

SFE1, a Constituent of the Fertilization Envelope in the Sea Urchin Is Made by Oocytes and Contains Low-Density Lipoprotein-Receptor-Like Repeats¹

Gary M. Wessel,² Sean Conner,³ Michael Laidlaw,⁴ Jacob Harrison,⁵ and Gary J. LaFleur, Jr.⁶

Department of Molecular and Cell Biology & Biochemistry, Brown University, Providence, Rhode Island 02912

ABSTRACT

At fertilization in most animals, cortical granules of the egg or oocyte secrete their contents, whose function it is to modify the extracellular matrix. This modified matrix then participates in the block to polyspermy and protection for early embryonic development. In the sea urchin, contents of the cortical granules are secreted within 30 sec of insemination. Several of these content proteins then bind to the nascent vitelline layer of the egg and lift off the cell surface to form a stable, impervious, fertilization envelope. At least six major proteins are present in the envelope, and recently we have identified cDNA clones of two, ovoperoxidase, and SFE9. Here we report on the identification and characterization of SFE1, a constituent of the fertilization envelope of the sea urchin *Strongylocentrotus purpuratus*, that has revealing characteristics of how the envelope might form and what protein interaction domains might predominate. We present the largest cDNA sequence we were able to identify representing approximately two thirds of the predicted protein coding region. The C-terminal half of the cognate SFE1 protein contains two different amino acid repeat motifs: a cysteine-rich (15%) motif of 40 amino acids that is tandemly repeated 22 times and is followed by a serine/threonine-rich (38%) repeat of 63 amino acids that is tandemly repeated 3.5 times. Surprisingly, just N-terminal to the cysteine-rich repeat region is a sequence of five repeats with similarity to repeats in another cortical granule protein, SFE9, and to the motif originally identified in the receptor of low-density lipoproteins, the LDLr motif. The amino acid composition deduced from the partial SFE1 cDNA is similar also to the composition of proteoliasin, a protein thought to tether the ovoperoxidase to the vitelline layer of the egg and thereby sequester the crosslinking activity of the ovoperoxidase to a limited population of proteins in the fertilization envelope. However, by use of monoclonal and polyclonal antibodies to SFE1 and proteoliasin, we show here that they are distinct gene products. We also show that SFE1 is packed selectively into the cortical granules and then is crosslinked into the fertilization envelope following fertilization. In situ RNA hybridization analysis shows that the mRNA of SFE1 (9 kilobases) is present in oocytes selectively and is turned over rapidly in the

oocyte following germinal vesicle breakdown. Our findings suggest that the gene encoding this major product of the egg is activated concomitantly with the other cortical granule-specific products already identified, and that a common LDLr-like motif of the fertilization envelope may reveal a structural mechanism for protein interactions in its construction.

developmental biology, fertilization, gamete biology, gametogenesis, ovary

INTRODUCTION

The fatal consequence of polyspermy was documented by Boveri [1] among others, who reported that fusion of more than one sperm with an egg typically leads to aberrant blastomere cleavage and early embryonic death. Thus, it was realized that a prevention or block to polyspermy must be a fundamental process of fertilization. Since then, the cortical granules, a specific group of exocytotic vesicles in the egg, have been identified as the organelle responsible for the conserved strategies used by animals throughout phylogeny to prevent polyspermy. The contents of cortical granules, once exocytosed following fertilization, modify the extracellular investments of the egg to form an impenetrable barrier to sperm. The zona pellucida in mice, and the vitelline layer in frogs and sea urchins are each modified in multiple and different ways to render it impervious to additional sperm.

Upon fertilization of a sea urchin egg, the contents of the cortical granules combine with the glycocalyx of the egg, the vitelline layer, to initiate assembly of the fertilization envelope. Formation of the fertilization envelope begins at the point of sperm fusion with exocytosis of the cortical granule contents and progresses throughout the circumference of the egg. The newly formed envelope is rapidly converted to a hardened matrix that is resistant to both mechanical and biochemical alterations. This conversion is largely the function of a peroxidase activity, first described by Katsura and Tominaga [2] and subsequently attributed to a 70-kDa protein termed ovoperoxidase [3]. Significant progress has been made by several investigators to understand the biochemistry and morphological changes that occur to form the fertilization envelope. For example, ovoperoxidase is now known to interact with proteoliasin (Pln), a protein believed to tether ovoperoxidase to the vitelline layer, where the peroxidase activity catalyzes the formation of dityrosine crosslinks between a subset of fertilization envelope polypeptides [4]. Thus, the assembly of an ovoperoxidase/Pln complex into the envelope serves as a key coordinating step for construction of the fertilization envelope. This entire process occurs within about 60 sec of insemination, and the structural changes that occur are documented in a series of studies using quick freeze fixation followed by deep etching [5–7].

