Journal of Cellular Biochemistry 93:1075–1083 (2004)

ARTICLES

Zymogen Activation and Characterization of a Major Gelatin-Cleavage Activity Localized to the Sea Urchin Extraembryonic Matrix

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Abstract The hyaline layer (HL) is an apically located extracellular matrix (ECM) which surrounds the sea urchin embryo from the time of fertilization until metamorphosis occurs. While gelatin-cleavage activities were absent from freshly prepared hyaline layers, a dynamic pattern of activities developed in layers incubated at 15 or 37°C in Millipore-filtered sea water (MFSW). Cleavage activities at 90, 55, 41, and 32 kDa were evident following incubation at either temperature. The activation pathway leading to the appearance of these species was examined to determine the minimum salt conditions required for processing and to establish precursor–product relationships. In both qualitative and quantitative assays, the purified 55 kDa gelatinase activity was inhibited by 1,10-phenanthroline (a zinc-specific chelator) and ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA). Calcium reconstituted the activity of the EGTA-inhibited enzyme with an apparent dissociation constant (calcium) of 1.2 mM. Developmental substrate gel analysis was performed using various stage embryos. The 55 and 32 kDa species comigrated with gelatin-cleavage activities present in sea urchin embryos. Collectively, the results reported here document a zymogen activation pathway which generates a 55 kDa, gelatin-cleaving activity within the extraembryonic HL. This species displayed characteristics of the matrix metalloproteinase class of ECM modifying enzymes. J. Cell. Biochem. 93: 1075–1083, 2004.

Key words: sea urchin; embryo; matrix metalloproteinase

The extracellular matrix (ECM) is increasingly recognized as a complex, dynamic structure which plays an important role in regulating the shape, migration, proliferation, and differentiation of cells through both direct and indirect interactions with the cell surface [Adams and Watt, 1993]. The functional roles played by the ECM depend on its composition, which is tightly regulated at several levels including the synthesis, modification, and degradation of constituent components. Proteinase activities have been recognized as important elements in controlling the composition of the ECM. Both matrix metalloproteinases (MMPs) and serine proteinases utilize cell surface proteins and components of the ECM as substrates [Werb, 1997; Werb and Yan, 1998; Egeblad and Werb, 2002]. The MMPs are a family of Zn^{2+}- and Ca^{2+}-dependent proteinases, which are secreted in zymogen form and activated in the ECM through proteolytic cleavage. MMPs are broadly classified into five subclasses based on their substrate specificities and primary structure: (i) gelatinases, (ii) collagenases, (iii) elastase, (iv) stromelysins, and (v) membrane-type MMPs. Controlled remodeling of the ECM is important for a number of physiological processes such as embryonic development, angiogenesis, wound healing, and immune responses. Increased levels of MMP activity in the ECM have been

Abbreviations used: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; DTT, dithiothreitol; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis (oxyethylenenitrilo) tetraacetic acid; MFSW, Millipore-filtered sea water; MMP, matrix metalloproteinase; HPF, hours post fertilization.

Grant sponsor: Natural Sciences and Research Council of Canada (to J.J.R.).

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Received 7 May 2004; Accepted 9 July 2004

DOI 10.1002/jcb.20268

associated with pathological conditions including metastatic cancer, arthritis, cirrhosis, nephritis, and cardiovascular disease.

The sea urchin embryo has been used extensively as a model system for developmental studies. This embryo is characterized by the presence of two extracellular matrices: the hyaline layer (HL) located on the apical surface and the basal lamina present on the basal surface of ectoderm cells. The vertebrate-like nature of the ECM components in the HL and basal lamina has been established [Eyre and Glimcher, 1971; Solursh and Katow, 1982; Wessel et al., 1984; Spiegel et al., 1989]. In addition, the HL and basal lamina are in intimate contact with different cell types in the embryo and are essential for development [Wessel et al., 1989; Ramachandran et al., 1993]. Clearly, the sea urchin embryo represents a useful model system for studying the regulation of ECM composition. To this end, we have begun to identify proteinase activities likely to utilize components of the sea urchin ECMs as substrates. We have previously described a 41 kDa collagenase/gelatinase, a serine-dependant class of proteinase, from the sea urchin egg [Robinson, 2000; Mayne and Robinson, 2002] as well as two higher molecular mass gelatin-cleavage activities of 90 and 94–117 kDa present in hyaline layers [Sharpe and Robinson, 2001]. In this report, we describe the characterization of a 55 kDa gelatinase activity and probe the proteolytic processing pathway leading to its activation.

