



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc



Conservation of sequence and function in fertilization of the cortical granule serine protease in echinoderms



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ARTICLE INFO

Article history:

Received 13 May 2014

Available online 27 May 2014

Keywords:

Cortical granules

Fertilization

Cortical granule protease

Egg receptor for sperm

Echinoderms

Sea star

ABSTRACT

Conservation of the cortical granule serine protease during fertilization in echinoderms was tested both functionally in sea stars, and computationally throughout the echinoderm phylum. We find that the inhibitor of serine protease (soybean trypsin inhibitor) effectively blocks proper transition of the sea star fertilization envelope into a protective sperm repellent, whereas inhibitors of the other main types of proteases had no effect. Scanning the transcriptomes of 15 different echinoderm ovaries revealed sequences of high conservation to the originally identified sea urchin cortical serine protease, CGSP1. These conserved sequences contained the catalytic triad necessary for enzymatic activity, and the tandemly repeated LDLr-like repeats. We conclude that the protease involved in the slow block to polyspermy is an essential and conserved element of fertilization in echinoderms, and may provide an important reagent for identification and testing of the cell surface proteins in eggs necessary for sperm binding.

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1. Introduction

Eggs are two – faced. At first they function to attract sperm, which enhances the fertilization potential and their future in making a new individual. To accomplish this task, the egg and its associated somatic cells release sperm attractants that are often highly selective for the homologous sperm. When the sperm gets close to the egg, generally the extracellular matrix then activates the sperm, enabling it to penetrate the matrix and reach the cell surface, whereupon the sperm and egg are now able to fuse.

Upon fusion however, the egg immediately changes tactics, and then employs its resources to block any subsequent sperm. Most often this means modifying its cell surface in a variety of ways, including construction of a new or modified extracellular matrix, removing sperm receptors from its plasma membrane, and electrical potential changes across the plasma membrane that are incompatible with sperm binding and/or fusion [1].

Although not universal, most eggs harbor secretory vesicles near their cell surface that are stimulated to release their contents upon sperm fusion with the egg [1]. These contents are variable,

depending on the species, but may include molecules that link to the extracellular layer matrix of the egg to block additional sperm from penetrating, hygroscopic polymers that mechanically force the extracellular layer of the egg away from the cell surface and thereby preventing sperm access to any sites for fusion, and the enzymes that modify the extracellular matrix and/or the egg cell surface. En toto, these modifications favor blocking subsequent sperm from fusing with the egg, a generally lethal condition, and is referred to as the block to polyspermy.

A prevalent feature of the block to polyspermy is a protease released by the cortical granules of the egg. This protease was originally postulated by Hagström in 1956 in sea urchins [2], and predicted to accomplish at least two separate molecular tasks. One task was in cleaving the presumed tethers of the extracellular matrix to the egg cell surface. Severing this link, the so-called delaminase, enables the extracellular layer to detach and lift off the cell surface to spatially separate subsequent sperm from the egg cell surface. The second task presumed by this protease is to cleave egg cell surface receptors for sperm and thereby reduce sperm access to the fusion mechanism for egg activation. This activity was referred to as the sperm receptor protease [3–5], although the definitive receptor for sperm is unknown for any species, with the exception of a few cases [6].

The cortical granule protease was characterized effectively in echinoderms and found to be an essential element in fertilization.

Abbreviations: LDLr, receptor for low density lipoproteins; SBTI, soybean trypsin inhibitor; FE, fertilization envelope.

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It was identified as a serine protease based on its sensitivity to protease inhibitors, was isolated by affinity chromatography, sequenced, and eventually molecularly cloned [7]. As predicted, its sequence (CGSP1 for cortical granule serine protease 1) encodes a serine proteinase with the classic catalytic triad. It is stored in the cortical granules as a large precursor form containing a series of LDLr-like repeats. The protease is autocatalytically activated upon release into the sea water (pH 8.0) from its acidic compartment (pH 4.5). In the sea urchin *Strongylocentrotus purpuratus* (Sp), it has multiple molecular targets, including other enzymes in the cortical granules, and a cell surface protein (p160) that appears to tether the eggs extracellular matrix, in this case called the vitelline layer, to the egg plasma membrane. However, this cortical granule protease is not present in mice, or in other organisms for which it was tested, e.g., frogs. Here we expand this search by testing if CGSP1 is conserved in sequence and function in echinoderms. This phylum has been a favorite for studying fertilization and early development ever since sperm were first reported to be important for fertilization [8].

2. Materials and methods

2.1. Animals

Patiria miniata were housed in aquaria with artificial seawater (ASW) at 16 °C (Coral Life Scientific Grade Marine Salt; Carson, CA). Gametes were acquired by opening up the animals. Oocytes were collected in filtered seawater. To obtain mature oocytes, the full-grown immature oocytes were incubated for an hour in filtered sea water containing 2 μ M 1-methyladenine [9,10].

S. purpuratus adults were housed in aquaria with artificial seawater (ASW) at 16 °C (Coral Life Scientific Grade Marine Salt; Carson, CA). Gametes were acquired by either 0.5 M KCl injection or by shaking. Eggs were collected in ASW or filtered seawater.

2.2. Protease inhibition

To determine which classes of proteases are required for the formation of the fertilization envelope, unfertilized eggs were pre-incubated for 30 min with inhibitors against three classes of endoproteases: SBTI (1 mg/ml; serine protease inhibitor), pepstatin (1 μ g/ml; aspartyl protease inhibitor), and E-64 (10 μ g/ml; cysteine protease inhibitor).

