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Calcium–Protein Interactions in the Extracellular Environment: Calcium Binding, Activation, and Immunolocalization of a Collagenase/Gelatinase Activity Expressed in the Sea Urchin Embryo

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Abstract We have purified and characterized a collagenase/gelatinase activity expressed during sea urchin embryonic development. The native molecular mass was determined to be 160 kDa, while gelatin substrate gel zymography revealed an active species of 41 kDa, suggesting that the native enzyme is a tetramer of active subunits. Incubation in the presence of EGTA resulted in nearly complete loss of activity and this effect could be reversed by calcium. Calcium-induced reactivation appeared to be cooperative and occurred with an apparent kd value of 3.7 mM. Two modes of calcium binding to the 41-kDa subunit were detected; up to 80 moles of calcium bound with a kd value of 0.5 mM, while an additional 120 moles bound with a kd value of 5 mM. Amino acid analysis revealed a carboxy plus carboxyamide content of 24.3 mol/100 mol, indicating the availability of substantial numbers of weak Ca^{2+}-binding sites. Calcium binding did not result in either secondary or quaternary structural changes in the collagenase/gelatinase, suggesting that Ca^{2+} may facilitate activation through directly mediating the binding of substrate to the enzyme. The collagenase/gelatinase activity was detected in blastocoelic fluid and in the hyalin fraction dissociated from 1-h-old embryos. Immunolocalization studies revealed two storage compartments in the egg; cortical granules and small granules/vesicles dispersed throughout the cytoplasm. After fertilization, the antigen was detected in both the apical and basal extracellular matrices, the hyaline layer, and basal lamina, respectively. J. Cell. Biochem. 71:546–558, 1998.

Key words: matrix metalloproteinase; sea urchin; development

The extracellular matrix (ECM) is now recognized as a dynamic structure that regulates the properties of cells. Through both direct and indirect interactions with the cell surface, the ECM can modulate such cellular properties as shape, migration, proliferation, and differentiation [Adams and Watts, 1993]. The functional role played by the ECM is related to its constituent components and factors that regulate the composition of the ECM also modulate its function.

The importance of proteolytic events during early embryonic development is becoming increasingly evident. The activation of the decapentaplegic gene product by tolloid, a member of the astacin protease family, is required for patterning along the dorsoventral axis of *Drosophila* [Shimell et al., 1991]. Recent evidence suggests that tolloid may act by cleaving the short gastrulation (sog) gene product, thereby releasing the decapentaplegic protein from an inhibitory sog/dpp complex [Marques et al., 1997]. Genes encoding additional members of the astacin family have been cloned in *Drosophila*, *Xenopus*, *C. elegans*, sea urchin, mouse, and *Aplysia* [Finelli et al., 1995; Liu et al., 1997; Hishida et al., 1996; Sarras, 1996; Lhmond et al., 1996; Takahara et al., 1996]; the encoded proteins all appear to be developmentally impor-

Abbreviations used: A, optical density; BMP, bone morphogenetic protein; CMFSW, Ca^{2+}, Mg^{2+}-free seawater; DTT, dithiothreitol; ECM, extracellular matrix; EDTA, ethylenediamine tetraacetic acid; MFSW, Millipore-filtered seawater; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; TCA, trichloroacetic acid; TGF, transforming growth factor; W/V, wgt/volume.

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tant and may function by activating latent forms of growth factors of the transforming growth factor-β (TGF-β) superfamily. In *Xenopus* the mesodermal ventralizing agent, bone morphogenetic protein-4 may be inhibited through complex formation with chordin. Xolloid, the *Xenopus* homologue of tolloid, may cleave chordin, allowing bone morphogenetic protein (BMP-4), to act [Piccolo et al., 1997]. BMP-1, also a member of the astacin protease family, has been shown to possess type I procollagen C-proteinase activity and cleaves the carboxy terminal propeptides from procollagen types I, II, and III [Kessler et al., 1996]. In addition, crayfish astacin has been shown to degrade type I collagen to small peptides [Stocker and Zwilling, 1995]. The matrix metalloproteinases (MMPs) are a family of Zn^{2+}- and/or Ca^{2+}-dependent endopeptidases that use individual components of the ECM as substrates [Matrisian, 1992; Bode et al., 1995/96; Basbaum and Werb, 1996]. The activities of the MMPs are tightly controlled at both the transcriptional and post-transcriptional levels [Stetler-Stevenson, 1993]. The cell surface location and substrate specificities of the MMPs suggest that they are intimately involved in remodeling the ECM.