MATERIALS AND METHODS

Growth of Embryos

Strongylocentrotus purpuratus were purchased from Seacology, Vancouver, Canada, and gametes were obtained by intracoelomic injection of 0.5 M KCl. Eggs were washed three times in ice-cold Millipore-filtered seawater (MFSW; 0.45 μm) and fertilized with a 100-fold numerical excessive of sperm. Embryos were cultured with constant aeration, at 12°C, in cylindrical chambers containing paddles rotating at 40 rpm. Samples were harvested at the times indicated after fertilization (HPF).

Isolation of Hyaline Layers

To facilitate the isolation of hyaline layers, unfertilized eggs were dejellied by titration with HCl to pH 5.5 and left on ice for 2 min. The pH was then returned to 8.0 with the addition of 1 M Tris-HCl (pH 8.0). After extensive washing in ice-cold MFSW , the eggs were resuspended in MFSW to give a final concentration of 10% (v/v) and their vitelline layer was disrupted with 10 mM dithiotheritol [Epel et al., 1970]. After extensive washing in ice-cold MFSW, the eggs were fertilized with a 100-fold numerical excess of sperm. Hyaline layers were isolated as follows. One-hour-old embryos were washed several times in ice-cold MFSW and resuspended to give a final concentration of 10% (v/v) in MFSW containing 10 mM benzamidine hydrochloride as a proteinase inhibitor. The resuspended embryos were homogenized with 100 strokes in a hand-held Dounce homogenizer (type A) at 0°C. The homogenate was passed by gravity filtration through a 28 μm-pore-size Nitex mesh (B, D, and H Thompson and Co., Mount Royal, Quebec), the retarded layers were resuspended in ice-cold MFSW and the filtration step repeated twice more. Each preparation was examined by phase-contrast microscopy and showed no evidence of contaminating cytoplasmic debris. The isolated hyaline layers were stored as pellets at −70°C.

Induction of Gelatin-Cleavage Activities in Hyaline Layers

Aliquots of isolated hyaline layers were resuspended in MFSW and incubated at 15 or 37°C. Following incubation, the layers were harvested by centrifugation at the times indicated.

Dissociation of Gelatin-Cleavage Activities From Hyaline Layers

Isolated hyaline layers, containing gelatin-cleavage activities, were incubated in 50 mM Tris-HCl, pH 8.0, containing 5 mM ethylenediaminetetraacetic acid (EDTA) for 24 h at 37°C. Following incubation, the suspension was separated into pellet and supernatant fractions. The supernatant contained the displaced gelatin-cleavage activities.

Substrate Gel Zymography

Substrate gel zymography was performed essentially as described previously [Huessen and Dowdle, 1980]. Gels containing sodium dodecyl sulfate were prepared by copolymerizing acrylamide and gelatin or a test substrate at a final concentration of 0.1% (w/v). Samples of embryos or the gelatinase(s) were dispersed for 30 min at room temperature in Laemmli

solubilizing solution from which both dithiothreitol (DTT) and bromophenol blue had been omitted [Laemmli, 1970]. Electrophoresis was performed at a constant current of 10 mA at 4°C for 4 h. After electrophoresis the gels were incubated for 60 min at room temperature in 50 mM Tris-HCl, pH 8.0, containing 2.5% (v/v) Triton X-100 followed by 16 h incubation at room temperature in 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$. The gels were stained with Coomassie Brilliant Blue R-250 using a standard protocol.

Gel Exclusion Chromatography

The supernatant containing the displaced gelatin-cleavage activities was applied to a 1 × 28 cm column of the gel exclusion resin, Agarose 1.5 M (BioRad, Richmond, CA). Chromatography was performed at a flow rate of 1.8 ml/h at 4°C and 0.3 ml fractions were collected.

Quantitative Gelatinase Assay

The quantitative assay was performed using fluorescein-conjugated gelatin (Molecular Probes, Inc., Eugene, Oregon). Cleavage activity was monitored by following the release of fluorescent peptides from the substrate. Reactions contained 10 μg of fluorescein-labelled gelatin and various aliquots of the purified gelatinase. The fluorescence was measured using a fluorescence microplate reader set to an absorption maximum of 490 nm and emission maximum of 520 nm. The rate of change in relative fluorescence unit (RFU/s) was determined using Softmax Pro software (Molecular Devices, Sunnyvale, California). The increase in fluorescence was monitored at 2 min intervals for a total of 60 min at 37°C.