The calcium ionophore A23187 (10 μ 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 connected to a Hamamatsu camera (orca-er). The images were taken using metamorph.

To test whether the protease activity was internal or external of the egg, the serine protease activity, SBTI was either present during activation of the eggs, or removed by washing 3 times with sea water (5 min each) before activation.

2.3. Protein alignment

The sequences of cortical granule serine protease 1 precursor in the different echinoderms were found by performing a tblastn using the Sp CGSP1 protein sequence (NCBI Reference Sequence: NP_999636.1) as a query and the ovary transcriptomes (Adrian Reich and Gary Wessel, unpublished data) from all the other echinoderms as subjects. The protein sequences were aligned using ClustalW (EMBL-EBI). Conserved Domain Database (CDD) was used to identify the protein domains [11].

2.4. Phylogenetic analysis

Phylogenetic trees were made using the program PhyML available on the website phylohyeny.fr [12].

3. Results

We first tested inhibitors used previously on sea urchin eggs to determine which type(s) of proteases were involved in the formation of the fertilization envelope in sea stars (Fig. 1A). Eggs from the sea urchin *S. purpuratus* were used as a control. As expected in the sea urchin, the serine protease inhibitor (SBTI) prevented the normal elevation of the fertilization envelope after activation of the eggs with calcium ionophore [7]. Eggs treated with pepstatin, an aspartyl protease inhibitor, exhibit little to no abnormalities (<1%). Similar results were obtained using sea star eggs. Pre-incubation with the serine protease inhibitor induced defects in the formation of the FE. Eggs activated in the presence of pepstatin underwent the cell surface transition exactly like the control eggs in absence of protease inhibitors. An additional inhibitor, E-64, targeting cysteine proteases was also tested in sea star, and did not affect the formation of the fertilization envelope (data not shown). These results indicate that in sea star, like in sea urchin, the formation of the fertilization envelope depends on the serine protease activity.

To test if the serine protease required for the FE is in the cortical granules or at the surface of the eggs before activation, the SBTI was washed out before addition of the calcium ionophore (Fig. 1B). When the serine protease inhibitor is washed out, the fertilization envelope forms normally, like in the control. This inhibitor needs to be present during the activation of the eggs to affect the elevation of the FE, suggesting that the serine protease might be protected in cortical granules before activation.

In the sea urchin, the cortical granule serine protease CGSP1 is the main protease required for the proper formation of the FE [7]. We tested for presence of the transcripts encoding CGSP1 in 15 echinoderm transcriptomes obtained from ovaries (Adrian Reich and Gary Wessel, submitted). We found that CGSP1 is present among Echinoderms: in feather stars, sea cucumbers, brittle stars, sea stars, sand dollar, pencil urchin and sea urchins (Fig. 2). Alignment of the CGSP1 protein sequences obtained among these 15 Echinoderms with the sequence previously defined in the sea urchin *S. purpuratus* show that this protease is highly conserved (Fig. 3). The protein contains a LDL-receptor-like motif at its N-terminus and a trypsin-like serine protease at its C-terminus. The LDL-receptor-like motif is composed by repeats of approximately 40 amino acids. Although the CGSP1 full length sequence is not available for all the species analyzed, the numbers and locations of the LDL-receptor-like repeats are presented for each species in the Supplemental Fig. 1. In the C-terminus of each sequence, the protease domain contains the three conserved amino acids involved in the formation of the active site triad. The arginine contained in the potential cleavage site, characteristic for serine protease zymogens, is also conserved among Echinoderms (Fig. 3).

4. Discussion

The results from these studies document that sea stars use the same protease for construction of its fertilization envelope. The results do not suggest that the CGSP1 ortholog is the only protease involved in this mechanism, only that it is an essential protease for the fertilization envelope to separate from the egg cell surface.

Phylogenetic analysis also documents that the CGSP1 ortholog is conserved throughout echinoderms. Given that the last common