Laidlaw and Wessel [8] identified cDNAs that encode proteins of the fertilization envelope. The identity of one of the cDNAs, termed SFE9 (for *Strongylocentrotus purpuratus* fertilization envelope protein 9) was further char-

¹This work was supported by grants to G.M.W. from the National Institutes of Health (HD28152) and the National Science Foundation (IBN 9816683).

²Correspondence: Gary M. Wessel, Department of Molecular and Cell Biology & Biochemistry, 69 Brown Street, Box G, Brown University, Providence, RI 02912. FAX: 401 863 1182; e-mail: rhet@brown.edu

³Current address: Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037.

⁴Current address: Temple University School of Medicine, 3340 North Broad Street, Philadelphia, PA 19140.

⁵Current address: Program in Cell and Molecular Biology, Duke University Durham, NC 27710.

⁶Current address: Department of Biology, Nicholls State University, Thibodaux, LA 70310.

Received: 31 May 2000.

First decision: 27 June 2000.

Accepted: 26 July 2000.

© 2000 by the Society for the Study of Reproduction, Inc.

ISSN: 0006-3363. <http://www.biolreprod.org>

acterized and found to encode a protein with low-density lipoprotein receptor (LDLr)-like domains and several other conserved repeating motifs [9]. During oogenesis, SFE9 is packaged specifically into cortical granules and following fertilization is crosslinked in to the fertilization envelope. In this paper we describe another protein of the fertilization envelope, termed SFE1. Surprisingly, it too contains LDLr-like motifs, and we postulate that this motif is important for protein interactions during the rapid construction of the fertilization envelope.

MATERIALS AND METHODS

Handling Embryos

Adult *S. purpuratus* were obtained from Marinus (Longbeach, CA). Gametes were obtained and fertilized as described [10] and fertilization envelopes were isolated using 3-aminotriazole [11].

Synthesis of Recombinant Proteins and Generation of Antisera

Recombinant SFE1 was constructed in pBluescript as described using the SFE1 cDNA [8]. Polyclonal antisera to this recombinant protein was made by mixing with Freund complete adjuvant and injecting s.c. into New Zealand White rabbits. Boost injections were made every 3 wk for 3 mo using Freund incomplete adjuvant, and 1 wk following the last boost, plasma was collected from ear veins [12]. Monoclonal antibodies against SFE1 from *S. purpuratus* were generated according to the manufacturer's protocol (Stem Cell Technology, Vancouver, BC, Canada), using cell surface complex [13] as an immunogen. The resulting hybridomas were cloned by limiting dilution using immunolocalization in situ and immunoblotting as screening mechanisms.

Complementary DNA Library Screens

A λ ZAP cDNA library (Stratagene, La Jolla, CA), constructed from *S. purpuratus* ovary poly(A)⁺ RNA [8], was used to screen for cDNAs encoding SFE1. SFE1 was isolated by immunoscreens, using polyclonal and monoclonal antibodies to native SFE1 as described [8]. Approximately 5×10^5 recombinants were screened.

To screen the ovary cDNA library by hybridization following random primer labeling with [³²P]dCTP [14], multiple overlapping cDNA clones were isolated that encompassed the coding region as well as a single cDNA clone that spanned the entire coding region. The consensus sequence of the cDNAs resulting from the above screens has been entered in the National Center for Biotechnology Information (NCBI, Bethesda, MD) database (accession no. U57753).

Sequencing of DNA

The DNA sequence was determined by the Sanger chain-termination method [15] using [³⁵S]dATP (Dupont, Boston, MA) and *Taq* DNA polymerase (Promega Biotech, Madison, WI). Sequence data were assembled and analyzed using the University of Wisconsin Genetic Computer Group (UWCG) sequence analysis package [16].

Gel Blots of RNA

Total RNA from ovaries, eggs, and embryos at several developmental stages was isolated and analyzed essentially

as described [17] using hybridization to random-primed oligonucleotide labeled cDNA fragments [14].