RESULTS

Using gelatin substrate gel zymography, a dynamic pattern of cleavage activities was identified in both the sea urchin egg and embryo [Mayne and Robinson, 1996]. However, no gelatin-cleavage activities were found in layers freshly prepared from 1-h-old-embryos [Flood et al., 2000]. In contrast, several gelatin-cleavage activities appeared when layers were incubated in MFSW at 15°C, the ambient temperature for *Strongylocentrotus purpuratus* (Fig. 1A). A 32 kDa species was the major activity detected after 24 h of incubation (lane 1), while a 41 kDa species was detected after 36 h of incubation (lane 2). A gelatin-cleavage activity at approximately 90 kDa was also detected after 24 h. After 96 h of incubation, one of the major activities detected was a species at 55 kDa (lane 7). When the incubation temperature was increased to 37°C, the 55 kDa activity was the major species detected (Fig. 1B). In addition, an approximately 90 kDa activity as well as a species greater than 200 kDa were observed. Comparative analysis of the activities seen at 15 and 37°C clearly showed that the induced patterns were similar. When incubations were performed in the presence of 10 mM benzamidine hydrochloride, no gelatin-cleavage activities were seen (data not shown).

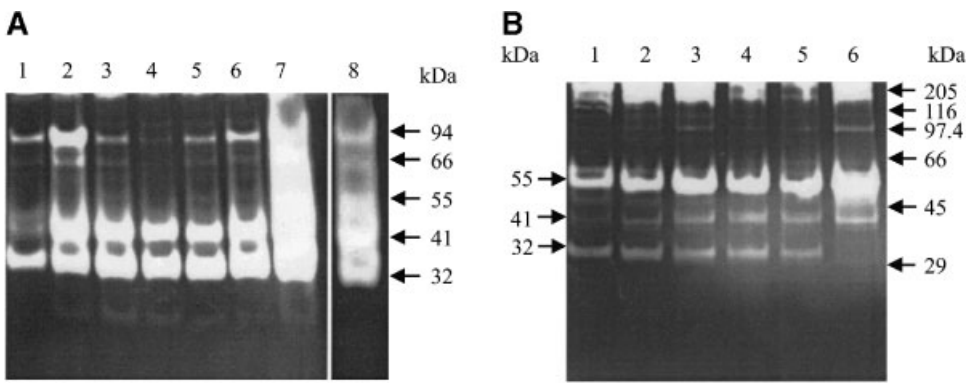

Fig. 1. Induction of the gelatin-cleavage activities in isolated layers following incubation in Millipore-filtered sea water (MFSW) at 15°C (**A**) or 37°C (**B**). Aliquots (15 μg) of isolated layers were incubated in MFSW for 24 h (**lane 1**), 36 h (**lane 2**), 48 h (**lane 3**), 60 h (**lane 4**), 72 h (**lane 5**), 84 h (**lane 6**), or 96 h (**lane 7**, **panel A**) or 24 h (**lane1**), 48 h (**lane 2**), 72 h (**lane 3**), 96 h (**lane 4**), 120 h (**lane 5**) or 149 h (**lane 6**, **panel B**) and fractionated in a 10% (w/v) polyacrylamide gel [Laemmli, 1970] containing 0.1% (w/v) gelatin. The gels were stained with Coomassie Brilliant Blue R-250 using the standard protocol. **Lane 8**, panel A, is a lower exposure of lane 7. Similar results were obtained when inductions were performed in Tris, pH 8.0-buffered MFSW (data not shown).

We examined the requirement for an intact HL. Newly prepared layers were resuspended in a solution of 0.5 M NaCl–5 mM EDTA and the supernatant and pellet fractions isolated. An aliquot of the supernatant was dialyzed against MFSW and aliquots of both dialyzed and non-dialyzed supernatant were incubated at 15°C for 72 h. When the dialyzed supernatant was fractionated in a gelatin substrate gel, three cleavage activities were seen at 90, 55, and 32 kDa (Fig. 2, upper panel). A small amount of cleavage activity was also seen at 41 kDa. The aliquot incubated at 15°C without prior dialysis against MFSW, was devoid of gelatin-cleavage activity (data not shown). This result suggested that the components of the processing pathway were dissociated from layers in the presence of high salt and EDTA and that induction of the cleavage-activities did not require an intact matrix. We further probed the induction process by placing a strip of gelatin substrate gel, containing the induced activities, in a solution of 50 mM Tris-HCl, pH 8.0–2% (w/v) SDS for 20 min at room temperature prior to loading onto a second dimension substrate gel. Following electrophoresis, several spots were visible, two of which migrated off the diagonal (Fig. 2). The anomalously migrating spots corresponded to species of 55 and 32 kDa and were derived from the 90 and 55 kDa species, respectively, seen in the first dimension gel strip. These results define precursor–product relationships between the induced activities and suggest that autocatalytic cleavage is at least partially responsible for producing the pattern of gelatin-cleavage activities.