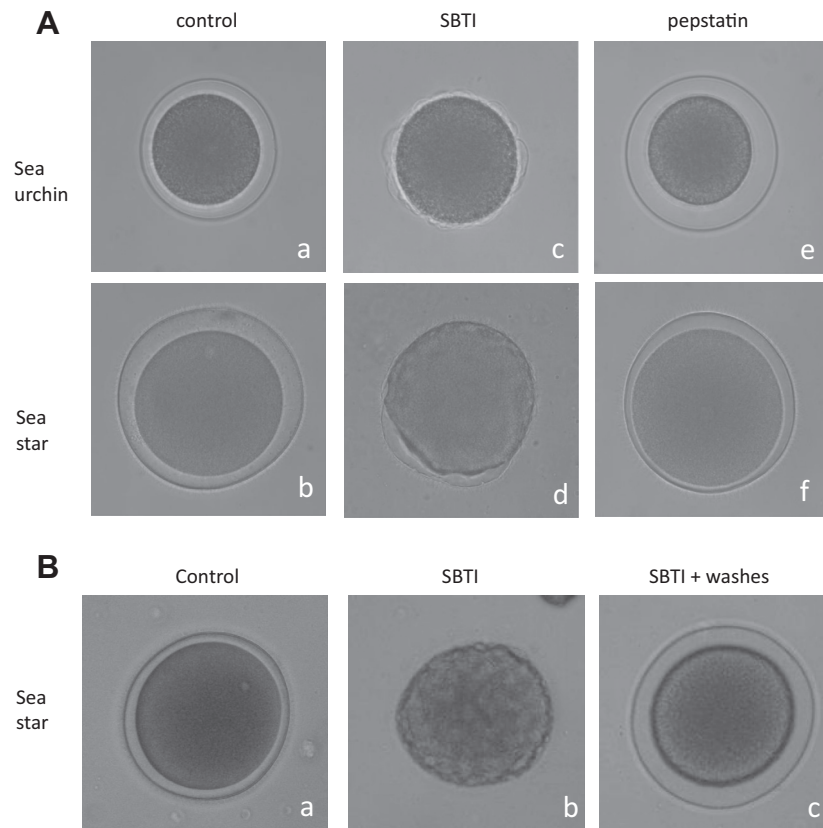


Fig. 1. Serine protease activity is required in both sea urchins and sea stars for the proper elevation of the fertilization envelope. (A) Unfertilized eggs from sea urchin (*S. purpuratus*) and sea star (*P. miniata*) were incubated with protease inhibitors and the fertilization envelope was observed after addition of calcium ionophore. (a and b) No inhibitor, (c and d) SBTI (serine protease inhibitor), (e and f) pepstatin (aspartic acid inhibitor). (B) In sea star, SBTI needs to be present during egg activation to affect the fertilization envelope: (a) no inhibitor, (b) SBTI, (c) SBTI followed by washes with sea water.

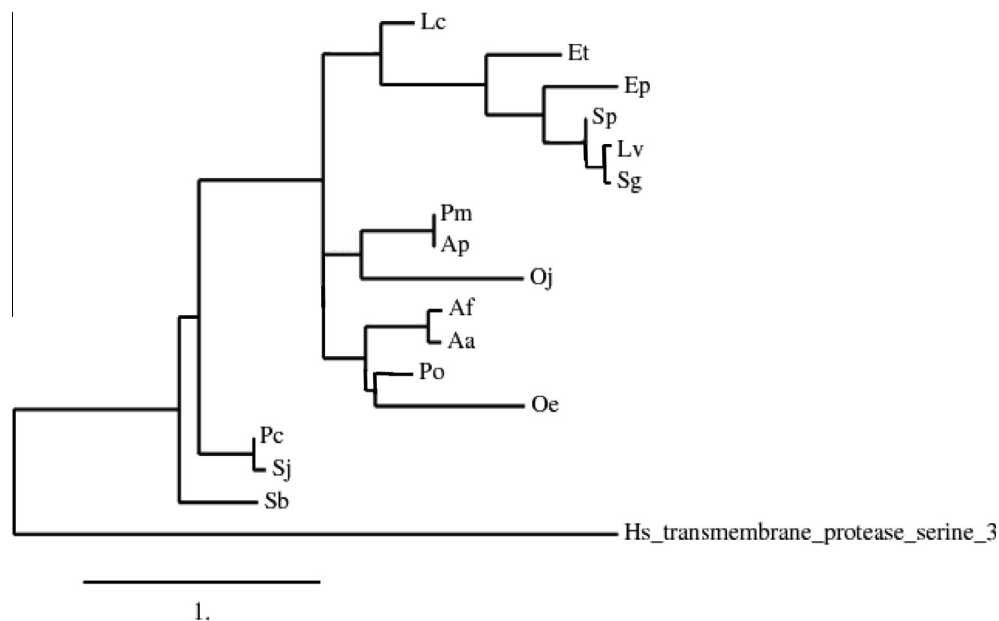


Fig. 2. Phylogenetic tree representing CGSP1 protein in Echinoderms. The amino acid sequences of CGSP1 identified in Echinoderms: feather star [*O. japonicus* (Oj)], brittle star [*O. echinata* (Oe)], sea stars [*A. amerensius* (Aa), *A. forbesi* (Af), *A. pectinifera* (Ap), *L. clathrata* (Lc), *P. miniata* (Pm)], sea cucumbers [*P. californicus* (Pc), *P. ochraceus* (Po), *S. briareus* (Sb), *S. japonicus* (Sj)], sand dollar [*E. parma* (Ep)], sea urchins [*E. tribuloides* (Et), *L. variegatus* (Lv), *S. granularis* (Sg), *S. purpuratus* (Sp)]. The *Homo sapiens* transmembrane protease serine 3 (NCBI Reference Sequence: NP_076927.1) was used as an outgroup. The scale bar indicates an evolutionary distance of amino acid substitutions per position.