In Situ RNA Hybridization

Whole mount in situ RNA hybridization was performed using digoxigenin-labeled RNA probes. The pSFE1 was linearized with either with *Bam*HI and transcribed with T7 RNA polymerase to yield an antisense RNA or with *Xho*I and transcribed with T3 RNA polymerase to yield a sense probe. Ovaries, eggs, and embryos were fixed, hybridized, and the signals detected essentially as described [18].

Electrophoresis and Immunoblot Analysis

Egg, embryos, or fertilization envelopes were subjected to SDS-PAGE [19] followed by immunoblot analysis essentially as described [20]. Protein samples were pelleted, resuspended in SDS-PAGE sample buffer containing 10 mM dithiothreitol and a protease inhibitor cocktail (consisting of a final concentration per ml of aprotinin, 1 TIU; benzamide, 10 μ g; soybean trypsin inhibitor, 10 μ g; antipain, 1 μ g; leupeptin, 1 μ g; bestatin, 0.5 μ g; E-64, 1 μ g; phosphoramidon, 1 μ g; PMSF, 10 μ g; chymostatin, 1 μ g; pepstatin), and denatured for 3 min at 100°C. The proteins were resolved on a 7.5% acrylamide gel and either stained with Coomassie blue or transferred to nitrocellulose for immunolabeling as described [20]. For immunolabeling, blots were washed twice for a total of 1 h in Blotto and then incubated for 1 h in Blotto containing anti-SFE1 (polyclonal rabbit antisera was diluted 1000 times, and monoclonal 4E4 supernatant was diluted 250 times) or anti-Pln [21] (polyclonal rabbit antisera diluted 1000 times). The blots were then washed three times in Blotto over 30 min and incubated in Blotto with either goat antirabbit or rabbit antimouse antibodies conjugated to alkaline phosphatase (Sigma, St. Louis, MO) diluted 30 000 times. Blots were washed in Blotto three more times over 30 min and finally washed in Blotto without milk. The secondary antibody was detected by 5-bromo-4-chloro-3-indoylphosphate toluidinium (salt)/nitroblue tetrazolium essentially as described [22]. Controls used in this experiment included blots incubated with preimmune antisera and secondary antibody alone. Each of these immunoblots showed no signal (data not shown).

In Situ Immunolocalization

Immunofluorescence localization of SFE1 was performed both on sections of embryos that were fixed, embedded, and labeled or on egg and embryos fixed for whole-mount immunolabeling [23]. Primary antibodies (mouse monoclonal antibody and rabbit polyclonal antibody to SFE1; rabbit polyclonal antibody to Pln [21], and mouse monoclonal antibody 2B7 to hyaline [24]) were diluted between 1:50 and 1:200. For double immunolabeling experiments, the secondary antibodies or Fab fragments were conjugated to fluorescein or rhodamine and labeled sequentially as described [25].

For electron microscopic immunolabeling, eggs and embryos were fixed with modified Karnovsky solution, post-stained with 0.01% OsO₄, and embedded in Spurr resin [26]. Silver-gold sections (approximately 90 nm) were placed on nickel grids and incubated for at least 10 min in PBS containing 10% fetal bovine sera (PBSF). Sections were then incubated for 1 h in PBSF containing either immune or preimmune SFE1 antisera (diluted 100 \times). After washing in



FIG. 1. Salient features of *S. purpuratus* SFE1 cDNA and predicted translation product. Twenty-seven tandem cysteine-rich repeats contain a segment that resembles the LDLr A domain (black bars), five of which pass the defined consensus pattern (*). The sequence between each LDL receptor A domain is variable in the first 9 repeats but highly conserved in the final 22 cysteine-rich repeats. The C-terminus contains 3.5 Ser/Thr-rich repeats, each containing 63 amino acids.

PBSF, sections were incubated for 1 h in goat antirabbit antibodies conjugated to 15-nm colloidal gold particles (diluted 30 \times in PBSF; Janssen-Amersham, Chicago, IL). Sections were then gradually washed into PBS, postfixed with 2% glutaraldehyde, and stained with uranyl acetate and lead citrate. Sections were visualized at 100 KeV with a Philips 410 electron microscope.

RESULTS

In our goal to identify constituents of the fertilization envelope in the sea urchin, two approaches converged on the same gene product that we refer to as SFE1. First, we made antibodies in rabbits to isolated fertilization envelopes. This polyclonal, polyspecific antisera was then used to screen a cDNA expression library made from ovaries of the sea urchin *S. purpuratus*. Positive plaques were characterized by sequence and restriction digest and grouped into overlapping clones. The overexpressed bacterial-derived fusion proteins were then injected into rabbits to obtain polyclonal antisera to a representative of each family of grouped plaques. These antisera were then used to test whether the cognate protein was indeed stored in the cortical granule prior to fertilization and incorporated into the fertilization envelope following insemination. The largest related family of plaques was referred to as SFE1, for *S. purpuratus* fertilization envelopes clone family 1.