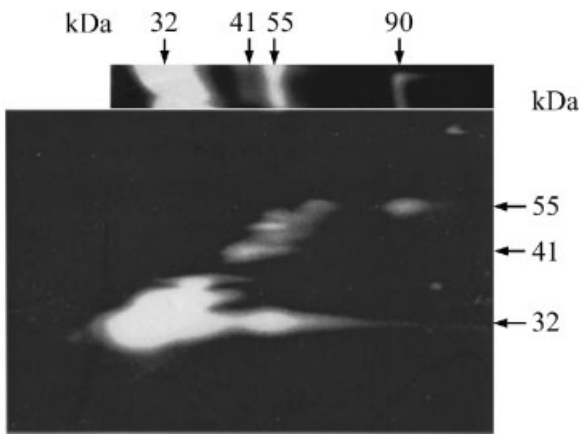

Fig. 2. An aliquot of freshly prepared hyaline layers was resuspended in a solution containing 0.5 M NaCl–5 mM ethylenediaminetetraacetic acid (EDTA), left on ice for 30 min and the pellet and supernatant fractions separated. The supernatant fraction was dialysed overnight at 4°C against MFSW and aliquots fractionated in a gelatin substrate gel. A strip of stained gel is shown in the **upper panel**. A second strip of gel was incubated for 20 min at room temperature in 50 mM Tris-HCl, pH 8.0–2% (w/v) SDS. Following incubation, the strip was placed on top of a second dimension gelatin substrate gel, fractionated by electrophoresis and the gel stained (**lower panel**).

We next investigated the minimum salt conditions required for the induction of the major gelatin-cleavage activities. Since sea water contains high concentrations of $CaCl_2$, $MgCl_2$, and NaCl, isolated hyaline layers were incubated at 15°C for 96 h in different salt solutions (Fig. 3A). Patterns of gelatinase activities were similar when hyaline layers were incubated in 50 mM Tris-HCl, pH 8.0, containing 500 mM NaCl (lane 4) or 50 mM Tris-HCl pH 8.0, 500 mM NaCl, and 50 mM $MgCl_2$ (lane 7). Both these lanes displayed a broad band of overlapping 55 and 41 kDa activities. The 32 kDa activity was found only in the presence of both 10 mM $CaCl_2$ and 500 mM NaCl (lane 6) or MFSW (lane 8). Further investigation revealed that induction of the 55 and 41 kDa activities required a minimum of 300 mM NaCl while induction of the 32 kDa species required the presence of both 3 mM $CaCl_2$ and 300 mM NaCl (data not shown).

We have found that the induced gelatin-cleavage activities can be dissociated from hyaline layers in the presence of the divalent metal ion chelator, EDTA. The dissociated activities were fractionated in an Agarose 1.5 M gel exclusion resin and the 55 kDa species isolated. We began our characterization of the 55 kDa species by determining the effects of metal ion chelators on the gelatin-cleavage activity (Fig. 3B). Gelatin substrate gel zymographic analysis revealed substantial inhibition of the 55 kDa gelatinase in the presence of the Zn^{2+}-chelator, 1,10-phenanthroline (lane 2). However, little or no effect was observed in the presence of serine protease inhibitors (lanes 3 and 4). In quantitative gelatinase assays, the Zn^{2+}-chelator resulted in $93.1 \pm 11.9\%$ inhibition of the 55 kDa activity while only $4.1 \pm 1.4\%$ inhibition was recorded in the presence of the serine proteinase inhibitor, benzamidine. We have also examined the effects of the metal ion chelators EDTA and ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA) on the 55 kDa activity. Both chelators were effective inhibitors of the gelatin-cleavage activity. The EGTA-driven inhibition suggested a specific requirement for Ca^{2+}. This was confirmed when Ca^{2+}