Lv	STPSDSLVSRSRVLI FLVIVATILHADCNNRINEQVPT--R-PRQSSSCHQDEFEC	57
Sg	STPSDLVISRSRVLI FLVIVATVLQADCNRIKEQVQII--R-PRQSPSCHQDEFQCD	57
Sp	MSLSTLLLSRRQVLAFLVIVLITLQADCTSRINGQVQTI--RRSRQSSSCHQDESQCD	58
Ep	--KYRFVVLV----LHLSSLIVVFLIANASGGYSSQGGSF--QRRQPNHVCAENEFEC	52
Et	---RRADNISFLVKLSFVIFSLLLGFSCTGTERREYKRI--GRHSSWAGCDEEYQCD	55
Pm	--MMAVHDSNNIRLTMHILMSLVLFSLHMLITASPV--FVKDKSKRCKAWEFACA	56
Ap	---VAVHDSNNIRFTHH--ILSLVLFSLH--LITASPV--FVKDKSKRCKAWEFACA	51
Lc	---AASSRGPCPCWTLCEAFLLLLIFGIWGSLEASPTSG--LVKDKTKGKPCGGPYPCR	55
Af	---AVLLKS----TLLHRVTVVIIVATLGIKLEASPLS--HVKDKS--RNCGLNGFTCS	48
Aa	---AVLLKS----TLLHRVTVVIIVATLGIKLEASPLS--HVKDKS--RNCGLNGFTCS	48
Po	---AVLLTHS----TLLCRVTLIIIVATFGINVKASPV--SKDKS--RHCSQNDFTCS	49
Oj	--ITLAVVT-----FFLFLICNIDFSFIRVDVGTNQS--YHRLSYTPCKDDVFHCL	49
Oe	-----	
Pc	DNIARIILISVICSTCCSGSLE-FQTTEISRYKRGQSNSLESCT-DVTGGCCLEQFRCD	58
Sj	DNLARIILISVICSTCCSGSLEIFQTADISRYKRGQSNSLESCTKDVTTGGCCLEQIRCD	60
Sb	ENTWCFVLVFLSFRYLKGQELPSSDEEISARGEDE-----CLPVQFHCN	47
Lv	DGSCIPSYRSCDWYLDCAADRSDGINCQYNG-----	88
Sg	DGSCIPSYRACDWYLDGDRSDGINCQYNG-----	88
Sp	DGSCIPAYLACDWYLDSDRSDGINCEYDG-----	89
Ep	DESCIPFDHACDWYLDCELDSDGDNCRYEAE-----	83
Et	SGECIAAELECDWYLDCAADSSDEHIHCHYEG-----	86
Pm	SGQCIDGDECDWYDCEDESDEGADCKYTG-----	87
Ap	SGQCIDGDECDWYDCEDESDEGADCKYTG-----	82
Lc	DGSCVNVSRLCDWYDCEDESDEHQCIGYSG-----	86
Af	DGTCIEAS-QCDWYDCNDQSDHQCIGYTG-----	78
Aa	DGTCIEAS-QCDWYDCNDRSDHQCIGYTG-----	78
Po	DGTCIEARLCDWYDCEDESDEHHCYTG-----	80
Oj	NGDCIYTEWECDFYDCADESDEANCPDLEG-----	80
Oe	-----	
Pc	ERKCLPPSWECGVIDCLDERDEPEGCPQ-----ND-----	89
Sj	ESKCLPPSWECGVIDCLDERDEPEGCPQ-----ND-----	91
Sb	DQRCLPPSWRCGDREDCDEGEDEPAGQKGFECFDGVIPLTSVCNEKDDCPHGEDEEQEC	107
Lv	--FECKSG--NNVSVEW--CDGFNDCEDESGDEE-----EEYCRDQANEVSKICPRISC	135
Sg	--FECKSG--NNVSLEW--CDGFNDCEDESGDEE-----EEYCRNQANEVSKICPRISC	135
Sp	--FECKSGDNMISLEWMDGSGYDCDDGSD--HQCENHA-SVTKVCPRISC	137
Ep	--FECHDG--LAISTEWVCDVVDCCDDGSD--ARYCKEDA-VIPKECPRISC	130
Et	--FHCTTG--TIVSTDWLDCGVQDCEDGSD--LQHCASTR---KDCPRISC	131
Pm	--FTCEEGT--MVSWSWLCGHADCKDKSDEK-----EANCLEAN---ACLTALC	131
Ap	--FTCEEG--TTVSNWLCGHADCKDKSDEK-----EANCLEAN---TCRTVSC	125
Lc	--FTCENG--AALVANSWLCGHQNDCEDESDE--EDNCHKEQR---ECKTWS	131
Af	--FTCEDG--QGLVADAWLCGQSDCQDNDSEK-----QENCIKPK-----CDSSK	121
Aa	--FTCEDG--QGLVADAWLCGQSDCQDNDSEK-----QENCIKPK-----CESSK	121
Po	--FTCDDG--KEIIAEAWLCGQSDCQDNDSEK-----QENCIKPK-----CESSK	123
Oj	--FSCDNG--VTIKLDWICDDIQCYDNTDEEGCDLSSYSFSSYSFSSYS---ECPYADC	134