The second approach we took was to use cortical granules as the immunogen in mice to generate monoclonal antibodies. Our reasoning was that because the majority of constituents from the fertilization envelope was derived from cortical granules, using cortical granules as an immunogen would enrich for hybridomas that identified fertilization envelope components prior to any changes in the protein that might occur at cortical granule secretion and activation of the cortical granule enzymes.

Using these two immunoscreen approaches we isolated 25 positive, overlapping plaques. Based on the calculation of frequency of in-frame positive plaques (25), and of screens by hybridization with the original SFE1 clone (resulting in 48 plaques), clones representing SFE1 in the library exceed 2.2% of the total library. Each cDNA clone was an independent isolate based on a different terminal sequence, yet each contained a similar overlapping region

of cysteine repeats and, in some cases, repeats rich in serine and threonine C-terminal of the cysteine-rich repeats (Fig. 1). We found no clones by hybridization that represented more N-terminal regions of the SFE1. Hybridization screens to identify the complete 3' coding and noncoding regions successfully identified the serine/threonine-rich repeats, the stop codon, 3' untranslated region, and a poly(A) tail. However, plaque hybridization experiments and 5' rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) protocols to identify 5' coding regions were not successful in recovering any more than a few hundred bases 5' of the cysteine repeats, and the existing consensus sequence is without an apparent initiating methionine and signal sequence. The cDNA clones we isolated in such screens (over 45) consistently contained the cysteine-rich sequence matching the existing repeat region and the non-cysteine-rich region immediately 5' of the LDLr-like domain (used to make the hybridization probe), but the 5' sequences consistently diverged from each other. The 5' RACE experiments to identify additional coding sequences were also unsuccessful. Here we present a consensus cDNA (3955 base pairs; NCBI accession no. AAB02256) that encodes the C-terminal 1297 amino acids, predicted to possess a mass of 136 751 kDa (Fig. 1).

The calculated amino acid composition derived from the cDNA translation of SFE1 is similar to that reported [4] for the amino acid analysis of native Pln (Table 1), though no cDNA sequence is available for Pln. Comparing the available SFE1 sequence with the amino acid analysis of isolated Pln, we find that both sequences contain high levels of Cys (10%) and Gly (10%), and a low composition of Met (0.1%). So, originally we believed that SFE1 encoded Pln. To test this, we performed immunoblots using antibodies to SFE1 (both monoclonal and polyclonal) and Pln (polyclonal) to compare the mobility of each target protein. As shown in Figure 2 the mobility and pattern of SFE1 and Pln are distinctly different. Recall that the antibody to Pln was against the whole native protein, whereas the SFE1 polyclonal antibody is only to the recombinant bacterial-expressed protein containing the LDLr-like domains and the Ser/Thr repeats. 4E4 is a monoclonal antibody that binds to an epitope in the LDLr-like repeats of SFE1 (based on immunoreactivity to different domains of bacterial-ex-

