among sea urchins.

In contrast, sequence identification of three cDNA orthologs of ovoperoxidase (LaFleur et al. 1998; Nomura et al. 1999), the only cortical granule-derived protein for which cross-species sequences are available, suggests that the mechanism of fertilization envelope construction is conserved. The sequence similarity in this enzyme across three sea urchin species suggests that at least some fraction of the cortical granule proteins are orthologous because (a) the target substrates for ovoperoxidase are specific to this population of proteins (Kay and Shapiro 1987) and (b) ovoperoxidase binds directly to proteoliaisin (Somers et al. 1989; Somers and Shapiro 1991), making the protein–protein interaction domain a potential site of purifying coevolution. These features of ovoperoxidase, combined with the morphological similarities between fertilization envelopes (Fig. 1C), suggest that despite the differences in cortical granule ultrastructure, the constituents of the physical block to polyspermy in sea urchins are also conserved.

To help resolve the dichotomy of diverse cortical granule ultrastructure versus fertilization envelope homogeneity, we asked whether the major fertilization envelope proteins are conserved between two species of sea urchin, _S. purpuratus_ and _L. variegatus_. Here we report the cloning of all the major fertilization envelope proteins of these two species and find that the structural components derived from the cortical granules contain regions of high sequence similarity, especially in protein-binding motifs. Amino acid identity, however, is not universal across all the constituents.

**METHODS**

**Animals**

Adult _S. purpuratus_ were obtained from Charles Hollahan (La Jolla, CA, USA) and kept in 15 °C artificial seawater (ASW) until needed. Adult _L. variegatus_ were collected from Tampa Bay, Florida (USA) and kept in 22 °C ASW until needed. ASW was generated from Instant Ocean premixed salts (Aquarium Systems, Mentor, OH, USA). Eggs and sperm were collected for use by intracoealomic injection of 0.5 M KCl. Eggs were passed twice over 80-μm Nyrex before using. Sperm were collected dry, pelleted at 1000 g for 30 sec, and stored on ice until needed.

**Isolation of SFEs**

SFEs were isolated from zygotes fertilized in the presence of the peroxidase inhibitor 3-aminotriazole (3-AT; Sigma Chemicals, St. Louis, MO, USA) by a method originally described in Showman and Foerder (1979). Briefly, eggs were collected, rinsed, and resuspended in 1 mM 3-AT diluted in ASW to block ovoperoxidase cross-linking activity. At 30 min after insemination, sperm were washed out using ASW and zygotes were passed through 80-μm (_L. variegatus_) or 64-μm (_S. purpuratus_) nylon mesh to separate the SFEs from the cells remaining. Cells were sedimented by gravity on ice, and the SFEs in the supernatant were collected by 10,000 g centrifugation and then stored as dry aliquots in microfuge tubes at −80 °C until needed.

**Electron microscopy**

Electron microscopic analysis was performed on eggs and embryos after fixation in modified Karnovsky’s solution, poststained with 1% OsO₄, and embedded in Spurr’s resin (Spurr 1969; Wessel et al. 1989). Silver-gold sections (90 nm thick) were placed on nickel grids, stained with lead citrate and uranyl acetate, and examined at 80 KeV on a Philips 410 electron microscope (FEI/Philips Electron Optics, Hillsboro, OR, USA). Images were recorded with a charged coupled device camera (Hamamatsu Corp., Bridgewater, NJ, USA) using AMT Advantage 4.0 software (Advanced Microscopy Techniques, Danvers, MA, USA).

**Production of polyclonal antisera**

Antiserum was raised against whole _L. variegatus_ SFEs dissolved in 50 mM Tris, 10 mM EDTA at pH 8.0. This soluble immunogen was mixed with Freund’s complete adjuvant (Sigma) and then injected subcutaneously into New Zealand white rabbits. Boost injections were made every 3 weeks over 3 months using Freund’s incomplete adjuvant (Sigma). Plasma was collected 1 week after boosts, and the serum was stored at −20 °C.

Antiserum raised to individual _S. purpuratus_ fertilization envelope components used methods similar to those above. Antiserum was generated, in some cases, by immunizing New Zealand white rabbits with bacterially overexpressed recombinant fragments subcloned downstream of a glutathione-S-transferase (GST) coding tag found in the pGEX expression vector (Amersham Biosciences Corp., Piscataway, NJ, USA). The following immunogens were used: (a) whole _S. purpuratus_ SFE from purified _S. purpuratus_ SFEs; (b) the cortical granule component of rendezvin (rendezvin®) from GST fusions to three distinct regions of the corresponding complementary DNA (cDNA) contig (unpublished data); (c) SFE9 from GST fusions to two distinct regions of the cDNA contig (Wessel 1995); (d) SFE1 from a GST fusion of a carboxy-terminal fragment of the cDNA contig (Wessel et al. 2000); and (e) proteoliaisin from SDS-PAGE electrophoroses of whole _S. purpuratus_ SFE (Somers et al. 1989; Somers and Shapiro 1991).