The sea urchin embryo is characterized by the presence of two ECMs: the hyaline layer on the apical surface and the basal lamina on the basal surface of ectoderm cells. Antisera prepared against vertebrate ECM components cross-react with species in both the hyaline layer and basal lamina [Wessel et al., 1984; Spiegel et al., 1989]. More recently, several collagen-encoding genes, expressed during sea urchin embryogenesis, have been cloned [Wessel et al., 1991; Exposito et al., 1993, 1994]. In addition, collagen deposition has been shown to be essential for gastrulation (Wessel and McClay, 1987; Wessel et al., 1989]. Arrested embryos can be rescued by the extracellular signaling molecules platelet-derived growth factor (PDGF-BB) and TGF-α [Ramachandran et al., 1993; Govindarajan et al., 1995]. These results suggest that a properly constituted ECM is required to facilitate the signaling events necessary for gastrulation. Both astacin- and MMP-like activities are present during sea urchin embryogenesis. Two astacin-like activities, BP-10 and SPAN, are expressed in blastula-stage embryos [LePage et al., 1992; Lhomond et al., 1996; Reynolds et al., 1992]. While substrates have not been identified both activities appear to be secreted into the ECM. Collagen and gelatin-degrading MMP-like activities have also been reported in the sea urchin embryo [Karakiulakis et al., 1993; Quigley et al., 1993; Vafa and Nishioka 1995; Mayne and Robinson, 1996; Robinson, 1997a]. All these activities are differentially expressed during development and have identifiable substrates in the ECM.

In this communication, we extend our previously reported study of a collagenase/gelatinase activity present in the sea urchin egg and early embryo. Our results suggest a somewhat novel mode of Ca^{2+}–protein interaction and clearly establish the bipolar export of this activity to both the hyaline layer and basal lamina. Collectively, our results establish the 160-kDa collagenase/gelatinase as an invertebrate MMP and afford us the opportunity to study ECM remodeling in a developing embryo.

MATERIALS AND METHODS

Growth of Embryos

Strongylocentrotus purpuratus (Sp) were obtained from Seacology (Vancouver, Canada). Gametes were obtained by intracoelomic injection with 0.5 M KCl. Eggs were filtered through a 300-μm nitex filter and washed four times in Millipore-filtered seawater (MFSW). Eggs were fertilized with a dilute suspension of sperm, and grown at 10–12°C in a cylindrical chamber with constant aeration. The cylinders contained paddles that rotated at 40 rpm. The efficiency of fertilization, determined by microscopic examination of the formation of the fertilization membrane, was 100%. Eggs and embryos at various stages of development were harvested by centrifugation at 2,000*g*. The eggs and embryos were washed twice in MFSW, centrifuged at 2,000*g*, and the pellets stored at −70°C.

Substrate Gel Zymography

Substrate gel zymography was carried out using the method of Heussen and Dowdle [1980]. Aliquots of eggs, embryos, or the purified collagenase/gelatinase were incubated for 30 min at room temperature in an equal volume of solubilizing buffer from which the bromophenol blue (BPB) and the reducing agent, dithiothreitol (DTT), had been omitted [Laemmli, 1970]. Electrophoresis was performed in 10% (w/v) polyacrylamide slab gels, prepared by copolymerizing acrylamide and 0.1% (w/v) gela-

tin, at a constant current of 8 mA and at 4°C. After electrophoresis, the gels were incubated in a solution containing 2.5% (v/v) Triton X-100 and 50 mM Tris-HCl, pH 8.0, for 1 h at room temperature. The gels were transferred to a solution containing 50 mM Tris-HCl, pH 8.0, and 10 mM $CaCl_2$ incubated for 16 h at room temperature stained with Coomassie brilliant blue R-250 and destained. Clear bands of proteolytic activity were seen against a blue background of stained intact gelatin.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [1970].