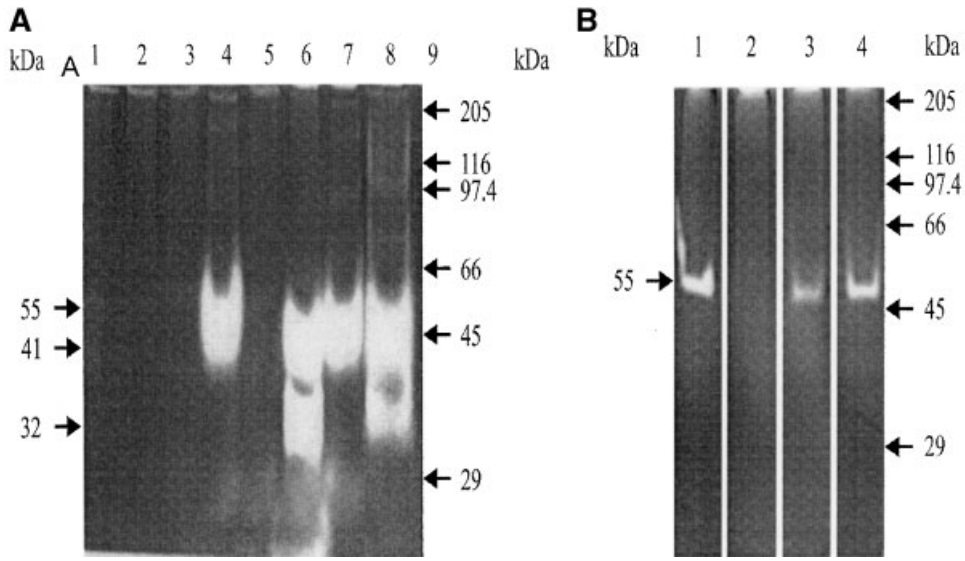

Fig. 3. **A**: Determination of the minimum ionic requirements for the induction of gelatin-cleavage activities at 15°C. Aliquots (15 μg) of isolated hyaline layers were incubated for 96 h at 15°C in 50 mM Tris-HCl, pH 8.0 (**lane 1**), 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ (**lane 2**), 50 mM Tris-HCl, pH 8.0, containing 50 mM $MgCl_2$ (**lane 3**), 50 mM Tris-HCl, pH 8.0, containing 500 mM NaCl (**lane 4**), 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ and 50 mM $MgCl_2$ (**lane 5**), 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$ and 500 mM NaCl (**lane 6**) 50 mM Tris-HCl, pH 8.0, containing 50 mM $MgCl_2$ and 500 mM NaCl (**lane 7**), or Millipore-filtered sea water (**lane 8**). Following fractionation in a gelatin substrate gel the bands of activity were visualized by staining with Coomassis Brilliant Blue R-250. **B**: The effects of various protease inhibitors on the 55 kDa gelatin-cleavage activity. Aliquots (0.5 μg) of the purified 55 kDa gelatin-cleavage activity were fractionated in a gelatin substrate gel. Following electrophoresis, the gel was dissected into strips which were processed overnight in 50 mM Tris-HCl, pH 8.0 (**lane 1**), 50 mM Tris-HCl, pH 8.0 and 2 mM 1,10-phenanthroline (**lane 2**), 50 mM Tris-HCl, pH 8.0 and 10 mM benzamidine hydrochloride (**lane 3**), 50 mM Tris-HCl, pH 8.0 and 0.6 mM [4-(2-aminoethyl) benzenesulfonylfluoride] (**lane 4**).

was shown to reconstitute gelatin-cleavage activity in the EGTA-inhibited species (Fig. 4). Reconstitution of activity occurred with an apparent Kd (Ca^{2+}) of 1.2 mM. Magnesium was ineffective at reconstituting cleavage activity in the EGTA-inhibited species (data not shown).