**Immunolocalizations**

Immunofluorescence localization was accomplished on live eggs and zygotes on ice. All reagents and buffers were also chilled to 4 °C before use. Eggs collected from females were washed extensively in ASW and then fertilized in the presence of 3-AT and washed again.
to remove excess sperm. Equal volumes of egg or zygote cultures were combined before antibody probing. Antiseras were diluted 1:100 in ASW and allowed to incubate with the cells for 1 h. Cells were washed in ASW and allowed to sediment by gravity. Rhodamine-conjugated affinity-purified, goat anti-rabbit IgG F(ab\')2 fragments (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were diluted 1:25 in ASW and incubated with the cells for 30 min. Cells were then washed three times in ASW. Just before imaging, cells were labeled using 1 μM FM1-43 (Molecular Probes, Eugene, OR, USA) diluted in ASW to counterstain the plasma membrane. Epifluorescence and Nomarski images were observed with an Axiosplan microscope (Zeiss Corp., Thornwood, NY, USA) and imaged with a Spotcam digital camera (Diagnostic Instruments, Sterling Heights, MI, USA).

For histological sections, eggs and zygotes from *S. purpuratus* and *L. variegatus* were fixed with Bouin’s fixative and embedded into paraffin blocks as previously described (Wessel et al. 1984). Thick tissue sections (10 μm) were rehydrated and then washed with BSA-TBST (1% bovine serum albumin [w/v], 170 mM NaCl, 50 mM Tris, 0.05% Tween20 [v/v], pH 8.0). Antisera were diluted 1:50 in BSA-TBST and incubated on the sections for 60 min. Cy3-conjugated, affinity-purified, goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) was diluted 1:300 in BSA-TBST and allowed to incubate on the sections for 30 min. Confocal and differential interference contrast micrographs were imaged with a Zeiss 410 laser confocal microscope using Renaissance 4.10 software (Microcosm Inc., Columbia, MD, USA).

### Electrophoresis and immunoblot analysis

SFEs dissolved in 50 mM Tris, 10 mM EDTA, pH 8.0, were diluted and subjected to SDS-PAGE on precast 4–20% polyacrylamide Tris-glycine gels (ICN Biomedicals, Irvine, CA, USA). The gels were then stained with Coomassie blue or transferred to nitrocellulose for immunoblotting. Blots were blocked in BLOTTO (3% nonfat dry milk [w/v], 170 mM NaCl, 50 mM Tris, 0.05% Tween20 [v/v]) and then probed with antisera diluted 1:100 in BLOTTO for 2 h. The blots were washed twice with BLOTTO and then incubated in alkaline phosphatase-conjugated, affinity-purified, goat anti-rabbit IgG (Sigma) diluted 1:30,000, in BLOTTO for 1 h. Blots were washed in TBST (170 mM NaCl, 50 mM Tris, 0.05% Tween20 [v/v], pH 8.0) and then in alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl2,100 mM Tris, pH 9.5). Immunoreactivity of the secondary antibody was detected by BCIP/NBT as previously described (McGadey 1970). ΔZAP II bacteriophages that contained cDNA encoding immunoreactive components were converted into pBluescript plasmid as per the manufacturer’s protocol (Stratagene) for sequencing.

Random-primed, biotinylated (BioPrime kit, Invitrogen, Carlsbad, CA, USA), or 32P-radioactive (Radprime kit, Invitrogen) probes were synthesized from the immunopositive clones. These probes were used to screen the same ΔZAP II cDNA *L. variegatus* ovary bacteriophage library by hybridization. Phage DNA associated with positive hybridizing plaques was converted into pBluescript plasmids in XL-1 Blue *Escherichia coli* cells according to the manufacturer’s protocol. These clones were then submitted for DNA sequencing.

### DNA sequencing and analysis

Sequencing was performed at the Brown University Sequencing Facility using an ABI 377 prism automated DNA sequencer (PE Biosystems, Foster City, CA, USA). Nucleotide sequence data were assembled and analyzed using Sequencher (GeneCodes, Grand Rapids, MI, USA). Contig cDNA sequences were deposited in GenBank with the following accession numbers: rendezvin (unpublished data); *S. purpuratus* SFE9 (U17377); *L. variegatus* SFE9 (AY540956); *L. variegatus* SFE1 (U57753); *L. variegatus* SFE1 (AY540955); *S. purpuratus* proteoliasin (AY540953); and *L. variegatus* proteoliasin (AY540954).

Motif predictions were made using the Simple Modular Architecture Research Tool (SMART) site to detect common motifs, modules, and domains (smart.embl-heidelberg.de). Translation alignment plots (dot plots) were generated from the open reading frames of each protein using the FASTA PALIGN program (fasta.bioch.virginia.edu/fasta/plalign2.htm; WR Pearson, University of Virginia). Alignments were made using the default MD20 matrix parameters and calculated with an upper E-value limit of 0.0001.

### Phylogenetic analysis of low-density lipoprotein receptor type A repeats

Low-density lipoprotein receptor type A (LDLRa) repeats were identified in sequences from our data or GenBank entries using SMART. Regions of interest were reverse translated to their primary nucleotide sequence and then aligned within MacVector (Accelrys, Burlington, MA, USA) according to their coding frames. The third nucleotide position of each codon and extraneous codons, found in fewer than 4% of the sequences, were ignored during the analysis. These alignments were used for analysis in PAUP (Swofford 2002) to generate the most parsimonious relationship among the individual LDLRA motifs. Heuristic searches were performed using the default TBR branch-swapping algorithm, and bootstrap scores were calculated from over 1000 reiterations to measure confidence levels for specific pairing.