Quantitative Gelatinase Assay

Gelatinase activity was assayed by quantitating the cleavage of gelatin into trichloroacetic acid (TCA)-soluble peptides. Aliquots of gelatinase were incubated in a total volume of 100 µl containing 50 mM Tris-HCl, pH 8.0, 0.1% (w/v) gelatin, and 10 mM $CaCl_2$ for 60 min at 37°C. A control was set up as above, except the gelatinase was omitted, to ensure that cleavage of the gelatin was dependent on the gelatinase. At the end of the incubations, the samples were precipitated with an equal volume of 20% (w/v) TCA at 0°C for 20 min. A control for measuring the total amount of protein before hydrolysis by the gelatinase contained all the reagents listed above and 20% (w/v) TCA was added at zero time. Samples were centrifuged in a microcentrifuge (Eppendorf) at 16,000*g* for 10 min and the proteins were determined by the method of Lowry [1951]. The percentage hydrolysis of the substrate was defined as:

$$\frac{\text{A 0 min}-\text{A 60 min}}{\text{A 0 min}} \times 100$$

Absorbance was recorded at 750 nm and a unit of gelatinase activity is defined as 0.1 µg gelatin cleaved/min. Quantitative gelatinase assays were performed to ensure that the assays described above were measured in the linear range for both gelatinase activity versus time and gelatinase activity versus concentration of gelatinase.

Electron Microscopy

Eggs and various stage embryos were fixed as previously described by Spiegel et al. [1989]. Fixation was carried out by adding aliquots of eggs and embryos of various stages to an equal volume of the stock fixative containing 4% (v/v) glutaraldehyde and 1% (v/v) paraformaldehyde in 75% (v/v) MFSW buffered with 0.15 M sodium cacodylate, pH 7.8, without osmium postfixation. Eggs and embryos were rinsed in 85% (v/v) MFSW containing 0.1 M sodium cacodylate, pH 7.6, for 3 × 30 min at room temperature. Samples were then rinsed for 3 × 30 min in 0.1 M sodium cacodylate, pH 7.6, dehydrated in an ethanol series and embedded in Spurr's resin overnight at room temperature in an evacuated container. The blocks were cured overnight at 70°C.

Thin sections (150 nm) of eggs and embryos of various stages were placed on nickel grids. Grids were incubated on drops of solution containing 1% (w/v) bovine serum albumin (BSA), 0.01 M phosphate-buffered saline (PBS), consisting of 0.01 M phosphate in 0.15 M NaCl), pH 7.4, and 0.5% (v/v) Tween-20 for 3 × 15 min at room temperature. Grids were then transferred to a drop of diluted anti-41-kDa collagenase/gelatinase antibody (1:200 dilution in 0.01 M PBS, pH 7.4, containing 1% (w/v) BSA and 0.5% (v/v) Tween 20) for 2 h at room temperature. After the antibody incubation, the grids were rinsed 5 × 5 min on drops of 0.01 M PBS, pH 7.4, containing 0.5% (v/v) Tween-20, then transferred for 3 × 5 min to drops of PBS, pH 7.4, containing 1% (w/v) BSA and 0.5% (v/v) Tween-20. Grids were incubated on drops of protein A-gold (Sigma Chemical Co., St. Louis, MO; 10 nm colloidal gold) diluted 1:50 in a solution of 1% (w/v) BSA containing 0.5% (v/v) Tween-20 for 60 min at RT. After several rinses in a solution containing 0.01 M PBS, pH 7.4, and then distilled water, the grids were allowed to air dry. Sections were stained with 2% (w/v) uranyl acetate in 50% (v/v) ethanol, followed by Reynold's lead citrate, and viewed in a Zeiss EM 109 transmission electron microscope at 80 kV.

Equilibrium Dialysis

Aliquots of the collagenase/gelatinase were brought to a final volume of 1 ml in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA. Samples were dialyzed against 100 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA, 20 µCi $^{45}CaCl_2$ (Amersham; 0.4 mCi/µmol) and various concentrations of $CaCl_2$. Free calcium concentrations use determined using the EQCAL

computer program from Biosoft (Cambridge, England). Dialysis was carried out for 72 h at 4°C. After dialysis 50 µl from each dialysis bag and an equal volume of dialysate were removed and added to 10 ml of Scinti Verse E liquid scintillation fluid. The samples were counted in a Beckman model LS 9000 liquid scintillation counter. The counting efficiency was determined to be 85%. All glassware used during these experiments were soaked overnight in 5 mM EDTA and washed extensively in deionized water. All plasticware was siliconized.

