

The Cortical Granule Serine Protease CGSP1 of the Sea Urchin, *Strongylocentrotus purpuratus*, Is Autocatalytic and Contains a Low-Density Lipoprotein Receptor-like Domain

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Trypsin-like activity is secreted from eggs of many species at fertilization, and this activity is believed to be critical for the block to polyspermy. Here we show that a cortical granule serine protease of sea urchins is the major and perhaps only protease family member important for fertilization. Zymography assays suggest that the cortical granules contain a single serine protease that can undergo autocatalysis and is secreted upon egg activation. We used this finding to identify a cDNA clone from a *Strongylocentrotus purpuratus* ovary cDNA library that encodes a 581-amino-acid-residue protein that we refer to as cortical granule serine protease 1 (CGSP1). The catalytic domain of the protein contains the essential residues of the catalytic triad characteristic of a member of the trypsin-like family of serine proteases and the N-terminus of CGSP1 resembles the ligand-binding domain of the low-density lipoprotein (LDL) receptor. Antibodies raised separately to both the protease and LDL receptor-like domains each localize to the cortical granules of unfertilized eggs. Furthermore, the full-length form of CGSP1, as well as intermediate and active forms of the protease, is detected in cortical granules by immunoblot analysis. Our evidence suggests that CGSP1 is activated at fertilization and is responsible for the protease-mediated reactions that follow cortical granule exocytosis and contribute to the block to polyspermy. © 1999 Academic Press

Key Words: fertilization; cortical granules; serine protease; block to polyspermy.

INTRODUCTION

A successful fertilization for most animals requires the fusion of a single sperm and egg. Multiple sperm fusions, or polyspermy, is usually lethal since additional paternal chromosomes and centrioles lead to abnormal cell divisions (Just, 1919). To block polyspermy, the eggs of all mammals, most other vertebrates, and many invertebrates have evolved specialized secretory organelles called cortical granules (Anderson, 1974). Cortical granules are secreted upon stimulation by sperm contact, and these Golgi-derived vesicles contain a variety of proteins that are exclusively packaged into the granule, including enzymes

and structural proteins (Shapiro, 1989). Once secreted, the proteins of the cortical granule modify the surface of the fertilized egg, making it impermeable to additional sperm (Anderson, 1974).

One protein activity that appears to be conserved in the cortical granule of animals is a protease. Once secreted from the egg at fertilization, the protease is believed to act in concert with other cortical granule proteins to modify the extracellular matrix (ECM)² of the egg and to cleave sperm receptors, making the egg refractory to binding by additional sperm. For example, hamster eggs release a trypsin-like activity during cortical granule exocytosis which may contribute to the block to polyspermy (Gwatkin *et al.*, 1973), since incubation of hamster eggs with trypsin prior to fertilization reduces the fertilizability of these eggs (Hartmann and Gwatkin, 1971). In addition, a trypsin-like

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² Abbreviations used: ECM, extracellular matrix; FE, fertilization envelope; CSC, cell surface complex; SBTI, soybean trypsin inhibitor; CGSP1, cortical granule serine protease 1.

protease is secreted from the cortical granules of the frog *Xenopus laevis* (Lindsay and Hedrick, 1989), which appears to cleave and activate another protease, ovoidism. This second protease is then believed to cleave proteins in the ECM of the fertilized frog egg, blocking additional sperm fusions (Lindsay and Hedrick, 1995).

Protease activity in the cortical granule was initially postulated by Hagström (1956) when he observed that sea urchin eggs fertilized in the presence of the serine protease inhibitor soybean trypsin inhibitor (SBTI) were polyspermic and displayed abnormal fertilization envelopes. The envelopes that form from treated eggs did not completely detach from the plasma membrane of the SBTI-treated eggs and instead formed irregular blebs around the cell. Vacquier *et al.* (1972) confirmed that the activity released at fertilization by *Strongylocentrotus purpuratus* eggs resembled that of a trypsin-like serine protease, and later this activity was biochemically localized to the cortical granule (Schuel *et al.*, 1973; Vacquier *et al.*, 1973). This cortical granule protease activity has been biochemically isolated, though little agreement exists as to the size or the number of proteins with this activity, even within the same species. Several groups have reported the isolation of a single serine protease from the cortical granule of *S. purpuratus*, but at divergent molecular weights, including 22.5 kDa (Fodor *et al.*, 1975) and 35 kDa (Alliegro and Schuel, 1988). In contrast, Carroll and Epel (1975) described proteolytic activity at 47 kDa and maintained that two separate proteases exist at this molecular weight: a vitelline delaminase that cleaves the protein anchor to the plasma membrane and a sperm receptor hydrolase that cleaves the sperm receptor. Later, Lois *et al.* (1986) recovered hydrolase activity at 60 kDa from cortical granule exudate. In addition, serine protease activity has been reported in a closely related sea urchin, *S. intermedius*, at 28 kDa (Sawada *et al.*, 1984). It is not yet clear why each group observes serine protease activity at a different molecular weight. It may be that multiple gene products encode different serine protease activities in the cortical granule, yet most groups report the presence of a single protease species. Here we present evidence that a single gene product encodes the serine protease activity present in the sea urchin cortical granule and that variability in reported molecular weights is the result of autocatalytic activation of the protein. We also demonstrate that serine protease activity is the major, and perhaps only, proteolytic activity required for the proper formation of the fertilization envelope.