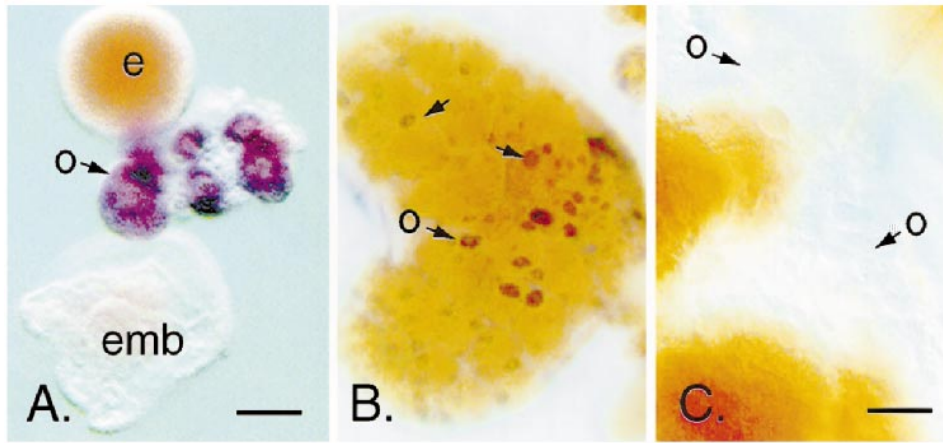


FIG. 4. In situ mRNA hybridization. A) Oocytes accumulate high levels of ovoperoxidase mRNA, shown within a matrix of somatic accessory cells of the ovary that are negative for ovoperoxidase mRNA. Late in oogenesis, the ovoperoxidase mRNA is turned over, so that it is no longer detectable in eggs or embryos. B) A low magnification view of a lobe of an ovary with many unlabeled eggs and associated developing oocytes that are strongly labeled. Within each oocyte, the nucleus is prominent by its lack of staining. C) Using sense strand transcripts, no signal is detectable in oocytes, eggs, or accessory cells of the ovary as shown in this segment of an ovarian lobe. e, Egg; o, oocyte; emb, embryo at early pluteus stage. Bar in A = 50 μm ; bar in B and C = 100 μm .

of an oocyte to an egg requires approximately 16 h in *S. purpuratus* (L. Berg, J. Brooks, and G. Wessel, personal observation), we conclude that the SFE1 mRNA in the maturing oocyte is degraded both rapidly and selectively.

SFE1 Protein Is Present Only in Cortical Granules and in the Fertilization Envelope

Two different antibodies to SFE1 were used to immunolocalize the cognate protein (Fig. 5). One polyclonal antibody was made to a recombinant section of SFE1 containing the cysteine-rich repeat region of the protein [8]. The second antibody was a mouse monoclonal antibody made to SFE1 from cortical granules. By mapping with fusion proteins from different regions of the cloned segments of SFE1, this monoclonal antibody binds an epitope found in several of the cysteine-rich repeats (data not shown). Both the monoclonal and polyclonal antibodies yield the same results. That is, SFE1 was present specifi-

cally in cortical granules in unfertilized eggs and in the fertilization envelope following fertilization. Both monoclonal and polyclonal SFE1 antibodies localize to the same 1- μm diameter vesicles and this immunolabeling overlaps the immunolabeling pattern of both Pln and hyaline, known content proteins of the cortical granules [21, 24]. In addition, electron microscopic immunolabel was performed on eggs and a definitive localization of SFE1 was made to the spiral lamellae of the cortical granules (Fig. 6). Following fertilization, SFE1 accumulates selectively in the fertilization envelope with none remaining in the egg nor within the hyaline layer. It is also clear that the SFE1 protein accumulates in the fertilization envelope, based on immunolocalization studies with the polyclonal anti-SFE1 antibody and by immunoblots on isolated fertilization envelopes. However, we note that the epitope recognized by the monoclonal antibody to SFE1 (4E4) was masked within the envelope and not accessible to the antibody. The protein is