Oe	-----	
Pc	--FDCDNG--ASIPLTSLCNGRVDCHEGEDEG-----ERC---QKHCPDFKC	129
Sj	--FDCDNG--ASIPLTSLCNGRVDCHEGEDEG-----ERC---RKHCPDFKC	131
Sb	AAFECDTG--ETQPASAVCDGQEDCEGGEDESR-----EQCSDRLSCAQLKC	153
Lv	DNNKRCVQEKELCDGIQDCSDGLDESDELCRAGKGCFCSD--GGLKCLEWDWVCDEIADC	194
Sg	DNNKRCVQEKELCDGIQHCSDGLDESDELCRAGKGCFCSD--GELKCLEWDWVCDEIADC	194
Sp	DNGTRCVQGEICDGTQHCSGLDESDELCSAGNVKFCSD--GGSKCLKWNWVCDEFADC	196
Ep	DNSTRICAESELCDGTTQCRDGFDESEELCSGEGGLCFSCD--GDTKCLEWDWVCDEIADC	189
Et	DNSTYICQDDICDGHQHCDFDESDELCLGGGGLCFSCD--EDSKCLQWDWVCDEIADC	190
Pm	DN--KCLKESEICDGVQCVGDADELLDNCKNI--RSCFSCR--SGEACLKWSWVCNADIAC	187
Ap	DN--KCLKESEICDGVQCVGDADELLDNCKNI--RSCFRCR--SGEACLKWSWVCNADIAC	181
Lc	DAGSKCLPEDDICDGTKHCEDESDSDYQDQ--RSCYCRNFNEGCISSWSWVCN--ADC	189
Af	DNGTVCLDDSQVCDGVKDCQDQDDEQEDCDNR--ATCFRCD--AGKRCIKSSWVCNLDAC	179
Aa	DNGTVCLTDSQVCDGVEDCLDGSDEQEDCDNR--ASCFRCD--ANKRCIKSSWVCNLDAC	179
Po	DNGVVCLTDSQVCDGVLDQDGTDEAPDHCSRNSACFCQD--SGEKCIPTPSWKCDNIADC	182
Oj	DDGTICLHEYDICIADICIDQDE---AESLCTHCWACD--GGTQCIDLDFVDCSIADC	189
Oe	----TCVEDNQICNGVQDCHQDTEHPAVCKHD---CFCLD--DITLCVEN--WRCDGVPCD	51
Pc	IDSSSCFSRDELCDGVSDCNDTDEAGCTAHT--TECFSCD--LGERCISFSLWLCDEIVDC	187
Sj	IDSSSCFSRDELCDGVSDCNDTDEAGCTAHT--TECFSCD--LGERCISFSLWLCDEIVDC	189
Sb	EDGSQCISRADFCNGVQDCDDGSDDEAYCSKRRG--PECFTCD--GGNVCVRYKWLCEDEF--DC	210
Lv	SD-ADELSGWCCTVFQRCWKGSYLCGHTHFCVPQRWRCDSDHDCGDDTDEEDCQT---D	249
Sg	SD-ADELSGWCCTVFQRCWKGSYLCGHTHFCVPQRWRCDSDHDCGDDTDEEDCQT---E	249
Sp	SDMADEFSGWCCTVFQRCWKGYLCGHTHFCVLQRWRCDSDHDCGDDTDEEDCET---D	252
Ep	SD-SDELS--DCGSVYQRCWKGSFLCGHTHYCVQRWRCDHQQDCGDDTDESFCDDENDNE	244
Et	RD-TDELS--ECGTVFQRCWRGSFLCGHHAHYCVQRWRCDHQQDCGDDTDEVSCDR---P	244
Pm	NDWSDETADICGTVEDRCWKGAFFPCHHQDFCVPGWRCDKDNDCGDSDETNCNV---K	243
Ap	NDWSDETADICGTVEDRCWKGAFFPCHHQDFCVPGWRCDKDNDCGDSDETNCNV---K	237
Lc	SDWSDEKATYCGTILERCWSGAF--CHHKDFCVPGWRCDGDDQDCGDSDEQCGV---E	244
Af	EDFSDEPETCGYDVNDRCTWGAFLCGHYFCVPQWRCDDEEDCDGNSDEVECDV---E	235
Aa	EDFSDEPETCGYDVNDRCTWGAFLCGHYFCVPQWRCDDEEDCDGNSDEVECDV---E	235
Po	DDISDEPDACGNDINKRCWTGGFLCDHGYFCVQWRCDGEGDCGDSDEVECKV---E	238
Oj	DDT--DENNDGCPPEVEDRCWNGAFLCHHSYFCIPRSW--CDNENDCGDNSEEIKC-----	240
Oe	QDLSDENKICGPVADRCWEGAFPCKHNYCVLQEWRCDDGDDCGDLSDELNCER---D	107
Pc	EDLADE--HPVCPVQDRCWGAFLCGHRLFCIRQYRCRGVNDGDSDEITCE---T	241
Sj	EDLSDE--HALCPPVQDRCWGAFLCDHRLFCIRQYRCRGVNDGDSDEIACD---T	243
Sb	QQQSDSHVLCPSVQRCRWGAFCRAHRLCYLRSYRCRGVNDGDSDEHDCDN---T	266