MATERIALS AND METHODS

All reagents were obtained from Sigma Co. (St. Louis, MO) unless otherwise noted.

Handling of Eggs

Adult *S. purpuratus* were obtained from Marinus (Long Beach, CA). Gametes were shed by intercoelomic injection of 0.5 M KCl,

and the eggs were dejellied and washed twice in Instant Ocean artificial seawater (ASW; Aquarium Systems, Mentor, OH). Cell surface complex (CSC) and cortical granules were isolated as previously described (Crabb and Jackson, 1985). Cortical granule exudate was isolated as previously described (Weidman and Kay, 1986).

To determine which classes of proteases are required for fertilization envelope formation, unfertilized eggs were diluted 1:2 in ASW and then incubated with inhibitors of the four classes of endoproteases: SBTI (100 $\mu\text{g/ml}$; serine protease inhibitor), E-64 (10 $\mu\text{g/ml}$; cysteine protease inhibitor), pepstatin (1 $\mu\text{g/ml}$; aspartyl protease inhibitor), *p*-aminobenzoyl-Gly-Pro-D-Leu-D-Ala hydroxamic acid (10 $\mu\text{g/ml}$; metalloprotease inhibitor) (Otake *et al.*, 1994), and EDTA (0.3 mM; metalloprotease inhibitor, data not shown). The calcium ionophore A23187 (10 $\mu\text{g/ml}$) was then added to induce cortical granule exocytosis and fertilization envelope formation. Elevation of fertilization envelopes was visualized using a Zeiss Axioplan microscope and recorded using Kodak T-max 400 film (Eastman Kodak Co., Rochester, NY).

Isolation of Protease and cDNA Cloning

Protease isolation was performed by soybean trypsin inhibitor chromatography, essentially as described by Fodor *et al.* (1975). Fractions were assayed for proteolytic activity by gelatin zymography (see below), and the fraction observed to contain the most enzymatic activity was concentrated by methanol-chloroform precipitation (Wessel and Flugge, 1984). The precipitated fraction was then electrophoresed on a 12.5% SDS-PAGE gel (Laemmli, 1970) and stained with Coomassie blue. Two bands, at 35 and 25 kDa, were excised and subjected to internal amino acid sequencing (Keck Foundation, New Haven, CT; and University of Florida, Gainesville, FL).

Degenerate PCR primers were designed from one of the obtained amino acid sequences: sense, 5'-GTI GAT/C TTT/C GTI CCI GAT/G GCI GG-3' "SH forward," where I is inosine, corresponding to the amino acid sequence VDFVPEAG; and antisense, 5'-GGA AAC AGC TAT GAC CAT GAT TAC-3' "pBS extended reverse," corresponding to the reverse primer region of pBluescript, and were used to screen a Lambda ZAP cDNA library constructed from *S. purpuratus* ovary (Laidlaw and Wessel, 1994). PCR products obtained with *Taq* DNA polymerase (Life Technologies Inc., Gaithersburg, MD) were ligated into the vector pGEM-T easy (Promega Corp., Madison, WI) and DNA sequencing was performed by the macromolecular sequencing facility at Brown University using an ABI 377 prism automated DNA sequencer. To obtain full-length cDNA encoding the protease, additional primers were constructed to the original protease sequence obtained and were used to rescreen the library by PCR. Several overlapping clones were sequenced; these data were analyzed by the University of Wisconsin Genetic Computer Group (UWGCG) sequence analysis package (Devereux *et al.*, 1984), and the signal sequence cleavage site was predicted by Signal P (Nielsen *et al.*, 1997). In the cDNA clones we have sequenced (greater than 10) we have noted less than 1% DNA sequence variation which we believe reflects the polymorphism of the wild-type population used to construct the cDNA library. We did not observe significant variations in the cognate amino acid sequence and no variation in the conserved residues of either the protease or the LDL receptor-like domains. The full-length sequence was thereafter referred to as cortical granule serine protease 1 (CGSP1).

Immunological Approaches

To generate polyclonal antisera to the protease, fusion proteins were made by ligating cDNA that encoded portions of the N-terminal LDL receptor-like domain (residues 147–333) and the catalytic domain (residues 334–529) into the pGEX expression vector (Pharmacia Inc., Piscataway, NJ), downstream of a coding region for glutathione *S*-transferase (GST). These two fusion proteins were expressed in *Escherichia coli* and isolated by elution from a 12.5% SDS-PAGE gel. Polyclonal antisera were obtained by using the recombinant proteins as immunogens by subcutaneously injecting two New Zealand White rabbits. Boost injections were made every 3 weeks for 3 months. Plasma was collected from the ears 1 week after the boosts (Harlow and Lane, 1988).