To rule out the possibility that the 55 kDa gelatin-cleavage activity was a nonspecific proteinase, we prepared substrate gels containing different proteins as test substrates (Fig. 5A). Equal amounts of a partially purified preparation of the 55 kDa activity were loaded in each gel. Cleavage activity was seen in the gel containing gelatin (lane 1). A substantially reduced level of cleavage activity was seen when casein was used as a test substrate (lane 2) and no cleavage activity was detected when bovine serum albumin or bovine hemoglobin were tested (lanes 3 and 4, respectively). These results are consistent with a previous finding that casein is a considerably poorer substrate for sea urchin MMP activities than gelatin [Mayne and Robinson, 1996]. These results clearly

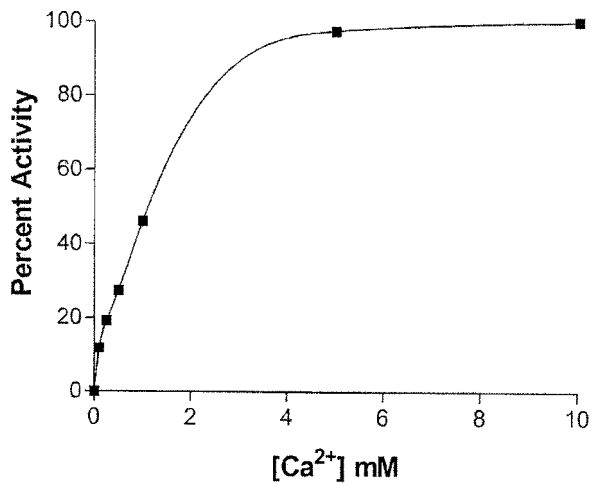

Fig. 4. Calcium concentration dependence of reactivation of the ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA)-inhibited 55 kDa gelatin-cleavage activity. An aliquot (25 μg) of the purified 55 kDa species was incubated in the presence of 5 mM EGTA. The EGTA was removed by dialysis against 50 mM Tris-HCl, pH 8.0, and aliquots assayed for gelatin-cleavage activity in the presence of various concentrations of $CaCl_2$. Curve fitting analysis was performed using Graphpad Prism, V 3.0.

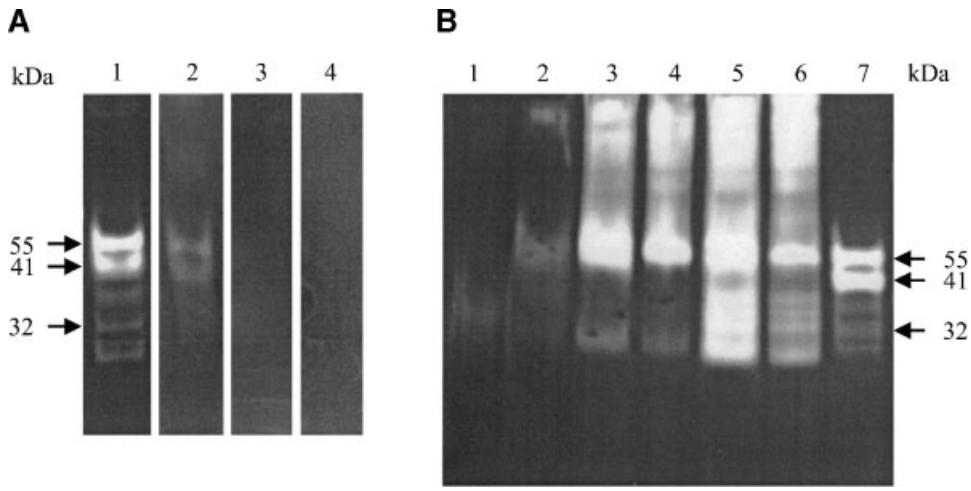

Fig. 5. **A**: Determination of the substrate specificity of the 55 kDa gelatin-cleavage activity. Aliquots of the partially purified 55 kDa species were fractionated in substrate gels containing 0.1% (w/v) gelatin (**lane 1**), 0.1% (w/v) casein (**lane 2**), 0.1% (w/v) bovine serum albumin (**lane 3**), or 0.1% (w/v) bovine hemoglobin (**lane 4**). Following electrophoresis, the gels were processed as described in ''Materials and Methods.'' **B**: Comparative substrate gel analysis of various stage embryos and the partially purified 55 kDa activity. Aliquots (15 μg) of whole embryos harvested at 22 hours post fertilization (HPF) (**lane 1**), 45 HPF (**lane 2**), 69 HPF (**lanes 3** and **4**), 93 HPF (**lanes 5** and **6**) were analyzed, in gelatin substrate gels, along side an aliquot (0.5 μg) of the partially purified 55 kDa species (**lane 7**). Mid-Blastula, early gastrula, late gastrula, and pluteus embryos were present at 22, 45, 69, and 93 HPF, respectively.

demonstrate that the 55 kDa cleavage activity was largely gelatin-specific. The physiological substrate(s) for this gelatin-cleavage activity has yet to be identified.