Fig. 3. Alignment of CGSP1 protein sequences found in the following Echinoderms: feather star [*O. japonicus* (Oj)], brittle star [*O. echinata* (Oe)], sea stars [*A. amerensius* (Aa), *A. forbesi* (Af), *A. pectinifera* (Ap), *L. clathrata* (Lc), *P. miniata* (Pm)], sea cucumbers [*P. californicus* (Pc), *P. ochraceus* (Po), *S. briareus* (Sb), *S. japonicus* (Sj)], sand dollar [*E. parma* (Ep)], sea urchins [*E. tribuloides* (Et), *L. variegatus* (Lv), *S. granularis* (Sg), *S. purpuratus* (Sp)]. The thrombospondin type 1 repeat is conserved among the species and underlined in blue. The protease domain identified from the *Sp* sea urchin sequence is underlined in red. The catalytic domain, highly conserved among serine proteases, is formed by three amino acids which are marked with a purple asterisk. The potential cleavage site of the protease domain from the LDL receptor like domain is represented by the conserved arginine marked with a green asterisk.

Lv	IAWTGSFGWSSWGDWSECHPSCGPGSRSRSRICESPA--ERCLGESQDEDEC-----	299
Sg	IAWTGSFGWSSWGDWSECHPSCGPGSRSRSRICESPA--ERCLGESQDEDEC-----	299
Sp	FAWTGSYGWSSWGDWSECHPSCGLGTRSRRFCASPG--GRCLGESQEEEC-----	302
Ep	HLWTGTFEWSEWSECHPACGPGTRSRVCSNPV--PICEGISTEEAC-----	297
Et	VEWSTGFHWQSWGSECDVTCGAGTRIRTRVCSVSG--GVCPGHEREAES--	294
Pm	SAWDPVYGSWSEWSEACNTDCGPGQQRQYRLCHELS--IACAGKEVQLESC-----	293
Ap	SAWDPVYGSWSEWSEACNTDCGPGQQRQYRLCHELS--IACAGKEVQLESC-----	287
Lc	SSWEPVYGWQWSAWSECSSTDCGP-----RDENEDNC-----	276
Af	SIWEPAYGWSSWSECSSTFCGPG--RTRYRTCHQLS--STCSGKEIASIEEC-----	284
Aa	SIWEPAYGWSSWSECSSTFCGPG--RTRYRTCHQLS--STCSGKEIASIEEC-----	284
Po	SSWQPVYGWGTWSEWSECSADCGPGERTFRSCHQLS--FTCAGKEIATEEC-----	288
Oj	---PDNDWSNWSEWSEICDADCGSGKKTRTRVCENPN--GVCKASSNEDKPC-----	286
Oe	ISWYVRQGTWSEWSECSNCGRGRSRTCLTSNPFKCSGKDRETEKCTEYGGCSS	167
Pc	VYWADSGGWSRWSPWSECSSTSCGPGTKIRRVCFPVV--QCSGPNDELLPC-----	290
Sj	VYWADSGGWSRWSPWSECSSTSCGPGTKIRRVCFPVV--QCSGPNDELLPC-----	292
Sb	QFWADQGGWNRSSWSECSSEPCGPGTRRRRCYQVQV--QCEGNDTETAC-----	315
*		
Lv	-----QQAECV-----EEKVIG-----CGIKQHIHFRDDGLALAEIVGGQPTDSAGDWP	344
Sg	-----QQAECV-----EDKVG-----CGIKQHIHLRDDGLALAEIVGGQPTVSG-DWP	343
Sp	-----EQVPCV-----DENVIA-----CGIKSHIHRDDGLALAEIVGGQPATAG-DWP	346
Ep	-----ETGLCFP-----HDIELG-----CGVKQHLHVRDDGLALAEIVGGQTSDEG-DWP	342
Et	-----EIVPCV-----EVEIG-----CGLQHLLLRDDGLALTEIVGGQASVSG-SWP	338
Pm	-----QQKECV-----PEKDVG-----CGTRQLPTWQP-----LFVKIVGGVEAVAG-SWP	334
Ap	-----QQKECV-----PEKDVG-----CGTRQLPTWQP-----LFEKIVGGVEAVAG-SWP	328
Lc	-----QLEECV-----VEKAAG-----CGTRKPQEGN-----IVKIVIGGKEADRG-GWP	323
Af	-----QLEECV-----VEKAAG-----CGTRKPQEGN-----IVKIVIGGKEADRG-GWP	323
Aa	-----QLKECV-----PEKARG-----CGTRNPQGSN-----FVRIVIGGKEAERG-SWP	327
Po	-----EKLNCV-----PEAKHG-----CGTRNTIPTQ-----KIVGGETATRG-SWP	323
Oj	GGKDESTEKCTE-----NGVCP-----PGFGLNCGSRHSDQLLHTIVGGQKAVHG-DWP	217
Oe	-----EITPCVNGNEPEKDS-----LGGCIRHIEGHTASP-----TTRIVIGGEQSVSG-SWP	337
Sj	-----EITSCD-----EEPEKDS-----LGGCIRHIEGHTASP-----TTRIVIGGEQSVSG-SWP	337
Sb	-----LVERCVNNTHYVSESGDILRVGCGVRHISGHEPS-----TARIVIGGVTSVIG-SWP	365
*		
Lv	WQAQLFYQ--TRGAWRPVCGGTLIDPQVVLTAACFCFSIEDLKSSDDARAPSRWQVHLGKH	403
Sg	WQAQLFYQ--TRGWRPVCVCGGTLIDPQVVLTAACFCF-----PATSRWQVHLGKH	391
Sp	WQAQLFYR--TRGSWQLVCGGTLIDPQVVLTAACFCFM-----PMMATSRWQVHLGKH	397
Ep	WQAQLYYR--QRGTWT--VCGGTLISPN-VLTAACFCF-----ANDASRWQVHFGKH	389
Et	WQAQLFYRFKKNTWAKVCGGTLINKD-VLTAACFCF-----SRTSRWQVHLGKH	387
Pm	WQAQLFHRFKSGRQVAICGGTLVRDNLVVTAAHCFCLKTMN-----DANSWKVHLGKH	386
Ap	WQAQLFHRFKNGRQVAICGGTLVRDNLVVTAAHCFCLKTN-----QANSWKVHLGKH	379
Lc	-----WQAQLIYKYSSSQYGAFCGGTLVHNNIVLTAACFCGTENE-----L-----WEVHLGKH	372
Af	-----WQAQLIYKYSSSHYGAVCGGTLVHNNIVLTAACFCGTENK-----L-----WEVHLGKH	372
Aa	-----WQAQLIYYSQGRSGAVCGGTLVHGNIVLTAACFCFKEIE-----TS-----WEVHLGKH	377
Po	-----WQAQLIWTYPSGARQAVCGGTLIQNRFILTAACFCFIDILD-----PPK-WKVHLGKH	374
Oj	-----WQAQLIWL--EKGHKYVSCGGTLIHPKIVVGAACFHAHN-----DRTRWLVLHLGKH	266
Oe	-----WQVQLWFKVAARRRELVCGGTLVSRIVITAAHCFHHKKS-----NRKGLWRVQLGKH	390
Pc	-----WQVQLWFKVAARRRELVCGGTLVSRIVITAAHCFHHKNS-----NRKGLWRVQLGKH	390
Sj	-----WQAQLWPKYKRNHILACGGTLISERIVVTAAHCFFLHYKG-----KDASRWTVDLGKE	418
Sb	-----	
*		
Lv	SIDF--VPETGSQHLRVREIIVHKKFNQNGGVGGDIALLLLD-EPVHQETGQINWACLDEG	461
Sg	SIDF--VPETGSQHLRVREIIVHKKFNQNGGVGGDIALLILD-EPVQETGQINWACLDEG	449
Sp	SVDF--VPEAGSQHLRVREIIVHKKFGEHGGVGGDIALLILD-EPVQETGQINWACLDEG	455
Ep	SKHP--SPEPGSQHLRVLEIVRHGEFDEHNLANDIAIVLVD-GTVSQESGQINWACLDEG	447
Et	NLNF--VPEEDESRTIHEIV-HPGFDGHHGVEDDLAILILD-APVRQEAQRINWACLDDG	444
Pm	TIDL--HLGSGEQEAQIKHIIKHKEFNKTMQNDLAVLVLD-RSLSLDGKQVNVACLDDK	443
Ap	TIDL--QLGSGEQEAQIKHIIKHKEFNKTMQNDLAVLVLD-RSLSLDGKQVNVACLDDK	435
Lc	-----	
Af	TIN--DLVNHEKRANIKRIIKHSGYDPRTRNDVAILILD-KPIELDGQHINTACLDR	428
Aa	TID--DLVDHEKRANIKRIIKHSGYDPRTRNDVAILILD-KPIELDGQHINTACLDR	428
Po	TISL--DLADHEKRANIKRIIKHSDYDPKTLKNDAILVLD-KSIP-DGQHINTACLDR	433
Oj	TIAS-IDKNIGEDDFDVNIIVHP--EFSTRNDIAVLVID-PAVQVQDN-INWACLDRQ	429
Oe	GINP--NLADNEVERRITHIEIHPEFSTTLTKNDIVILVLNSPAVESPTTHIRWACLDRG	324
Pc	KYKKTVDPTTGEIQSKITDIIYTESLDQFSVWHDIAILVLK-TPITQFTETVSPAC----	445
Sj	KYRKTVDPTTGEIQSKITDIIYTESLDQFSVWHDIAILVLK-TPITAFTERVSPACVDNR	449
Sb	RSSRSLYTSQGHRSRLKIYHYDTFTAFDLWQDAI-VLR-NPVTSTTKISPAIDNR	476