Immunofluorescence localization of the two fusion proteins was performed on sections of *S. purpuratus* eggs that were fixed and processed as previously described (Wessel *et al.*, 1984). Preimmune sera and the primary antisera of the anti-LDL receptor-like domain were diluted 1:10, the anti-catalytic domain antisera were diluted 1:180, and the secondary antibody (Cy3-conjugated affinity-purified goat anti-rabbit IgG; Jackson Research Laboratories, Westgrove, PA) was diluted 1:200. To test antisera specificity, anti-catalytic domain antisera were incubated either with an irrelevant protein, bovine serum albumin (BSA) (100 μ g/ml), or with purified CGSP1 fused to GST prior to immunolocalization. Signals were visualized either by epifluorescence with a Zeiss Axioplan microscope or by laser-scanning microscopy with a Zeiss LSM 410 confocal microscope.

For immunoblot analysis, cell surface complex (20 μ g total protein) and concentrated, precipitated fractions from SBTI chromatography (10 μ g) were subjected to SDS-PAGE and immunoblotting essentially as described (Towbin *et al.*, 1979). Samples for analysis were pelleted, resuspended in 2 \times sample buffer (20% sucrose, 5 mM Tris-Cl, pH 6.8, 2% SDS, 0.05% bromophenol blue), and denatured for 5 min at 100°C. The proteins were then resolved by SDS-PAGE and blotted. Blots were washed twice for 30 min in blotto (50 mM Tris-Cl, pH 7.5, 0.18 M NaCl, 0.05% Tween 20, 3% nonfat dry milk) and then incubated for 3 h in blotto containing anti-protease domain diluted 1:1000. The blots were then washed three more times over 1 h and incubated with goat anti-rabbit antibodies conjugated to alkaline phosphatase diluted 1:30,000. Blots were washed in blotto three more times over 30 min and then washed twice in blotto without milk. Immunolabel signals were detected by BCIP/NBT colorimetric development as described (Harlow and Lane, 1988; Promega Corp.). Controls used in this experiment included blots incubated with preimmune sera or secondary antibody alone. Each of these controls showed no signal (data not shown).

Detection of Proteolytic Activity by Gelatin Zymography

Proteolytic activity in the CSC and cortical granule exudate of *S. purpuratus* eggs was detected by gelatin zymography as follows. SDS-PAGE gels were prepared as previously described (Laemmli, 1970), except that they were copolymerized with gelatin at a final concentration of 0.3%. CSC (1 μ g) in 2 \times sample buffer, unreduced and unboiled, was electrophoresed at 4°C. Following electrophoresis, the SDS was removed by soaking the gel in 2.5% Triton X-100 for 2 h at room temperature. The gels were subsequently incubated in calcium activity buffer (200 mM NaCl, 40 mM Tris-Cl, 10 mM CaCl₂, pH 7.5) for 12 h at room temperature. The gels were then

stained in Coomassie blue and destained, and proteolytic activity was detected by clear zones of gelatin lysis against a blue background.

To determine which classes of proteases are present in the CSC, samples of CSC (1 μ g) were incubated with PMSF (1 mM; serine protease inhibitor), EDTA (10 mM; metalloprotease inhibitor), pepstatin (1 μ M; aspartyl protease inhibitor), or E-64 (10 μ M; cysteine protease inhibitor) and then assayed by zymography. The samples were incubated for 30 min on ice prior to loading the gel. Protease inhibitors were included in all buffers during zymography.

For the rezymography assays (Kleiner and Stetler-Stevenson, 1994) areas of the zymography gel previously observed to contain proteolytic activity were excised from a 12.5% SDS-PAGE gel, electroeluted, and concentrated in a Centricon 10 filter unit (Amicon Inc., Beverly, MA). The concentrated samples were then assayed by zymography as described above.

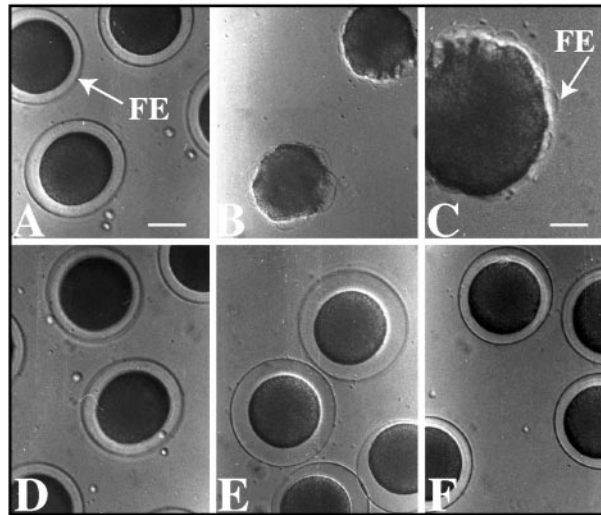
RESULTS

Serine Protease Activity Is the Only Detectable Class of Protease Activity of the Cortical Granules Necessary for the Formation of the Fertilization Envelope