Sequences selected for use in the comparative LDLRA analysis include *Caenorhabditis elegans* low-density lipoprotein receptor-related protein-1 (gi|1359554) motifs #04 (amino acid residues 181–220) and #10 (1186–1225); *C. elegans* gene similar to chicken low-density lipoprotein receptor (gi|1483242) motif (36–76); *C. elegans* MUA-3 (gi|1067000) motifs #04 (130–163) and #05 (164–210);
8.1 mM NaH2PO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.4) and 30 min. Excess biotin was dialyzed against 10 mM Tris, 1 mM added to the proteins and allowed to incubate in the dark for 30 min. Excess biotin was dialyzed against 10 mM Tris, 1 mM EDTA, and 2 mg/ml glycine at 4°C overnight. After dialysis, an aliquot of proteins was diluted serially to measure biotinylation efficiency using a modified ELISA protocol described previously (Feder et al. 1996). Briefly, samples were diluted in ELISA coating buffer (32 mM Na2CO3, 68 mM NaHCO3, pH 9.5) and allowed to bind to polystyrene plates at 4°C overnight. Plates were washed five times in 1× phosphate-buffered saline (PBS) (140 mM NaCl, 8.1 mM NaH2PO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.4) and then blocked in BSA-PBS (1% bovine serum albumin [w/v] diluted in 0.1 M NaHCO3) containing 10 mM EDTA to a concentration of about 5 mg/ml. Five volumes of 0.5 mg/ml Sulfo-N HS-LC-Biotin (Pierce Biotechnology Inc., Rockford, IL, USA) diluted in 0.1 M NaHCO3 were added to the proteins and allowed to incubate in the dark for 12 h/C2 overnight. Plates were washed extensively in ASW. Eggs from both species were fertilized with sperm from each species added to half of the egg mixture, and washed again. ASW was replaced by Extravidin-Cy3 (Sigma) and allowed to settle by gravity. Eggs and zygotes were then mixed three times in ASW and then imaged using a Zeiss LSM410 confocal laser scanning microscope using Renaissance 4.10 software (Microcosm Inc.).

**Biotinylation and quantification of biotin labeling efficiency by a modified ELISA**

Isolated SFEs or gelatin were resuspended in 0.1 M NaHCO3 containing 10 mM EDTA to a concentration of about 5 mg/ml. Five volumes of 0.5 mg/ml Sulfo-N HS-LC-Biotin (Pierce Biotechnology Inc., Rockford, IL, USA) diluted in 0.1 M NaHCO3 were added to the proteins and allowed to incubate in the dark for 30 min. Excess biotin was dialyzed against 10 mM Tris, 1 mM EDTA, and 2 mg/ml glycine at 4°C overnight. After dialysis, an aliquot of proteins was diluted serially to measure biotinylation efficiency using a modified ELISA protocol described previously (Feder et al. 1996). Briefly, samples were diluted in ELISA coating buffer (32 mM Na2CO3, 68 mM NaHCO3, pH 9.5) and allowed to bind to polystyrene plates at 4°C overnight. Plates were washed five times in 1× phosphate-buffered saline (PBS) (140 mM NaCl, 8.1 mM NaH2PO4, 2.7 mM KCl, 1.5 mM KH2PO4, pH 7.4) and then blocked in BSA-PBS (1% bovine serum albumin [w/v] diluted in 0.1 M NaHCO3). The proteins were incubated for 60 min in Extravidin-AP diluted 1:300,000 in BSA-PBS. Plates were washed again, three times in 1× PBS and two times in ELISA alkaline phosphatase buffer (1 M diethanolamine, 0.5 mM MgCl2, pH 9.8). Enzymatic activity of alkaline phosphatase on p-nitrophenyl-phosphate (Sigma) was measured for 15 min and then stopped with 0.1 volumes of 1 M EDTA. The quantity of substrate conversion was detected spectrophotometrically with a 405-nm filter using a Vmax spectral plate reader driven by SoftMax Pro software (Molecular Devices Corp., Sunnyvale, CA, USA). All samples displayed similar slopes across their dilution curves.

**In vivo biotinylated protein incorporation during fertilization envelope assembly**

All work on live cells was carried out on ice, and all reagents and buffers were chilled to 4°C before use. Eggs collected from females were washed extensively in ASW. Eggs from both species were mixed together and then resuspended in a 1:10 dilution of biotin-labeled proteins in 1 mM 3-AT made up in ASW. A mix of dilute sperm from each species was added to half of the egg mixture, allowed to rock at room temperature for 20 min, and followed by rocking on ice for 40 min. Cells were washed twice with ASW and allowed to settle by gravity. Eggs and zygotes were then mixed and washed again. ASW was replaced by Extravidin-Cy3 (Sigma) diluted 1:300 in ASW and incubated for 30 min. Cells were washed three times in ASW and then imaged using a Zeiss LSM410 confocal laser scanning microscope using Renaissance 4.10 software (Microcosm Inc.).

**Quantification of biotin incorporation within envelopes**

Images from the biotin competition assay (see above) were analyzed with Metamorph software (Universal Imaging Co., Downingtown, PA, USA) at the focal equator of both S. purpuratus and L. variegatus fertilization envelopes. Fluorescence intensity of the fertilization envelope was used to quantify incorporation of the biotinylated components: Total fluorescence intensity (I) and total area (A) were measured in circular regions 5 pixels inside (z−) and outside (z+) the fertilization envelopes. Measurements for each species were normalized according to the following formula:

Fluorescence intensity of the fertilization envelope equator = \( \frac{(I_{z+}-I_{z-})}{(A_{z+}-A_{z-})} \).

Averages of five to eight individual zygotes of each species, from each sample, are reported from a single experiment. A two-tailed Student’s t-test was conducted to assess significance.

