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

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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.

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 μM 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 μg/ml; aspartyl protease inhibitor), and E-64 (10 μg/ml; cysteine protease inhibitor).

The calcium ionophore A23187 (10 μg/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 phyloheny.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 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