Although it has been shown that sea urchin eggs fertilized in the presence of the serine protease inhibitor SBTI exhibit abnormal fertilization envelopes and are polyspermic (Hagström, 1956; Vacquier *et al.*, 1973), the involvement of other classes of proteases has not been analyzed. We tested the hypothesis that other classes of proteases are required for the activation of the serine protease or that the serine protease functions by cleavage and activation of another protease, analogous to procollagenase activation by mast cell tryptase (Gruber *et al.*, 1988). Eggs were activated in the presence of inhibitors of the four classes of endoproteases: serine, cysteine, aspartyl, and metalloprotease. SBTI was observed to inhibit FE elevation (>99%), consistent with previous observations (Hagström, 1956), while eggs treated with the inhibitors of the other classes of proteases exhibited little to no abnormalities (<1%) (Fig. 1), even when used at their upper limit of suggested concentration for inhibition (Beynon, 1989). These results have focused our attention on the functional characterization of the cortical granule serine protease.

The Cortical Granule Serine Protease Is Secreted at Fertilization

We tested cortical granule exudate and the CSC, which is enriched in cortical granules, of *S. purpuratus* eggs by gelatin zymography. Zymography is a sensitive electrophoretic technique that has been used to detect the four classes of endoproteases (serine, cysteine, aspartic, and metalloproteases) (Heussen and Dowdle, 1980). When cortical granule exudate and CSC samples are electrophoresed through zymograms at 4°C, three distinct bands are detected at 35, 30, and 25 kDa (Fig. 2A). When the same



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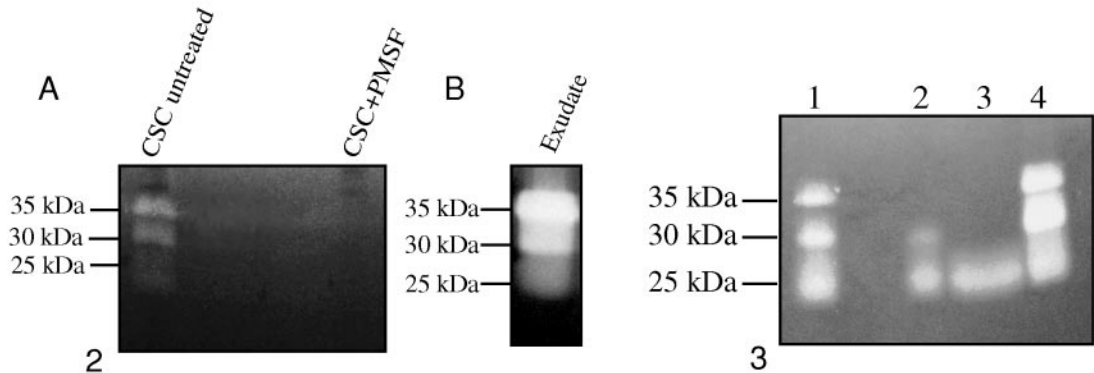


FIG. 1. Proper formation of the fertilization envelope is prevented by a serine protease inhibitor, but not by inhibitors of other classes of proteases. Unfertilized *S. purpuratus* eggs were incubated in the indicated inhibitor, and then 10 $\mu\text{g/ml}$ A23187 was added and FE formation analyzed. (A) No inhibitor, (B) 100 $\mu\text{g/ml}$ SBTI (serine protease inhibitor), (C) magnified image of FE inhibition by SBTI, (D) 1 $\mu\text{g/ml}$ pepstatin (aspartic acid protease inhibitor), (E) 10 $\mu\text{g/ml}$ *p*-aminobenzoyl-Gly-Pro-D-Leu-D-Ala hydroxamic acid (metalloprotease inhibitor), and (F) 10 $\mu\text{g/ml}$ E-64 (cysteine protease inhibitor). Images were visualized by Nomarski microscopy. Bar, 40 μm (A, B, D-F), 10 μm (C).

FIG. 2. Serine protease activity is present in the cell surface complex of unfertilized *S. purpuratus* eggs and is secreted from the egg upon activation. (A) Detection of proteolytic activity in the CSC. Three major proteolytic bands are observed in these samples; all three bands are inhibited by the serine protease inhibitor PMSF. Lane 1, 1 μg untreated CSC; lane 2, 1 μg CSC treated with 1 mM PMSF. (B) The same pattern of proteolytic activity is present in the cortical granule exudate.

FIG. 3. Gelatin zymography demonstrates that the proteolytic bands observed in the CSC are autocatalytic products of one protease. Lane 1, 35-kDa band; lane 2, 30-kDa band; lane 3, 25-kDa band; and lane 4, control CSC.

samples are treated prior to electrophoresis with PMSF, an irreversible inhibitor of serine proteases, all activity is abolished (Fig. 2A). When CSC is treated with inhibitors of other classes of proteases, no inhibition is detected

(data not shown). This observation suggests that the only detectable proteolytic activity in cortical granules is a serine protease, and this activity is secreted from the egg at fertilization.