**RESULTS**

**Epitopes of fertilization envelope-specific components are shared between species**

Most fertilization envelope components derive from the cortical granules of sea urchin eggs. To compare the proteins that contribute to such different ultrastructure between *S. purpuratus* versus *L. variegatus* cortical granules (Fig. 1B), we raised polyclonal antiserum to *L. variegatus* whole SFE constituents. We then compared the immunoreactivity of this new antiserum with our existing *S. purpuratus* SFE polyclonal antiserum (Fig. 2). Both antibodies detect epitopes exclusive to the fertilization envelopes of live zygotes but are not exposed at the surface of live eggs of either species (Fig. 2, cortical details). The minor fluorescence associated with some, but not all, eggs is attributed to autofluorescence of the glycoprotein jelly coat secreted with the eggs during spawning.

We then used antisera raised against each individual major structural fertilization envelope protein—the cortical granule component of rendezvin (rendezvin CG), SFE9, SFE1, and proteoliasin—to further test which epitopes might be conserved between the species. We detected cross-species, envelope-specific staining for rendezvin in live zygotes (Fig. 3) and within cortical granules of each species (Fig. 4). Preparations of whole SFEs were separated by SDS-PAGE and probed for each protein. These immunoblots show immunoreactivity for individual proteins in the SFEs of both species (Fig. 5).

**Cloning and assessment of *Lytechinus variegatus* fertilization envelope homologs**

The immunoreactivity of antisera for each of the four major structural components suggests that some fertilization envelope epitopes or motifs of each component are conserved between species. Yet the distinct protein sizes from fertilization envelope preparations of each species (Fig. 1D) and a failure to detect cross-species transcripts by RNA gel blots (M. Laidlaw and G. M. Wessel, unpublished data) imply a lack of sequence similarity. To resolve these contrary
observations, we sought to clone the major SFE structural proteins from *L. variegatus*.

We used the antiserum raised against complete *L. variegatus* SFEs to immunoscreen a λZAPII bacteriophage library made from *L. variegatus* ovary cDNA. This method yielded multiple clones containing LDL receptor motifs—namely homologs to *S. purpuratus* SFE9, SFE1, and proteoliaisin. Neither rendezvin cg nor ovoperoxidase were identified in these *L. variegatus* SFE immunoscreens. Hybridization screening of the same library using probes generated from the original *L. variegatus* SFE1 or SFE9 cDNA clones resulted in near-complete SFE9 (Fig. 6A) and SFE1 (Fig. 6B) orthologs, including the initiating methionine and signal sequence identification in both species. Most clones for proteoliaisin, however, were obtained from both high- and low-stringency plaque hybridization screens using the 5' most sequence of an *S. purpuratus* clone originally obtained using degenerate PCR amplification from the amino-terminal amino acid sequence (Somers and Shapiro 1991; V. Zaydfudim, personal communication). Our inability to clone...
the carboxy-terminal end and stop codons for proteoliasin in either species limits our analysis of the protein’s sequence similarities between orthologs, but the existing 61.1% amino acid identity between clones suggests true orthology. This value is consistent with the rendezvin orthologs (Table 1) (unpublished data).

**SFE1 and SFE9**

SFE1 and SFE9 are fertilization envelope structural proteins that contribute to the physical block to polyspermy in *S. purpuratus*. Similar initiating methionines, signal sequences, and amino-terminal motifs have been mapped between species for both sequences, showing 50.2% and 53.7% residue identity for SFE1 and SFE9, respectively (Fig. 6, B and C). The primary differences in the amino-terminal regions between species are the number of tandem LDLrA repeats and the frequency of residue substitutions within these repeats. Many of the repeats contain conserved acidic residues clustered around cysteine IV and cysteine V (Fig. 7) (Esser et al. 1988). Each set of aspartate (D) or glutamate (E) residues is predicted to be involved in coordinating a single calcium ion per folded repeat motif, whereas an asparagine (N) substitution for aspartate may be involved in stabilizing the tertiary structure of the tandem arrays (Atkins et al. 1998; Bieri et al. 1998; Andersen et al. 2000; Koduri and Blacklow 2001). One such set of substitutions is clustered within tandem arrays in each sequence (Fig. 7). Coiled-coil motifs, alpha-helical domains believed to be involved in protein–protein interactions, present in both SFE1 and SFE9 and whey-acidic protein domains in SFE9 act as anchor points that mark the end of the orthologous sequences.

Significant species differences are found in the carboxy-terminal portions of SFE1 and SFE9 (Fig. 8), just beyond the coiled-coil motifs in each protein. Within each species, the carboxy-terminal sequence is organized as a concatemeric repeat of variable unit length and composition. Within a gene, these repeats appear to be homogenizing (Fig. 8, repeat sequences marked by bars). For example, *L. variegatus* SFE1 contains the consensus 17-residue repeat “AST . . . V,” whereas the *S. purpuratus* ortholog contains a 63-residue repeat of “TST . . . T.” The organization of SFE9 repeats also retains this concatemeric organization but spans two different sequences of repeats with varying degrees of sequence similarity among individual repeats. The first octamer repeat of each SFE9 species is enriched in proline and tyrosine residues. In *L. variegatus*, the repeats are less well conserved but retain a general “PYE” sequence across about eight residues, whereas *S. purpuratus* repeats strictly follow an eight-residue “QPY” sequence. This enrichment in tyrosine residues suggests that SFE9 is a major target of ovoperoxidase-dependent di-tyrosine cross-linking. The second carboxy-terminal region shows more significant divergence across a definitive repeat unit: *L. variegatus* contains a nine-residue “PYE” repeat that might be a continuation of its first SFE9

![Fig. 4.](image-url)
repeat region, whereas *S. purpuratus* possesses an eight-residue “NHL” repeat (Wessel 1995). Such divergence in carboxyterminal repeats is reminiscent of short repeat regions in two other sea urchin genes: the spicule matrix protein SM50, which contains a homogenized seven-residue “QPG(M/F/V)GG(R/Q)” repeat disrupted by a triplet “PNN” repeat found exclusively in three closely related species (Meeds et al. 2001), and bindin, an extracellular sperm protein containing a seven-residue “QGM(G/S/I)(G/M/I)(Q/P)(P/H)” repeat under positive selective pressure (Minor et al. 1991; Metz and Palumbi 1996; Biermann 1998; Palumbi 1999).