We have examined various stage, sea urchin embryos for the presence of a gelatin-cleavage activity of apparent mol mass 55 kDa. Embryos, between 22 and 93-h-old, were fractionated in a gelatin substrate gel alongside a mixture containing the 41 and 55 kDa species (Fig. 5B). A comigrating species was first detected in 45-h-old embryos (lane 2). Between 45 and 69 h post-fertilization the 55 kDa activity was the major gelatin-cleaving species detected and persisted until at least 93 h post-fertilization (lanes 5 and 6). This result suggests that the induced 55 kDa species is most likely an endogenous activity present in 45-h-old and later stage embryos. A 32 kDa, gelatin-cleavage activity was also seen in 93-h-old embryos (lanes 5 and 6) suggesting that the 55 and 32 kDa species are the physiological end-products of the processing pathway.

DISCUSSION

The sea urchin embryo is characterized by the presence of two extracellular matrices, the hyaline layer and basal lamina. The biogenesis, composition and function of the apically located hyaline layer has been extensively studied. The layer is assembled within 1 h following fertilization through the timed, sequential release of components stored within the egg [Alliegro and McClay, 1988; Alliegro et al., 1988]. The assembled layer is a multi-component entity which is organized into two distinct structural domains [Robinson, 1990, 1991]. Functionally, the layer is required to maintain the structural integrity of the early embryo and antibodies directed against hyalin, a major protein component of the layer, result in the arrest of development prior to gastrulation [Adelson and Humphreys, 1988]. In addition, failure to deposit cross-linked collagen into the ECM resulted in the arrest of development [Wessel and McClay, 1987]. Furthermore, micromeres have been shown to undergo a developmentally programmed loss of affinity for the layer at a time coincident with their migration into the blastocoel [McClay and Fink, 1982]. The biological importance of the interactions occurring between the apical ECM and the developing embryo, suggest a requirement for careful control of the composition of the hyaline layer.

The matrix metallo- and serine proteinase families are important elements in the control of ECM composition. Characteristic properties of MMPs include their synthesis and secretion in zymogen form followed by their activation, by proteolytic processing, at the cell surface and

a requirement for Zn^{2+} or Zn^{2+} and Ca^{2+} for activity. Using the sea urchin as a model, we and others have identified gelatin and collagen cleaving activities in this embryo [Quigley et al., 1993; Vafa and Nishioka, 1995; Mayne and Robinson, 1996]. The study reported here follows from an earlier investigation which showed that newly isolated hyaline layers were devoid of gelatin-cleavage activities [Flood et al., 2000].

We isolated hyaline layers from 1-h-old embryos and stored them at −70°C. As expected, freshly prepared hyaline layers had no associated gelatin-cleavage activities. We found that these activities appeared when layers were incubated in MFSW at either 15 or 37°C. To rule out the possibility that the cleavage activities derived from microbial contamination of sea water, we used only sea water which had been both UV-irradiated and Millipore-filtered. In addition, gelatin-cleavage activities were detected in hyaline layers following their incubation in Tris-buffered, partially reconstituted artificial sea water but not in Tris-HCl containing $CaCl_2$ and/or $MgCl_2$ (Fig. 3A). While the final patterns of gelatin-cleavage activities induced at 15 and 37°C were similar, differences were evident in the temporal sequences of appearance of the 90, 55, 41, and 32 kDa activities. This result suggests a temperature-dependent variation in the processing pathway. Interestingly, gelatin-cleavage activities failed to appear when layers were incubated in MFSW containing 10 mM benzamidine hydrochloride, suggesting that serine proteinase activity is a necessary component of the processing pathway. Studies have shown that vertebrate MMPs are secreted as inactive zymogens, which are proteolytically processed to active forms. Plasmin, a serine proteinase cleaves the N-terminal propeptide domains of proMMP-2, proMMP-9, and proMMP-12 to form active MMPs [Carmeliet et al., 1997; Mazzieri et al., 1997]. Thrombin is also a serine proteinase, which has been implicated in progelatinase A (proMMP-2) activation in vascular endothelial cells [Zucker et al., 1995]. The results reported here suggest that the sea urchin embryo generates active MMP species using a pathway similar to that found in vertebrates: a zymogen(s) form is secreted into the ECM followed by the production of an active species as a result of serine protease activity. The final composition of gelatin-cleaving activities is then determined by auto-proteolytic activity (Fig. 2). This latter step appears to be a feature of MMP activation in the sea urchin embryo which is not shared with vertebrates.