FIG. 4. Nucleotide sequence and amino acid translation of CGSP1 cDNA. The signal sequence is underlined by a broken line. The LDL receptor-like domain (residues 30–333) is delineated by horizontal arrows. The potential cleavage site of the protease domain from the LDL receptor-like domain is represented by an arrowhead. The catalytic triad, highly conserved among serine proteases, is formed by H378, D430, and S526, which are circled. The sequences obtained by internal amino acid sequencing are underlined with a solid line. The termination codon is marked with an asterisk.

The Cortical Granule Serine Protease Is Autocatalytic

When the CSC is electrophoresed in a zymogram gel at room temperature, a smear of proteolytic activity is visible from 35 to 25 kDa (data not shown). However, when the gels are run at 4°C, three distinct bands are present at 35, 30, and 25 kDa (Fig. 2A), suggesting a temperature-dependent autocatalysis (Lantz and Ciborowski, 1994). When samples are treated with reversible inhibitors of serine proteases such as benzamidine, larger bands of proteolytic activity are faintly visible as well, particularly at 45 kDa (data not shown). Reversible serine protease inhibitors thus apparently slow down autocatalysis and reveal possible precursor forms not normally seen.

To distinguish whether the three bands of proteolytic activity we observe represent three separate proteases or are the autocatalytic products of one protein, we performed a rezymography assay (Fig. 3) (Kleiner and Stetler-Stevenson, 1994). When the 35-kDa band of activity is rezymographed, it exhibits all three major forms of activity, i.e., 35, 30, and 25 kDa. When the 30-kDa band is rerun it exhibits the 30- and 25-kDa activities, and when the 25-kDa band is rerun it remains at 25 kDa. We conclude from this experiment that the activity in the CSC originates from one serine protease and that it undergoes an autocatalytic activation into smaller, proteolytically active forms.

The Cortical Granule Serine Protease Is a Trypsin-like Protease with an N-Terminal LDL Receptor Motif

The cortical granule serine protease was isolated from whole eggs by SBTI chromatography (Fodor *et al.*, 1975) and assayed for activity by gelatin zymography as described above. Multiple proteolytic bands were apparent in peak fractions eluted from SBTI affinity columns, similar to the bands observed from CSC. After protein concentration and separation by SDS-PAGE electrophoresis, the most prominent bands visible by Coomassie staining were at 35 and 25 kDa, consistent with sizes of activity by zymography. These bands were excised and subjected to internal amino acid sequencing, which resulted in one amino acid sequence from the 35-kDa band (HSVDFVPEAGSQHRLVR) and two sequences from the 25-kDa band (HSVDFVPEAGSQHR and MPLIPR). The longest sequence, HSVDFVPEAGSQHR, was present in both bands, further supporting the conclusion that the different-sized activities seen by zymography are products of a single gene.

A PCR screen of a *S. purpuratus* ovary cDNA library, using degenerate primers designed from these sequences, yielded an 800-bp product whose sequence resembled human elastase and other serine proteases. Subsequent PCR screens using gene-specific primers resulted in the isolation of a 1743-bp open reading frame, coding for a 581-amino-acid polypeptide (Fig. 4). The predicted molecular weight of the full-length protein is 64,416 (61,203 following removal of the signal sequence), and the predicted *pI* is 4.7. The potential open reading frame begins with a methionine

which fits a consensus-initiating methionine (Kozak, 1986), followed by a hydrophobic signal sequence and a signal sequence cleavage site predicted to be between residues 29 and 30 (Nielsen *et al.*, 1997). From BLAST analysis, we determined that the cDNA encodes a mosaic protease, with an LDL receptor-like motif at its N-terminus and a trypsin-like serine protease at its C-terminus (Fig. 4). The LDL receptor-like motif is characterized by repeats of approximately 40 amino acids, containing six cysteines that form three disulfide-bonded loops, and a cluster of acidic residues in each repeat that participate in either ligand or calcium binding (Fass *et al.*, 1997; Krieger and Herz, 1994) (Fig. 5A). The protease domain resembles other serine proteases, including plasminogen (Marti *et al.*, 1985) and hepsin (Leytus *et al.*, 1988) (Fig. 5B), and contains the conserved amino acid residues involved in the formation of the active-site triad, e.g., His-378, Asp-430, and Ser-526. In addition, the sequence contains the RIVGG sequence at residues 333–337, which is a characteristic activation site for serine protease zymogens. We have chosen the name cortical granule serine protease 1 (CGSP1) to represent the protein encoded by this sequence which reflects its historic name and its functional contribution to the egg at fertilization.

CGSP1 Is Stored in the Cortical Granule

Polyclonal antisera were raised separately against recombinantly expressed protease and LDL receptor-like domains and both sera selectively label the cortical granules of unfertilized *S. purpuratus* eggs (Figs. 6A–6F). This result shows that the protease encoded by the cDNA identified here is the cortical granule serine protease and that the LDL receptor-like domain remains with the protease domain following targeting to the granule. Following fertilization, CGSP1 immunolabel is secreted from the egg as expected and is not detected at the two-cell stage or later stages of development (data not shown). Specificity of the antibodies is demonstrated when immunolabel is competed away by preincubation with the fusion protein CGSP1–GST (Figs. 6E and 6F), but not with an irrelevant protein, bovine serum albumin (Fig. 6B). Preimmune sera used at the same concentration as immune sera gave no significant signal (Fig. 6A). These results suggest that CGSP1 is stored specifically in the cortical granule until fertilization, when it is secreted and cleaves its targets to participate in the block to polyspermy.