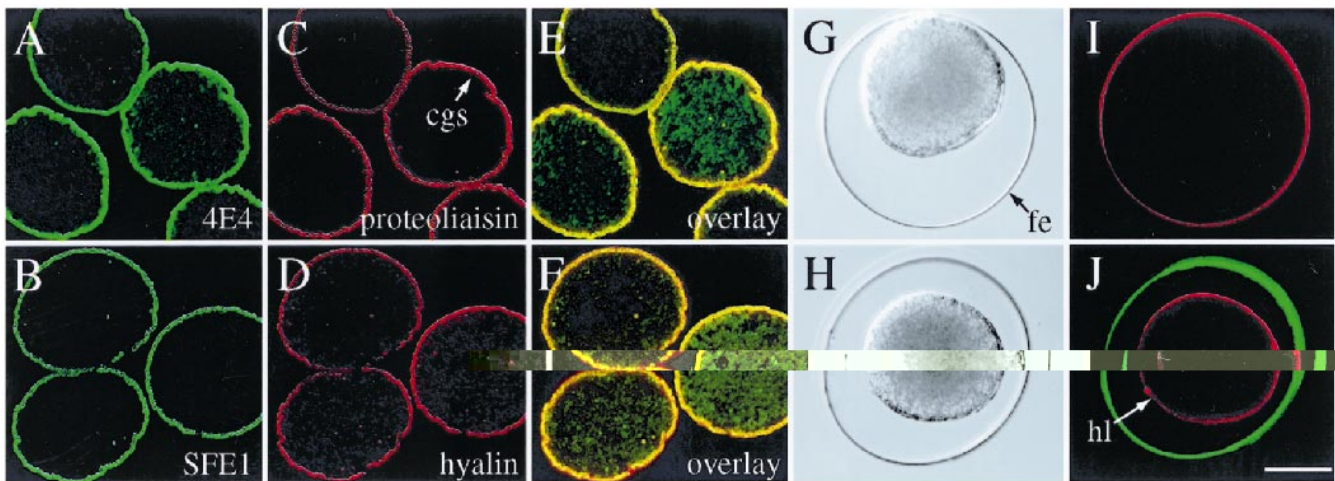


FIG. 5. Coimmunolocalizations of cortical granule proteins. Shown are both the monoclonal antibody 4E4 to SFE1 and the polyclonal antibody to recombinant SFE1 colocalized with other known cortical granule proteins Pln and hyaline, in both sections of unfertilized eggs (A–F) and whole mount immunolabeling of fertilized eggs (G–J). Note the cortical granule labeling in unfertilized eggs for each antibody. Following fertilization however, the 4E4 epitope is masked in the fertilization envelope (G, I). Using the polyclonal antibody SFE1 though, SFE1 is readily detectable in the envelope and clearly distinguishable from the hyaline protein labeling in the hyaline layer (H, J). cgs, Cortical granules; hl, hyaline layer; fe, fertilization envelope. Bar = 50 μm .

12. Harlow E, Lane D. *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1988.
13. Kinsey WH. Purification and properties of the egg plasma membrane. *Methods Cell Biol* 1986; 27:139–152.
14. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 1983; 132:6–13.
15. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1977; 74:5463–5467.
16. Devereux J, Haeblerli P, Smithies O. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* 1984; 12:387–395.
17. Bruskin AM, Tyner AL, Wells DE, Showman RM, Klein WH. Accumulation in embryogenesis of five mRNAs enriched in the ectoderm of the sea urchin *pluteus*. *Dev Biol* 1981; 87:308–318.
18. Ransick A, Ernst S, Britten RJ, Davidson EH. Whole mount in situ hybridization shows *Endo 16* to be a marker for the vegetal plate territory in sea urchin embryos. *Mech Dev* 1993; 42:117–124.
19. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–685.
20. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 1979; 76:4350–4354.
21. Somers CE, Battaglia DE, Shapiro BM. Localization and developmental fate of ovoperoxidase and proteoliasin, two proteins involved in fertilization envelope assembly. *Dev Biol* 1989; 131:226–235.
22. Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal Biochem* 1984; 136:175–179.
23. Conner SD, Wessel GM. Syntaxin is required for cell division. *Mol Biol Cell* 1999; 10:2735–2743.
24. Wessel GM, Berg L, Adelson DL, Cannon G, McClay DR. A molecular analysis of hyalin—a substrate for cell adhesion in the hyaline layer of the sea urchin embryo. *Dev Biol* 1998; 193:115–126.
25. Wessel GM, McClay DR. Two embryonic, tissue-specific molecules identified by a double-label immunofluorescence technique for monoclonal antibodies. *J Histochem Cytochem* 1986; 34:703–706.
26. Spurr A. A low viscosity epoxy resin embedding medium for electron microscopy. *J Ultrastruct Res* 1969; 26:31–43.
27. Somers CE, Shapiro BM. Functional domains of proteoliasin, the adhesive protein that orchestrates fertilization envelope assembly. *J Biol Chem* 1991; 266:16870–16875.
28. Yamamoto T, Davis CG, Brown MS, Schneider WJ, Casey ML, Goldstein JL, Russell DW. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell* 1984; 39:27–38.
29. LaFleur GJ Jr, Horiuchi Y, Wessel GM. Sea urchin ovoperoxidase: oocyte-specific member of a heme-dependent peroxidase superfamily that functions in the block to polyspermy. *Mech Dev* 1998; 70:77–89.
30. Bieri S, Djordjevic JT, Jamshidi N, Smith R, Kroon PA. Expression and disulfide-bond connectivity of the second ligand-binding repeat of the human LDL receptor. *FEBS Lett* 1995; 371:341–344.
31. Shapiro BM, Somers C, Weidman PJ. Extracellular remodeling during fertilization. In: Schatten H, Schatten G (eds.), *Cell Biology of Fertilization*. San Diego: Academic Press; 1989: 251–276.
32. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 1988; 240:622–630.
33. Haley SA, Wessel GM. The cortical granule serine protease CGSP1 of the sea urchin, *Strongylocentrotus purpuratus*, is autocatalytic and contains a low-density lipoprotein receptor-like domain. *Dev Biol* 1999; 211:1–10.