Circular Dichroic Spectra

Circular dichroic (CD) spectra in the far-ultraviolet (UV) region from 190–280 nm were recorded at room temperature on a model J500-A JASCO spectropolarimeter and processed with a DP-500N data processor. Aliquots (0.5 mg/ml) of the purified collagenase/gelatinase were dialyzed against 50 mM Tris-HCl, pH 8.0 containing 0.1 mM EGTA. Spectra were measured in the absence and presence of 10 mM $CaCl_2$. The measurements were carried out in a quartz cell with a pathlength of 0.1 cm. Background spectra were recorded for each buffer and were subtracted from the recorded spectra of the protein. Secondary structure was calculated from the CD spectra using the method of Chen et al. [1974]. Protein concentrations were determined by the method of Lowry et al. [1951].

Amino Acid Analysis

Amino acid analyses were performed after hydrolysis of the collagenase/gelatinase for 72 h in 6 M HCl at 100°C. Liberated amino acids were fractionated with a Beckman 121 amino acid analyzer. Trypophan content was not determined.

Isolation of Blastocoelic Fluid

Blastocoelic fluid was isolated from mid-blastula-stage embryos as described by Kiyomoto and Tsukahara [1991], except that centrifugation was at 16,000*g* for 6 min. Embryos were grown to 30 h postfertilization, washed 3× in Ca^{2+}, Mg^{2+}-free seawater (CMFSW), and concentrated suspensions centrifuged at 16,000*g* for 6 min. The supernatant was retained and dialyzed against 50 mM Tris-HCl, pH 8.0, for 1.5 h at 4°C. Fifteen microlitre aliquots were solubilized in the absence of reducing agent and bromophenol blue and fractionated in a gelatin substrate gel as described above. In additional experiments, we varied the centrifugation time between 2 and 6 min and analyzed the supernatants by gelatin substrate gel zymography. The amount of activity in the supernatants increased with centrifugation time, probably reflecting the extent of flattening of the embryos.

Dissociation of the Collagenase/Gelatinase from the Extraembryonic Hyaline Layer

As described previously, 1-h-old embryos were derived from eggs treated with dithiothreitol [Robinson, 1991]. The embryos were washed twice in MFSW (0.45 µm), followed by washing for 2 × 10 min in CMFSW containing 5 mM ethylenediamine tetraacetic acid (EDTA). The CMFSW washes were combined, cleared by centrifugation at 20,000*g* for 15 min at 4°C, and the supernatant made 100 mM in $CaCl_2$ to precipitate hyalin [Robinson, 1988]. An aliquot of the hyalin precipitate was analyzed by gelatin substrate gel zymography as described above.

Gel Filtration Chromatography

A 100-µl aliquot of the purified collagenase/gelatinase was dialyzed at 4°C against 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA and fractionated on a Sephacryl S-200 gel filtration column (1 − 50 cm) equilibrated in 50 mM Tris-HCl, pH 8.0. Ten-drop fractions were collected at a flow rate of 1 drop/30 s. The column was calibrated with the following molecular mass markers: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), yeast alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Collagenase/gelatinase activity was assessed using the quantitative gelatinase assay.

Preparation of Antisera and Protein Gel Blot Analysis

Aliquots (50 µg) of the purified collagenase/gelatinase were boiled for 2 min in an equal volume of 2% (w/v) SDS. The SDS bound to the collagenase/gelatinase was removed by acetone precipitation and the protein resuspended in 50 mM Tris-HCl, pH 8.0, and an equal volume of incomplete Freund's adjuvant added. A New Zealand white rabbit was injected subcutaneously with this sample. The rabbit was twice

boosted at 2-week intervals and blood was withdrawn 10 days after the last injection. Antiserum was prepared as described by Robinson [1990]. Aliquots of eggs and various stage embryos were precipitated in an equal volume of 20% (w/v) TCA on ice, the pellets were solubilized and fractionated in a 10% (w/v) polyacrylamide gel [Laemmli, 1970]. The protein was transferred onto nitrocellulose by electroblotting at 60 V for 1.5 h and probed with the anti-collagenase/gelatinase antiserum. Western blot analysis was performed using methodology described previously [Robinson, 1992].