The localization of CGSP1 to the cortical granule is further supported by immunoblot analysis of cell surface complex with the anti-protease domain antibody. In these immunoblots, the full-length form of CGSP1 and an intermediate (45 kDa) and an active form (35 kDa) are detected (Fig. 6G). The 35-kDa band is the major active form detected in the cortical granule exudate and is also the major band detected by affinity chromatography with soybean trypsin inhibitor, used for the original protein microsequence. The intermediate (45 kDa) band was predicted

A

CGSP1	QTIRRSRR-QSSSCHQDESQCDDGSCIPAYLACDWYLDSCDRSDE----G-INCEYDGF	90
LRP	EDVKYCKDGKQKPVCAAKKFQCDNHRCIPEQWKCDSDNDCGDGSDKLEMCNATCAANQF	1193
	: : : * * : ***: *** ** * * * * * * * * * * * * * * :	
CGSP1	ECKSGDNMISLEWMCDSYDCDDGSDHEDHQYCENHASVTKVCPRISCDNGTRCVQEGEIC	150
LRP	SCANGR-CIPIYWLCDGDNDYDGTDEDKERCP-PVQCSALQFR--CANGRQCVPPLRNHC	1249
	* * * : * : *** ** * : * : * : * * * * * * * * * * * * * * :	
CGSP1	DGTQHCSDDLDESDELCSAGNVK----FSCDGGSKCLKWNVWCDEFADCSDMADEFSGW	206
LRP	DGQSDCEDGSDE-D-SCAVTAESCTPDQFKCVSSGLCIPASWKCDGQDCDDGSDPEKFG	1307
	** * * * * * * * : * :	
CGSP1	CGTVFQRCWKAYLFCGHTHFCVLQRWRCNNHDDCGDDTDE--ED-CETDF-AWTGSYG-W	261
LRP	C-TSGRQCSSDQFKCG-NGRCILNNWLCDGENDCGDGSDESSERGCKTSMNARKCPFEHV	1365
	* * : * : * :	
CGSP1	SSWDWSEC--HPSCGLGTRSRSRFCASPGGRCLG--ESQEEEECEQVPCVD-EN--VI	313
LRP	ACENDQETCIPHLQLCDGKTHCPG--GTDEGGRCARDLCSADRAGCS-FKCHNSPNGPIC	1422
	: * * * * * * * * : * :	
CGSP1	ACGIKSHIHFRRDGLLALAER-	333
LRP	SCPFGEQLVNKTKCEPENEC-	1442
	: * : : : * :	

B

CGSP1	RIVGGQPATAGDWPWQAQLFYRTRGWSQLVCGGTLIDPQVVLTAACHFMGPMMATSRWQV	392
PLMN	RVVGCVSIPHSWPWQISLRVRYRG--HFCGGTLISPEWVLTAKHCLE-KSSSPSSYKV	615
HEPSIN	RIVGGRDTSLGRWPWQVSLRY--DG--AHLCCGSLLSGDWVLTAAHCFPERNRVLSRWRV	217
	* : * * * : * :	
CGSP1	HLG--KHSVDFVPEAGSQHRLVREIIVHK---KFGHEHGGV--GCDIALLILDEFPVQETG	447
PLMN	ILG--AHEEYHLGEG-----VQIEDVSK---LFKEPS---EADIALLLKLSSPA-VITD	659
HEPSIN	FAGAVAQASPHGLQLG-----VQAVVYHGGYLPFRDPNSEENSNDIALVHLSSPL-PLTE	273
	* : : : * : * : * :	
CGSP1	QINWACLDEG-MPLNDRTECYISGWGVTEMGGNGPDVLEHARMPLIPRRICNYKKSYPNGK	506
PLMN	KVIPACLPTPNYVADRTACYITGWGETK-GTYGAGLLKEARLPVIEKVCNRYEYLGK	718
HEPSIN	YIQPVCLPAAGQALVDGKICTVTGWGNTQYYGQAGVLQEARVPIISNDVCNGADFYGNQ	333
	: ** : * :	
CGSP1	IEKIMLCAGHLEGGIDACQGDSSGGPLSCLG---PDDHWYVVGVTSWGHGCAIANKPGVYT	563
PLMN	VSPNELCAGHLAGGIDSCQGDSSGGPLVCFE---KDKYILQGVTSWGLGCALPNKPGVYV	774
HEPSIN	IKPKMFCAGYPEGGIDACQGDSSGGPFVCEDSISRTPRWRLCGIVSWGTCALAQKPGVYT	393
	: : * * * : * :	
CGSP1	KVSSYLDWIDEMIHHLHHE	581
PLMN	RVSRFVTWIEEIMRRN	790
HEPSIN	KVSDFREWIFQAIKTHSEAS-	412
	: * * : * * : :	