Fig. 3 (continued)

ancestor of extant members of this phylum is ~540 million years old [13], the extensive conservation of the CGSP1 is pronounced, particularly when considering that this enzyme is involved in a reproductive process that specializes in diversifying its genes [14].

Recently other members of the fertilization envelope in sea stars were identified [15]. This included SFE9, shown to be within the cortical granules prior to fertilization, then in the FE following fertilization. Although the sequence of SFE9 between sea urchins and sea stars has differences, they are extensively similar. Both have tandemly repeated LDLr-like motifs that are postulated to be involved in protein–protein recognition important for the rapid construction of the fertilization envelope upon release of the monomers from the cortical granules. Importantly, the CGSP1 of sea stars and other echinoderms also has LDLr repeats, suggesting

the functional significance perhaps for binding and targeting the proteinase for cleavage of its target proteins.

Other orthologs of the sea urchin cortical granules were seen throughout the echinoderms, including proteolisin (LDLr repeats), rendezvin (CUB repeats), and ovoperoxidase (cross-linking enzyme), but not one of the targets of the CGSP1, p160 (CUB domains). The protein p160 was originally identified by following its release at fertilization from the cell surface – eggs were biotinylated prior to fertilization [16]. Following egg activation, the released biotinylated cell surface proteins were collected and analyzed by mass spectrometry. Antibodies to the subsequently cloned p160 (so named by its molecular mobility in SDS–PAGE gels) blocked cleavage of the protein, and blocked raising of the fertilization envelope, mirroring the phenotype of a protease null egg.