Purification of the Collagenase/Gelatinase

The collagenase/gelatinase was purified from unfertilized eggs as described by Mayne and Robinson [1996]. Eggs were suspended to 10% (v/v) in CMFSW; 500 mM NaCl, 10 mM KCl, 2 mM $NaHCO_3$, and 3 mM Na_2SO_4 made 50 mM in Tris-HCl, pH 8.0, and 20 mM in benzamidine-HCl and homogenized at 0°C. The homogenate was centrifuged at 2,000*g* at 4°C for 8 min. The supernatant was loaded onto a 10–40% (w/v) sucrose step gradient. The gradient was centrifuged in a SW 41 rotor at 160,000*g* at 4°C for 18 h. Sucrose density fractions containing the collagenase/gelatinase were pooled and loaded onto a 3 × 35-cm column of the gel exclusion resin, Bio-Gel P-200 (Bio-Rad) equilibrated in 50 mM Tris-HCl, pH 8.0. Sixty-drop fractions were collected at a flow rate of 1 drop/2 min. P-200 fractions containing the collagenase/gelatinase were combined and concentrated in an Amicon-10 Concentrator. Ion-exchange chromatography was performed in a 1 × 4 cm column of DEAE-cellulose (Sigma) equilibrated in 20 mM Tris-HCl, pH 8.0. The column was fractionated with a step gradient from 0.05–0.45 M NaCl in 20 mM Tris-HCl, pH 8.0. The collagenase/gelatinase eluted in 0.30–0.45 M NaCl. All chromatographic procedures were carried out at 4°C.

RESULTS

We have previously reported a dynamic pattern of gelatinase activities during sea urchin embryonic development [Mayne and Robinson, 1996]. Several gelatinase activities, ranging in apparent molecular masses from 27.5 to 86.9 kDa, were seen. The predominance of the 41-kDa species during early development prompted us to purify and characterize this activity. As previously reported this species possesses a selective cleavage activity toward sea urchin collagen in addition to a less species-specific gelatinase activity [Robinson, 1997b]. In addition, we have reported a calcium requirement for activity [Mayne and Robinson, 1996].

To analyze the relationship between the 41-kDa species seen in SDS-containing gelatin substrate gel zymograms and the native activity we determined native molecular mass by gel exclusion chromatography. The elution profile from a sephacryl S-200 column is shown in Figure 1. The native activity eluted with a molecular mass of approximately 160 kDa, suggesting a tetrameric structure. This tetramer is composed of subunits, which, as seen in gelatin substrate gel analysis, possess gelatinase activity. The native molecular mass was not altered by the presence of 10 mM Ca^{2+} (data not shown). Subsequent experiments were performed using the native, tetrameric enzyme, which is the form of the purified collagenase/gelatinase. Since the seawater environment contains Ca^{2+} concentrations as high as 10 mM we have analyzed the Ca^{2+} concentration dependence of reactivation of the EGTA-inhibited enzyme. At concentrations of Ca^{2+} of <3 mM there was little reconstitution of activity (Fig. 2). However, at 3.5–4 mM Ca^{2+} there was a dramatic