FIG. 5. (A) The LDL receptor-like domain of CGSP1 shares sequence similarity with the lipoprotein receptor (LRP) of *C. elegans* (Yochem and Greenwald, 1993), as shown by this ClustalW alignment (Thompson *et al.*, 1994). Asterisks denote identities; residues that share charge similarity are denoted by colons. (B) The catalytic domain of CGSP1 shares sequence similarity with other extracellular serine proteases, including plasminogen (Marti *et al.*, 1985) and hepsin (Leytus *et al.*, 1988).

based on detection of low proteolytic activity by zymography when CSC is isolated and run in the presence of reversible serine protease inhibitors, e.g., benzamidine (data not shown). The 68-kDa, full-length form of CGSP1 is slightly larger than the 61.2 kDa expected by DNA se-

quence analysis following removal of the signal sequence and probably reflects a posttranslational modification of the protein often observed in these secreted proteins. We do not routinely detect immunoblot activity at 30 and 25 kDa, even though significant proteolytic activity is present in

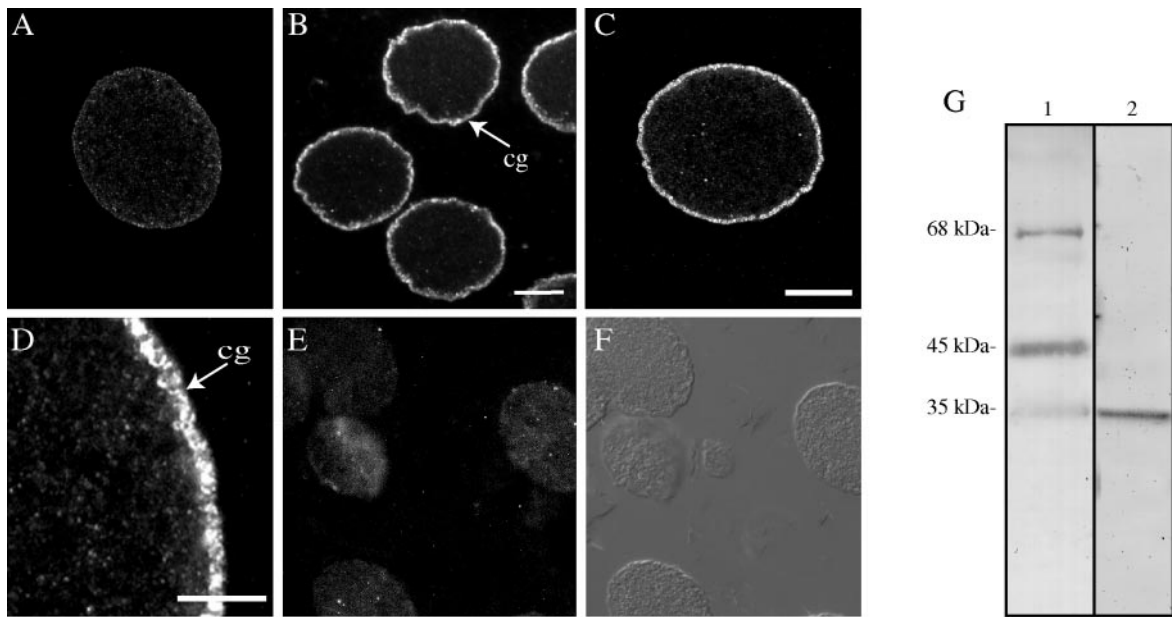


FIG. 6. *In situ* immunolocalization shows that CGSP1 protein is present in the cortical granules of unfertilized eggs of *S. purpuratus*. (A) Preimmune serum, (B) anti-catalytic domain serum, (C) anti-LDL receptor-like domain serum, (D) magnified image of the egg cortex, (E) anti-catalytic domain sera preincubated with full-length fusion protein, and (F) brightfield image of E. Images were visualized either by laser-scanning microscopy with a Zeiss LSM 410 (A, C, D) or by epifluorescence with a Zeiss Axioplan microscope (B, E, F). Bar, 40 μ M (A, B, C, E, F), 15 μ M (D). cg, cortical granules. (G) Immunoblot analysis of the protease domain of CGSP1 indicates that this protease is present in cortical granules and is selectively isolated by SBTI chromatography. Lane 1, cell surface complex, 20 μ g/lane. Lane 2, affinity-purified protease extract, 10 μ g/lane.

these forms, likely because of the superior sensitivity of zymography and the instability of the proteolytically active forms. In conjunction with the zymography data (Figs. 2 and 3), we conclude that the full-length, inactive form of CGSP1 is present in the cortical granules and must be autocatalyzed to a smaller form to be active. Secretion at fertilization initiates the subsequent autocatalysis to the 35-, 30-, and 25-kDa forms of activity.