Lv	-KL--NAKTECYISGWGVT--GGKSPQVLHEA-KPLIPRRICNYKKSNGKIERT-LCA	514
Sg	IKL--NSKTECYISGWGVT--GGKAPHVLHEA-KPLISRRVCNYKKSNGKIEKT-LCA	503
Sp	MPL--NDRTECYISGWGVT--GGNGPDVLHEARMPLIPRRICNYKKSNGKIEKTMLCA	512
Ep	IEL--QSDIECYISGWGVTQK-GKQADYLRQA-RPLIPRSQCNRPSSYNGLIQNT-LCA	502
Et	-RL--SQLTNCYITGWGVT--GNQGSNVLHEA-HPIISRAECNAPKAYDG-IKRT-LCA	497
Pm	LDM--GDKTHCFVTGWGLTE--GGRQARKLQEAAPVPIILRSQCNHKKDVYKGMVRDTMMCA	500
Ap	LD--GDKTHCFVTGWGLTE--GGRQARKLQEAAPVPIILRSQCNHKKDVYKGMVRDT--CA	487
Lc	-----	-----
Af	LD---INKIPCFVTGWGLTE--GGTQAPKLQQAQVPIIHQKICSHQDVYS---HTDGICA	480
Aa	LD---VNKIPCFVTGWGLTE--GGTQAPNLQQAQVPIIHQKICSHKDVYS---HTDGICA	480
Po	LD---INKIPCFVTGWGLTE--GGNQAYTLQQAQVPIIHP-VCNHRAVYSN-VVTDGFCA	486
Oj	FVL--TETSYCFVTGWGVT--NGFQAVELQQAQVVPFIPYYICRSPHYVGN-VITENICA	484
Oe	IN---IIEDDCFISGWGITE--GGSQAHYLQEAAPTILKSAVCNRRGDIYGGGLVEDS-LCA	378
Pc	-----	-----
Sj	NVNVEDENLLCYATGWGITQIVSGRRAQWLQEARLPIINHQTCHENVYENSIEDD-FCA	508
Sb	TVDVSDENVLCYATGFGVTQILTQGRASHLQEARLPIIDHVCNRAEVYSNAVTED-FCA	535
<hr/>		
Lv	GH-EGGIDACQGDGGGPLSCLG-PDDQWYVVGVTSWGHCALANKPGVYTRVSSYLEWIH	572
Sg	GHLEGGIDACQGDGGGPLSCLG-PDDHWYVVGVTSWGHCALANKPGVYTKVSSYLDWIH	562
Sp	GHLEGGIDACQGDGGGPLSCLG-PDDHWYVVGVTSWGHCALANKPGVYTKVSSYLDWIH	571
Ep	GH-EGGIDACQGDGGGPLSCVG-PDGHWVVGVTSWGHCALANKPGVYTRVSRIDWIH	560
Et	GH-SGGIDACQGDGGGPLSCVG-SDGRWVVGVTSWGHCALANKPGVYTRVSKYVDWIH	555
Pm	GYLAGGIDACQGDGGGPLICEL-SDGRWHLVGVTSWGRCAMANKPGVYTKVAHFAQWIE	559
Ap	GYLAGGIDACQGDGGGPLICEL-SDGRWHLVGVTSWGRCAMANKPGVYTKVAHFAQWIE	545
Lc	-----	-----
Af	GYLHGQVDACQGDGGGPLVCEL-DDGRWHVVGITSWGRGCALPLNPGVYTKVAYYQQWID	539
Aa	GYLHGQVDACQGDGGGPLVCEL-EDGRWHVVGITSWGRGCALPLNPGVYTKVAYYQQWID	539
Po	GYLTGQIDACQGDGGGPLICE--EDGRWHVVGITSWGRGCALPHKPGVYTKVAYYQWID	544
Oj	GHLSGGIDACQGDGGGPLQCEG-EDGHWYLVGVVSWGRGCARENKPGVYTKVSAYADWIL	543
Oe	GY-EGGIDACQGDGGGPLSCKNPEDGLWYLVGITSWGRGCALPKKPGIYTRVT-FYDWIL	436
Pc	-----	-----
Sj	GFLEGRVDACKGDGGGPVCLNTAEDRWFLVGITSWGRGCALPYKPGVYTNVSYYSQWID	568
Sb	GFLEGEIDACKGDGGGPYICHQPVEDRWYLVGITSWGRGCALENKPGVYTNVSYQVFNWID	595
<hr/>		
Lv	---ELHHH-LHH--- 580	
Sg	---ELHHH-LHH--- 570	
Sp	---EMIHHYLHHE--- 581	
Ep	RVIEEHHHIHHVK--- 573	
Et	EVIEQHT----- 562	
Pm	DHIKKHGL----- 567	
Ap	DHIKKHGL----- 553	
Lc	-----	
Af	NLIFEHGVQPDN--- 551	
Aa	NLIFEHGVQPDN--- 551	
Po	NLIQEHGSQ----- 553	
Oj	SITEVINA----- 551	
Oe	QTVDKYL----- 443	
Pc	-----	
Sj	YVIQNYQLP----- 577	
Sb	NIVNVAVQLLSREEN 611	

Fig. 3 (continued)

Thus, from its position within the cell, its CUB tandem repeats, and its sensitivity to the cortical granule serine protease, the p160 protein is thought to be a tethering protein linking the vitelline layer to the egg cell surface. An alternative (or additional) model for its function is that it binds sperm directly through its CUB motifs. The lack of p160 in other echinoderms may indicate a recent invention for fertilization in sea urchins, or a rapidly diversifying cell surface receptor for sperm on the egg. The proteins targeted by the CGSP1 ortholog in sea stars may be important in identifying those proteins exposed to sperm, and deemed important for removal promptly following fertilization.

Acknowledgments

We thank members of PRIMO for stimulating discussions and the National Institutes of Health (HD028152), and the National Science Foundation (IOS-1120972). We are particularly grateful to Professor William J. Lennarz for the wonderful training opportunities he provided to members of his laboratory and for being such a special person.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2014.05.098>.

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