**Proteoliaisin**

Named for its function as a tether between ovoperoxidase and the vitelline layer, proteoliaisin sequence was first described by a microsequence of a tryptic fragment obtained by C. Somers (Somers et al. 1989; Somers and Shapiro 1991). We used this peptide sequence to clone cDNAs encoding the amino-terminal portion of this multifunctional protein first from *S. purpuratus* and then from *L. variegatus*. Similar to the sequences of SFE1 and SFE9, the predominant domain encoded by the cDNA of the protein is the LDLrA repeat.

Comparing the proteoliaisin translations of both species clearly suggests that the cloned amino-terminal regions of this protein are orthologous (Fig. 6C). Most of the proteoliaisin open reading frame consists of concatemers of LDLrA repeats, with each ortholog containing a single break in this chain. Based on its position with the *S. purpuratus* sequence, we speculate that this break corresponds to a site of trypsin accessibility first identified by Somers and Shapiro (1991), implicating the LDLr repeats amino-terminal to this break as involved in vitelline layer and ovoperoxidase binding. Although nine motifs longer than the *S. purpuratus* proteoliaisin, an analogous break is present in the *L. variegatus* ortholog. This longer series of amino-terminal LDLrA repeats suggests that more surface area is available to tether *L. variegatus* ovoperoxidase to its cognate vitelline layer proteins. Another difference between the orthologs is the duplication of a region spanning nine LDLrA repeats in *L. variegatus* (Fig. 6C, repeats 20–28 and 29–38). This duplication contains nearly 99% nucleotide identity between the regions but shows more divergence in the flanking sequences. The minimal divergence in coding sequence suggests that this duplication occurred quite recently in the *L. variegatus* proteoliaisin gene.

**Phylogeny of repeat motifs**

The abundance of protein–protein interaction motifs and repeats found within *S. purpuratus* and *L. variegatus* cortical granule orthologs (Table 1) is consistent with the primary role of these proteins as structural proteins. We asked how similar the orthologs and their repeats are with respect to themselves and to each other using translation alignment plots (dot plots) generated by FASTA PALIGN (Fig. 9). As expected, the tandem stretches of LDLrA repeats in proteoliaisin, SFE1,
and SFE9 show the highest identity within species and between orthologs, suggesting that the location and organization of these motifs have been structurally conserved within the folded protein. Finally, as noted within the sequence alignments (Figs. 6 and 8), the C-terminal repeats of SFE1 and SFE9 are identical within a species’ sequence but completely divergent between orthologs.

To determine the evolutionary relationship of each repeat with respect to the others involved with fertilization envelope assembly, we looked further at the LDLrA repeats contained...
within each protein. We asked whether a single LDLrA repeat or a string of repeats from one gene might be the ancestral motif that gave rise to the other genes through paralogous duplication events. To assess this, we assembled an unrooted phylogenetic tree from nucleotide sequences of each LDLrA repeat defined by the SMART tool. We included LDLrA motifs from other extracellular proteins found in *C. elegans*, *X. laevis*, and *H. sapiens* as representatives of the sequence diversity in other families of proteins that may have derived from the original ancestral domain. These additional LDLrA repeat motifs belong in two categories: cell-surface receptor proteins and enzymes functional during development. The former category includes proteins such as *C. elegans* low-density lipoprotein receptor-related protein-1 (motifs #04 and #10, of 14 total); *C. elegans* homolog to the chicken low-density lipoprotein receptor, *C. elegans* MUA-3 (motifs #04 and #05, of 5 total); *H. sapiens* low-density lipoprotein receptor (motifs #03 and #04, of 7 total); and *H. sapiens* receptor protein sorLA-1 (motifs #04 and #09, of 11 total).

The latter category is represented by *S. purpuratus* cortical granule serine protease, CGSP1 (motifs #03 and #08, of 14 total) and *X. laevis* embryonic serine protease-2 (motifs #03 and #08, of 8 total).

We searched for the most parsimonious tree among assembled repeats, but heuristic searches yielded over 300 different configurations. Closer analysis of these trees show only positional swaps in SFE1 repeats 11–15 and SFE9 10–12, suggesting that these repeats have homogenized in their respective...
Fig. 6. (Continued).
Table 1. Structural identity of the major fertilization envelope proteins derived from the cortical granules

<table>
<thead>
<tr>
<th>Lytechinus variegatus</th>
<th>Strongylocentrotus purpuratus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length (amino acids)</strong></td>
<td><strong>Type</strong></td>
</tr>
<tr>
<td>Ovoperoxidase</td>
<td>804&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rendezvin</td>
<td>1801</td>
</tr>
<tr>
<td>SFE9</td>
<td>1285</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SFE1</td>
<td>1044&lt;sup&gt;1&lt;/sup&gt;</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteoliasin</td>
<td>1921</td>
</tr>
</tbody>
</table>

Qualities and lengths of the predicted open reading frames of each protein are listed, as well as the number and types of repeat motifs found in each protein. Amino acid length of previously undefined repeats is indicated in parentheses. Percentage of amino acid residue identity between each ortholog is also listed. Low-density lipoprotein type A (LDLrA) repeats and CUB domains have been previously defined (see text).