The unique ionic composition of sea water prompted us to examine the role of Na^{+}, Ca^{2+} and Mg^{2+} in the induction process. Our results demonstrated a compatibility, both quantitative and qualitative, between the salt requirements for the processing pathway and those present in the sea water environment. In the presence of 500 mM NaCl, the concentration found in sea water, sufficient processing occurred to generate the 55 and 41 kDa activities (Fig. 3A, lane 4). Interestingly, as described in the "Results" section, when a NaCl–EDTA extract of freshly prepared layers was incubated at 15°C for 72 h no induction occurred. These contrasting results suggest that one or more steps in the processing pathway leading to the 55 and 41 kDa species required the presence of endogenous Ca^{2+} which is likely removed by the 5 mM EDTA present in the NaCl–EDTA extraction medium. Calcium was clearly required to generate the full spectrum of cleavage-activities (lane 6) and Mg^{2+} was unable to substitute for Ca^{2+} (lane 7). Additional experiments will be required to delineate the Na^{+} and Ca^{2+} requirements for the serine protease-dependent and autoproteolytic components of the processing pathway.

The 55 kDa activity was purified by gel exclusion chromatography and its catalytic nature explored. The serine protease inhibitors, benzamidine hydrochloride and aminoethylbenzene sulfonyl fluoride, were largely ineffective (Fig. 3B, lanes 3 and 4, respectively) while the Zn^{2+}-specific chelator 1, 10-phenanthroline completely abolished activity (lane 2). In a series of quantitative assays, 2 mM phenanthroline resulted in $93.1 \pm 11.9\%$ ($n = 3$) inhibition while 10 mM benzamidine resulted in $4.1 \pm 1.4\%$ ($n = 3$) inhibition. These results identify a requirement for Zn^{2+} and attest to the metalloproteinase nature of the 55 kDa activity. The Ca^{2+}-specific chelator EGTA, also inhibited the 55 kDa species and the lost activity could be restored by exogenous Ca^{2+} (Fig. 4). Reconstitution of activity occurred with an apparent Kd of 1.2 mM which reflects the high concentration of this cation (10 mM) present in sea water. Collectively, these results point to a dual requirement for Zn^{2+} and Ca^{2+} to maintain the catalytic activity of the 55 kDa species. The Zn^{2+}

is most likely accommodated in a high affinity site and probably participates directly in the catalytic reaction. In contrast, Ca^{2+} clearly occupies a low affinity site(s) which more likely contributes indirectly to catalytic activity by stabilizing the structure of the enzyme.

Since the physiological substrate(s) for the 55 kDa activity has not yet been identified, we wanted to eliminate the possibility that this species was a non-specific proteinase with little substrate preference. We prepared a series of polyacrylamide gels containing different proteins as test substrates. Using a preparation which contained both the 55 and 41 kDa activities we found that cleavage activity was limited to gelatin (Fig. 5A, lane 1) with little or no activity when casein, bovine serum albumin or bovine hemoglobin (lanes 2, 3, and 4, respectively) were tested as substrates. The physiological relevance of the 55 kDa activity was examined by comparing the migration of this species with those of gelatin-cleaving activities present in various stage embryos. We have previously shown that the developing embryo displays a dynamic pattern of cleavage activities [Mayne and Robinson, 1996]. When embryos, ranging from 22- to 93-h-old, were analyzed alongside a mixture of the 55 and 41 kDa activities, a co-migrating species of apparent mol mass 55 k was seen (Fig. 5). This activity was first detected in 45-h-old embryos (lane 3) and persisted until at least 93 h post-fertilization (lane 6). The presence of the 55 kDa species in later stage embryos, grown at 15°C, is predicted from the induction data in Figure 1A which demonstrated that prolonged incubation in MFSW at 15°C was required for the appearance of this activity. However, when isolated layers were incubated at 15°C, the appearance of the 32 kDa activity preceded that of the 55 kDa species a result which contrasts with that seen in the sea urchin embryo where the 55 kDa activity appeared first (Figs. 1A and 5B, respectively). These findings probably result from small differences in the timed sequence of the processing events occurring in isolated layers and at the surface of the developing embryo. The order of appearance of these species in the developing embryo support the 55/32, precursor/product relationship suggested by the data in Figure 2. While both species coexist in the embryo, a portion of the pool of 55 kDa activity most likely serves as the source of the 32 kDa , gelatin-cleaving activity.

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