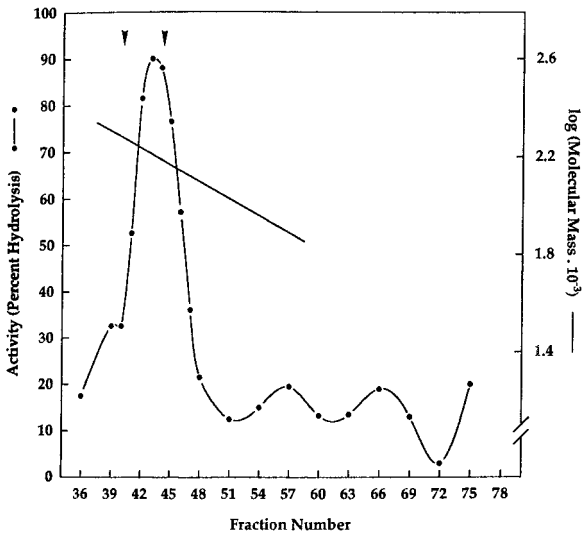

Fig. 1. Gel exclusion chromatographic analysis of the collagenase/gelatinase in a column of Sephacryl-S200 in the absence of Ca^{2+} was performed as described under Materials and Methods. Enzymatic activity is presented as percentage of gelatin hydrolyzed. The column was calibrated using carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (BSA) (66 kDa), yeast alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa): Arrows, elution profiles for β-amylase and alcohol dehydrogenase.

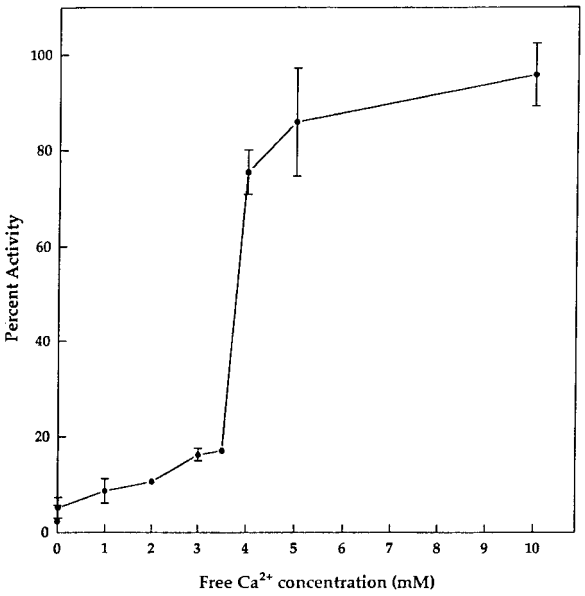

Fig. 2. Calcium concentration dependence for reactivation of the EGTA-inhibited collagenase/gelatinase. The collagenase/gelatinase was dialyzed overnight against 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA at 4°C. After dialysis, aliquots of the collagenase/gelatinase were incubated with $CaCl_2$ to give the final concentrations of free calcium indicated. Free calcium concentrations were determined using the computer program EQCAL from Biosoft (Cambridge, England). Gelatinase activities were measured in the quantitative assay. Values were normalized with respect to an aliquot of enzyme, which was assayed in the presence of 10 mM Ca^{2+} and was not dialyzed before assay. Values presented represent means ±SEM (n = 4).

recovery of activity; the apparent kd for reactivation was 3.7 mM. At 10 mM Ca^{2+}, 98% of the uninhibited activity had been restored. These results suggest that reconstitution of activity is mediated by the weak binding of Ca^{2+} to the collagenase/gelatinase; the apparent binding constant is of the order of $2–3.10^2$ M^{-1}. The sharp transition from the inactive to active states suggests that a critical Ca^{2+} concentration must be present to initiate reactivation; beyond this Ca^{2+} concentration reactivation occurs rapidly, suggesting positive cooperativity. This result is similar to that reported for the Ca^{2+}-dependent human interstitial collagenase [Zhang et al., 1997], but contrasts with the noncooperative Ca^{2+}-induced reactivation of the 87-kDa gelatinase activity present in the gastrula stage sea urchin embryo [Robinson, 1997a]. Additional experimentation will be required to accurately define the mechanism of the Ca^{2+}-induced reactivation of the collagenase/gelatinase. To further analyze Ca^{2+} binding, we have performed equilibrium dialysis measurements over the range of Ca^{2+} concentrations required for reactivation of the EGTA-inhibited enzyme (Fig. 3). Biphasic Ca^{2+} binding was detected; up to 80 mol of Ca^{2+} bound per mole of 41-kDa subunit with a dissociation constant of 0.5 mM. An additional 120 moles of Ca^{2+} bound with a dissociation constant of 5 mM. In control experiments, we quantitated Ca^{2+} binding to troponin C. Our results were similar to those reported in the literature; two classes of Ca^{2+}-binding sites, each binding two Ca^{2+} ions, were detected with dissociation constants of 1.10^{-7} and 4.10^{-5} M [data not shown; Potter and Gergely, 1975]. Substantial reactivation of the EGTA-inhibited collagenase/gelatinase coincided with saturated binding to the first set of sites. This massive, Ca^{2+}-binding capacity is most likely mediated through oxygen ligands serving as weak Ca^{2+}-binding sites. To address this point, we performed amino acid compositional analysis on the purified collagenase/gelatinase (Table 1). This analysis revealed a 24.3 mol/100 mol carboxy plus carboxyamide residue content. The carbonyl oxygen atoms in these residues would be expected to contribute weak Ca^{2+}-binding sites. In addition, the carbonyl oxygen atoms in the peptide bonds and the oxygen atoms in alcohol side chains would