<sup>1</sup>Represents a complete open reading frame, including a signal sequence and in-frame STOP codon.

<sup>2</sup>Two additional CUB domains are predicted from the open reading frame but may not be translated due to splicing.

Fig. 7. Alignment of low-density lipoprotein type A (LDLrA) repeat residues predicted to be involved with calcium and ligand binding. Amino acids clustering around cysteine<sup>39</sup> and cysteine<sup>1</sup> (labeled IV and V, respectively) are aligned for each protein containing LDLrA repeats. (A) SFE9. (B) SFE1. (C) Proteoliasin. Numbers in left column correspond to the position of each repeat within the original parent tandem array of LDLrA repeats (see text and Fig. 6). Arrows indicate the acidic residues predicted to be involved with calcium binding (Atkins et al. 1998; Rong et al. 1998); arrowheads indicate the residues predicted to be involved with ligand specificity (Rong et al. 1998). Nonacidic residue substitutions in the calcium-binding positions are indicated in bold type. In proteoliasin sequences, the bracketed LDLrA repeats highlight a possible region of genetic duplication, with approximately 99% nucleotide identity between the regions.
species. Therefore, we present the consensus tree (Fig. 10). The individual motifs are distributed among all the major branches of the tree. None of these branches is monophyletic for a gene, but a rough grouping may be made: Branch A is primarily SFE1, branches B and E are proteolisin, and branch D is SFE9. The outgroup motifs included in our analysis are also polyphyletic among the five branches.

Most of the aligned repeat motifs from the echinoderm cortical granule proteins segregate together, with the interspecies L. variegatus and S. purpuratus orthologous motifs diverging most terminally within the tree. This suggests that most interrepeat variation within each of the genes has been preserved subsequent to the divergence of these two species. The two exceptions to this interspecies sequence similarity can be found with LDLr repeat #03 of SFE1 and repeat #01 of SFE9 (Fig. 10, arrows and arrowheads). Both of these motifs cluster more closely with LDLr motifs from different motifs from the same gene (SFE1 #03) or from another gene. This distinction suggests the influence of recombination and strong selection at these specific sites.

**Functional conservation**

Given the high degree of sequence identity among the specific cortical granule-derived fertilization envelope proteins, we sought to test whether the repetitive structural motifs were functionally conserved between species. We first biotinylated whole SFE components from both L. variegatus and S. purpuratus and then tested their ability to interact with either live unfertilized eggs or eggs undergoing fertilization.

![Fig. 8. Comparison of SFE1 and SFE9 carboxy-terminal repeats from Lytechinus variegatus versus Strongylocentrotus purpuratus. Side-by-side comparison of eight- and nine-residue divergent repeats found downstream of the conserved low-density lipoprotein receptor repeats for SFE9 (top) and SFE1 (bottom) for L. variegatus (left column) and S. purpuratus (right column). The last SMART-defined LDLrA repeat for each protein are bounded by dashed lines; whey acid protein (WAP) domains are highlighted with gray arrowheads; coiled-coil domains are outlined in black lines. Dashes indicate shifts in the translation to accommodate repeat structure. Repeats undergoing homogenization are highlighted with a line to the left.](image-url)
(Fig. 11). All eggs were exposed to equal specific activities of biotinylated SFE, regardless of the total protein concentration. An excess of biotinylated gelatin over biotinylated fertilization envelope was used as a binding control (data not shown). Neither the egg nor fertilizing egg appeared to bind this protein nonspecifically (Fig. 11A).

Biotinylated SFE components bind to the *S. purpuratus* egg vitelline layer and incorporate into fertilization envelope both homo- and heterospecifically (Fig. 11A). Surprisingly, the reciprocal is not true for *L. variegatus* cells: No biotinylation signal is observed on the surface of *L. variegatus* eggs yet signal is found specifically in the *L. variegatus* fertilization envelopes. Because these assays test whole populations of fertilization envelope proteins against one another, little may be deduced about specific protein–protein interactions. These binding results do, however, suggest that the high percentage of sequence identity retains substantial functional conservation between sea urchin proteins comprising the vitelline layer and packed within the cortical granules.

To test whether or not the individual binding capacity of proteins varied between species, we quantified the fluorescence intensity of biotinylated fertilization envelopes using mixed samples from above (Fig. 11B). To assess the envelope-specific incorporation, we subtracted integrated fluorescence intensity of the zygote, hyaline layer, and perivitelline space from the integrated intensity of the zygote and its envelope. We see no significant difference between the incorporation of *L. variegatus* SFE components (P = 0.05) or *S. purpuratus* SFE components (P = 0.25) into the fertilization envelopes of either zygote.

**DISCUSSION**

What makes the cortical granules between *S. purpuratus* and *L. variegatus* so different: A difference in their constituents? Variability in the ratio of homologous proteins? Alternative packing strategies? Our results suggest that a combination of these factors plays a role in the final ultrastructural organization of each species’ cortical granules. The sequence orthology of the five major cortical granule-derived fertilization envelope proteins implies that the evolution of structural components...
necessary for the physical block to polyspermy is constrained by interactions of specific domains in a preestablished order.