DISCUSSION

A trypsin-like activity has been observed in the cortical granule of eggs and oocytes from a number of different animals, yet a definitive identification of the protease(s) has not been made. Here we have characterized a serine protease in the sea urchin cortical granule, CGSP1, and provide evidence that it is the major, if not the only, protease in the cortical granules. Since we tested both cell surface complex, which contains intact cortical granules, and cortical granule exudate, which contains activated cortical granule contents, and still observe only serine protease activity attributable to CGSP1, we believe it is the serine protease involved in the block to polyspermy in the sea urchin. This is different from *X. laevis* in which at least two serine protease activities are present and activated in a catalytic cascade at fertilization (Lindsay and Hedrick, 1995). Al-

though we cannot exclude the possibility that other types of proteases are present in sea urchin cortical granules whose activities are not detectable by our assay, CGSP1 is clearly an essential activity for normal fertilization. Key to the resolution of a single trypsin-like activity in the sea urchin cortical granule is the observation that CGSP1 is autocatalytic. It is possible that the different methods of protease purification by other groups resulted in the preferential isolation of one autocatalytic product size over another. Since the purified protease was reported to autocatalyze from 35 to 23 kDa when stored for a prolonged time, with a coordinate change in substrate specificity (Alliegro and Schuel, 1985; Carroll and Epel, 1975), the processing we observe could be of biological importance. Perhaps when the protease is first secreted, the larger form has a narrower substrate specificity, which progressively becomes broader and cleaves more substrates as it diffuses from the cell surface. This change in specificity could explain the separate delaminase and hydrolase activities observed by the protease in previous studies (Carroll and Epel, 1975).

CGSP1 contains a large N-terminus that resembles the ligand-binding domain of the LDL receptor. In addition to being found in the LDL cell surface receptor, such motifs are present at the N-terminus of other extracellular serine proteases, including enterokinase (Kitamoto *et al.*, 1994) and complement factor I (Catterall *et al.*, 1987). The function of the motif in these proteases is not yet known, but it

may mediate substrate specificity or stabilize enzymatic activity. Surprisingly, two other proteins of the sea urchin cortical granule share this motif, SFE1 and SFE9 (Wessel, 1995). The exact amino acid sequences of the LDL receptor-like repeats are different in the various cortical granule proteins, but the cysteines that create the disulfide-bonded loops and the acidic amino acids are conserved. SFE1 and SFE9 appear to be structural proteins without enzymatic activity, so it may be that this domain contributes to intramolecular interactions between the proteins that form the fertilization envelope. Disulfide-bonded loops have been shown to play a role in protein sorting in the trans-Golgi network (Cool *et al.*, 1995), so the LDL receptor-like domains may also play a role in selectively sorting the proteins before they are packaged in the granule.

The catalytic domain of CGSP1 resembles other extracellular serine proteases, including trypsin, hepsin, and plasminogen. Previous work has shown that the cortical granule protease will preferentially cleave C-terminal to arginine residues (Green, 1986; Sawada *et al.*, 1984), though the natural substrate(s) of CGSP1 in the egg are not yet known. Since the fertilization envelope appears to retain contact with the plasma membrane when the egg is activated in the presence of SBTI, the putative vitelline post-protein is a likely substrate for CGSP1. CGSP1 may also cleave the sperm receptor (Foltz *et al.*, 1993; Hirohashi and Lennarz, 1998), thereby removing supernumerary sperm and preventing further sperm binding. Furthermore, CGSP1 may cleave other content proteins upon exocytosis. For example, the fertilization envelope protein SFE9 appears to be proteolyzed following fertilization (Wessel, 1995) and we observe that p90, a newly characterized fertilization envelope protein, is processed before incorporation into the envelope, and its N-terminus is cleaved after an arginine residue, consistent with trypsin-like protease activity (F. Molina and G. LaFleur, unpublished observations). CGSP1 may also cleave and activate other enzymes of the cortical granule, such as ovoperoxidase, so they can contribute to the formation of the FE. One form of ovoperoxidase is cleaved after an arginine residue before incorporation into the FE (LaFleur *et al.*, 1998), though the effect of this proteolysis on peroxidase activity is not known. Identification of the natural protease substrates in the egg will be essential to understand the fertilization process and the full-length cDNA clone that encodes CGSP1 described here should be helpful for that goal.

Our current model of the sea urchin cortical granule protease is that CGSP1 is packaged in the cortical granule and secreted from the egg in its 61-kDa proform, similar to the size observed in native PAGE gels from cortical granule exudate by Lois *et al.* (1986). Following exocytosis, CGSP1 apparently undergoes autocatalysis to its mature, enzymatically active form(s), since we do not observe proteolytic activity at 61 kDa. It is possible that part of the N-terminus of CGSP1 must be removed before the protease is enzymatically active. It is likely that the protein is cleaved between residues R-333 and I-334, as the sequence that surrounds these residues is a con-

served "activation domain" sequence of trypsin-like serine proteases (Rawlings and Barrett, 1994). A cleavage at this site would yield the catalytic domain, which has a predicted molecular weight of 27 kDa, close to the 25-kDa size we observe by zymography. We are not sure where the cleavages take place that yield the 35- and 30-kDa bands of activity and if these cleavages are sequential or random. It is possible though that the processing we observe will influence the substrate specificity of CGSP1 and its role in the block to polyspermy following fertilization.

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