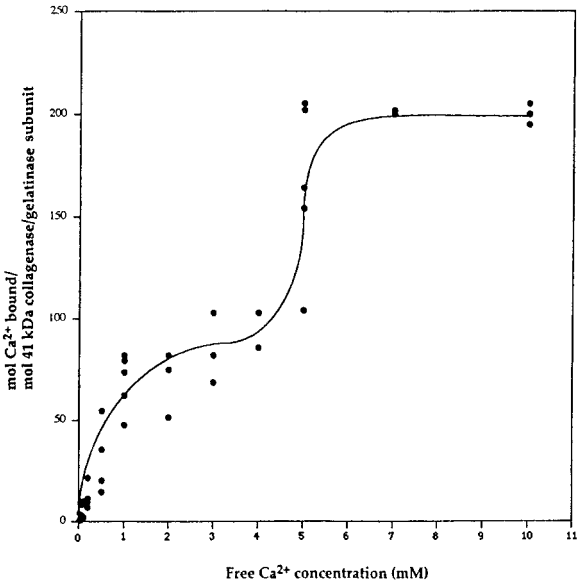

Fig. 3. Quantitation of Ca^{2+} binding to the collagenase/gelatinase. Calcium binding was quantitated by equilibrium dialysis as described under Materials and Methods. The results presented represent a composite of 12 independent experiments. Free calcium concentrations were determined using the computer program EQCAL from Biosoft (Cambridge, England).

TABLE I. Amino Acid Composition of the Collagenase/Gelatinase

Amino acid	Residues per 1,000
Asx	127
Thr	69
Ser	54
Glx	116
Pro	56
Gly	112
Ala	70
Val	78
Cys	0
Met	2
Ile	59
Leu	81
Try	30
Phe	40
Lys	49
His	17
Arg	40

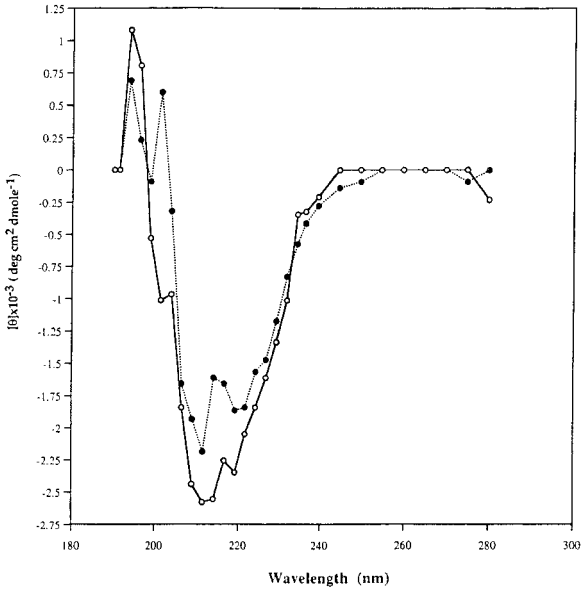

Fig. 4. Circular dichroic spectra of the collagenase/gelatinase in the presence (closed circles) and absence (open circles) of 10 mM $CaCl_2$. Spectra were measured as described under Materials and Methods.

also be expected to contribute weak Ca^{2+}-binding sites [Williams, 1977].