**Conservation of motifs involved with the physical block to polyspermy**

The comparative sequence data presented here suggest that synonymous nucleotide substitutions cluster in specific regions of each structural fertilization envelope protein, namely the LDLrA repeats of SFE9, SFE1, and proteolaisin. Attributes that may have favored the retention and expansion of LDLrA repeats within the sea urchin include their ability to chelate calcium (Bieri et al. 1998; Thielens et al. 1999; Andersen et al. 2000; Koduri and Blacklow 2001), which aids in the precipitation of these proteins to form the physical barrier,
and their role in protein–protein interactions (Esser et al. 1988; Russell et al. 1989; Rong et al. 1998). The observed phylogenetic distribution of these LDLrA repeats into branches that partly segregate with individual fertilization envelope proteins (Fig. 9) suggests that multiple duplication events have occurred early in their evolution. The resultant stability in protein structure may have been retained or enhanced through selection (Heringa 1998).

The tandem organization of these LDLrA repeats within a single protein, a genetic organization common to the animal kingdom (Tolleshaug et al. 1983; Yamamoto et al. 1984; Jacobsen et al. 1996; Yochem et al. 1999; Andersen et al. 2000; Yamada et al. 2000; Bercher et al. 2001), suggests that the quantity of imperfect repeats may regulate a protein’s affinity for its binding partner. For example, the presence of 7 LDLrA repeats in *S. purpuratus* proteoliaisin versus 18 repeats in *L. variegatus* between the signal sequence and a predicted trypsin-accessible break in the amino-terminal tandem repeats provides more possible binding domains for *L. variegatus* proteoliaisin. Hence, the *L. variegatus* proteoliaisin may have a higher affinity for vitelline layer proteins or for ovoperoxidase than its *S. purpuratus* ortholog (Somers and Shapiro 1991). Such a partnering preference is analogous to the ability of the human low-density lipoprotein receptor to distinguish between

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**Fig. 11.** Partial functional conservation of soft fertilization envelope proteins between species. Solubilized protein from purified soft fertilization envelopes (SFEs) from either *Lytechinus variegatus* or *Strongylocentrotus purpuratus* were biotinylated and then tested for localized binding on live eggs or for incorporation into the emerging fertilization envelopes in the presence of 1 mM 3-AT in ASW. (A) Binding of various biotinylated proteins on eggs and zygotes. Fluorescence images of Extravidin-Cy3 fluorochrome are shown on the left; DIC images on the right. *Lytechinus variegatus* eggs (Lg) and zygotes (Lz); *S. purpuratus* eggs (Sp) and zygotes (Sz). Arrows indicate eggs that have bound biotinylated protein. Bar, 100 μm. (B) Quantitation of fluorescence intensity within the zygote fertilization envelope of each species for biotinylated purified SFE from *L. variegatus* and *S. purpuratus*. Fluorescence images overlaid on DIC images for SFE reactions shown in A, included as a visual comparison for envelope quantitation. Chart displays mean envelope fluorescence per unit area of envelopes at the equator for each species’ fertilization envelope used. Bars indicate standard deviation. Number of individuals used per measurement is indicated.
apolipoproteins B-100 and apoE based on which LDLrA repeats are present and active (Esser et al. 1988; Russell et al. 1989). Thus, differences in motif sequence, position of the motif within the concatemer, and quantity of such imperfect repeats are responsible for the specificity of protein–protein interactions, defining which proteins are included or excluded—as in the case of CGSP1 (Haley and Wessel 1999), an enzyme containing 14 LDLrA repeats most similar to those from SFE9 in branch D (Fig. 10)—from the final fertilization envelope.

Our observation of chimeric fertilization envelopes (Fig. 11) suggests that whereas protein–protein binding specificity may segregate at the level of individual cortical granule proteins, this does not occur to a detectable degree between orthologs. An analogous mosaic matrix has been reported for vertebrate eggs using orthologous zona pellucida proteins (Kimloch et al. 1992; Rankin et al. 1998; Dore et al. 1999), suggesting that assembly, and possibly modification, of an extracellular matrix uses a common series of steps during fertilization. Such uniformity is not predicted for a normally radiating reproductive process (Lee et al. 1995; Metz and Palumbi 1996; Vaquier et al. 1997; Swanson et al. 2001), indicating possible negative selective pressures, which are more often associated with essential processes, at work.

The prefertilization matrix acts in a species-specific manner to qualify the courting sperm as suitable or not for fusion. Thus, any components that interact with these diverging regions must coevolve to compensate for the changes, yet retain domains necessary for the postfertilization modification events. In the case of the sea urchin, we would expect any cortical granule proteins directly binding to the species-specific vitelline layer proteins to vary at the region of contact. Conversely, the remainder of the protein in contact with other cortical granule proteins would be highly conserved to maintain its ease of incorporation into a rapidly assembled fertilization envelope.

**Similarity in the midst of sequence diversity**

Three candidate modifications could account for species-specific differences in cortical granule ultrastructure between *L. variegatus* and *S. purpuratus*. First, nonsynonymous mutations at residues necessary for proteolysis, as observed in rendezvin*E* (unpublished data), or posttranslational processing would alter the number and types of interactions achievable during biogenesis. Second, the quantity of a conserved motif, such as the tandem LDLrA repeats of SFE9, SFE1, and proteoliaisin, would result in variability in length or mass, quantity, and types of binding sites. These would be amplified through modifications in binding affinities and partners, resulting in alternative packaging and protein polymerization schemes. Finally, insertions or deletions of nucleotides or entire domains would result in regions of complete nonsynonymous coding, such as seen at the carboxy-terminal eight- and nine-residue repeats of *S. purpuratus* versus *L. variegatus* SFE9 and SFE1 clones. The folding spaces of these tandem repeats could serve to seed different packing schemes in the context of the other orthologs during cortical granule biogenesis, resulting in the species-specific granule ultrastructure (Fig. 1B). This last scenario follows the prediction that radical nonsynonymous amino acid sequences in orthologs may behave as functionally independent units (Heringa 1998).

The organization of these unique repeats implies that they play a very specific role, distinct from that of the conserved LDLrA repeats or CUB domains found elsewhere. The residue length, tandem organization, and species variability of these carboxy-terminal repeats in SFE9 and SFE1 is reminiscent of the “QPG(M/F/V)GG(R/Q)” repeat found in the skeletal matrix protein SM50, which is thought to be undergoing concerted evolution in response to the structural constraints of mineralized tissue (Meeds et al. 2001), and the radiating “QGM(G/S/I)(G/M/I)(Q/P)(P/H)” repeat of bindin, which is also under positive selective pressure (Biermann 1998). Might both constraints be at work in these cortical granule-derived proteins? On the one hand, the homogenization and amplification of a repeat sequence in any one of these cortical granule proteins is an indication that its particular primary structure and fold are essential for fertilization envelope assembly. The diversity in repeat sequence between orthologs, however, suggests the multimerization occurred after the species radiated (Meeds et al. 2001). This event is similar to the lower percentage identity observed among the repeats of different species in an abalone sperm receptor (Swanson and Vaquier 1998; Meeds et al. 2001; Galindo et al. 2002), the spider silk protein flagelliform (Hayashi and Lewis 2000), and bindin (Biermann 1998). Thus, as proposed for the coevolution of the bindin repeats in response to mutations in its egg receptor (Minor et al. 1991; Lopez et al. 1993; Biermann 1998), the retention of a unique repeat sequence in SFE9 and SFE1 may reflect constraints imposed by their species-specific binding partners. If true, then comparing the degree of repeat homogenization to the radiation in repeat sequence should correlate with the distance separating each species. Additional cloning of fertilization envelope orthologs from other genera of urchins must be completed to clarify whether or not these hypothetical modifications parallel speciation events. In particular, analysis of other genera of sea urchins, as well as other echinoderms outside of the urchin clade, will be critical to further characterize the radical carboxy-terminal divergence we observe in SFE9 and SFE1 across phylogeny.

**How universal is this block-to-polyspermy mechanism?**

To answer this question requires knowledge of an overlapping set of proteins involved in blocks to polyspermy across many
classes of animals. Extracellular matrices in vertebrates have been studied in depth, but little is known about their cortical granule contents. Conversely, most of the cortical granule proteins have been identified in echinoderms (data present herein and Wessel et al. 2001), whereas little is known about their vitelline layer composition. In the absence of complete information about this process, two hypotheses may be posed. First, the vast number of paralogs and orthologs to the three glycosylated zona pellucida proteins discovered in mammals, marsupials, birds, teleosts, and amphibians (Spargo and Hope 2003) compared with the estimated 25 unique sea urchin vitelline layer components (Gache et al. 1983; Niman et al. 1984) implies that vertebrates and invertebrates do not share a common mechanism to physically block polyspermy; rather, the assembly and hardening event is a consequence of convergent evolution. Alternatively, if all fertilization events have similar origins and the extracellular and cortical granule components continued to coevolve, then we would expect to find a handful of urchin vitelline layer components containing at least one zona pellucida module. Analogously, we should expect to see functional cortical granule homologs to ovoperoxidase, rendezvin, SFE1, SFE9, and/or proteoliasin, in species from other phyla.

Evidence suggesting that a common block-to-polyspermy process exists within the animal kingdom comes from (a) the high degree of conservation among the zona pellucida family of extracellular matrix proteins in vertebrates (Moller and Wassarman 1989; Hatanaka et al. 1992; Moos et al. 1994; Tatemoto and Terada 1999), (b) detectable peroxidase (Hall 1978; Gulyas and Schmell 1980a,b; Deits et al. 1984; Kudo 1988; Nomura and Suzuki 1995; Li et al. 1996; LaFleur et al. 1998; Nomura et al. 1999) and proteinase activity (Carroll and Epel 1975b; Gulyas and Schmell 1980a; Moller and Wassarman 1989; Haley and Wessel 1999) in all species studied to date, and (c) the identification of at least one whey-acidic protein protease inhibitor domain from a cortical granule-derived protein in both invertebrates (data present herein) and vertebrates (Chang and Huang 2002). This common list of enzymatic activities and shared motifs suggests that the physical block to polyspermy has evolved along a gradient that bridges two extremes: systems that rely heavily on the egg extracellular matrix to supply the structural material versus systems whose major contribution comes from cortical granules.

Our results clearly show a genetic and functional conservation in cortical granule content proteins responsible for establishing the physical block to polyspermy within a single class of echinoderms. Identification of these orthologs allows us to understand sea urchin fertilization envelope assembly at a more general level, defining a common currency that may be used to translate events from one urchin species to another. The implications of our work on fertilization in other animals, specifically vertebrates, cannot be inferred to date because so little is known about their cortical granules and, conversely, so little is known about the sea urchin vitelline layer. If homologs or more common motifs are discovered in egg cortical granule proteins of other species, then our continued characterization of the assembly process in echinoderms should accelerate the understanding of the universal physical block to polyspermy.

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