The data in Figures 2 and 3 collectively suggest that activation of the collagenase/gelatinase requires the binding of ≤80 mol of Ca^{2+} per mol of 41-kDa subunit. The mechanism of action of Ca^{2+} was examined by measuring the circular dichroic spectra of the collagenase/gelatinase in both the presence and absence of 10 mM Ca^{2+} (Fig. 4). In the presence of Ca^{2+}, the α-helical content was determined to be 6.1 ± 1.2 % (n = 3), while in the absence of Ca^{2+} the α-helical content was 7.1 ± 0.1% (n = 3). These results suggest that Ca^{2+} is not inducing significant changes in the secondary structure of the collagenase/gelatinase. Since the quaternary structure of the collagenase/gelatinase is also unaltered by Ca^{2+}, it seems likely that the activating effect of this cation is not mediated through conformational change.

In order to assess the biological relevance of the collagenase/gelatinase activities we determined the site(s) of localization of this species within the egg and various stage embryos. Initially, blastocoelic fluid was examined by gelatin substrate gel zymography (Fig. 5). Blastocoelic fluid, isolated by centrifuging blastula-stage embryos, exhibited gelatinase activity (lane 2) that comigrated with the purified collagenase/gelatinase species (lane 3). These results suggested that the collagenase/gelatinase activity was localized to the blastocoel of the embryo and could access substrates in the basal lamina.

We also analyzed the association of the collagenase/gelatinase with the apically located extraembryonic matrix, the hyaline layer. Washing 1-h-old embryos, prepared from eggs treated with the reducing agent dithiothreitol, in the absence of Ca^{2+} and Mg^{2+}, results in the dissociation of hyalin from the layer, while other components of the hyaline layer remain associated with the apical surface of the embryo [McClay and Fink, 1982; Robinson, 1988]. Hyalin is a major protein component of the layer and when an aliquot of the dissociated protein, isolated by Ca^{+2} precipitation, was analyzed by gelatin substrate gel zymography a gelatinase activity, migrating with an apparent molecular mass of 41 kDa was detected (lane 1). The remaining supernatant also contained the 41-kDa gelatinase activity (data not shown). Clearly, disruption of the hyaline layer resulted in at least partial dissociation of the collagenase/gelatinase from the surface of 1-h-old embryos, indicating that this activity is associated with the apically located extracellular matrix. The nature of the interactions that retain the 41-kDa gelatinase/collagenase within the hyaline layer remain to be elucidated. Collectively, these results suggest that the collagenase/gelatinase may be involved in remodeling both the hyaline layer and the basal lamina. To further define the localization of the collagenase/gelatinase

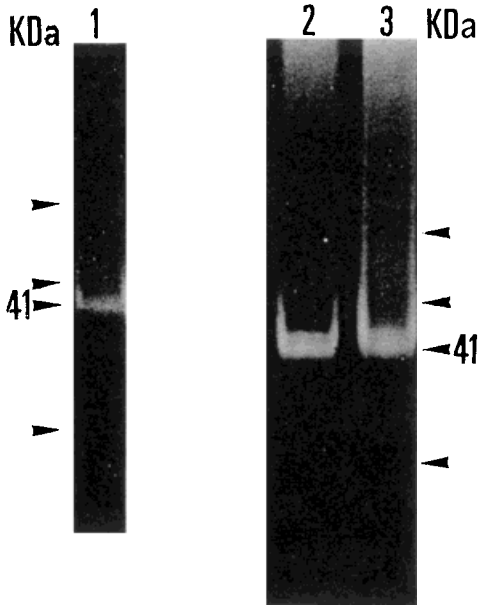

Fig. 5. Gelatin substrate gel zymography of the hyalin fraction dissociated from 1-h-old embryos and the blastocoelic fluid isolated from mid-blastula-stage embryos. An aliquot (5 µg) of the hyalin fraction **(lane 1)**, an aliquot (15 µl) of blastocoelic fluid **(lane 2)**, or an aliquot (1 µg) of the purified collagenase/gelatinase **(lane 3)** were analyzed by gelatin substrate gel zymography as described under Materials and Methods. The gelatinase activity in lane 1 comignated with the purified 41-kDa collagenase/gelatinase (data not shown): Arrows, positions of molecular mass makers, bovine serum albumin (BSA)(66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

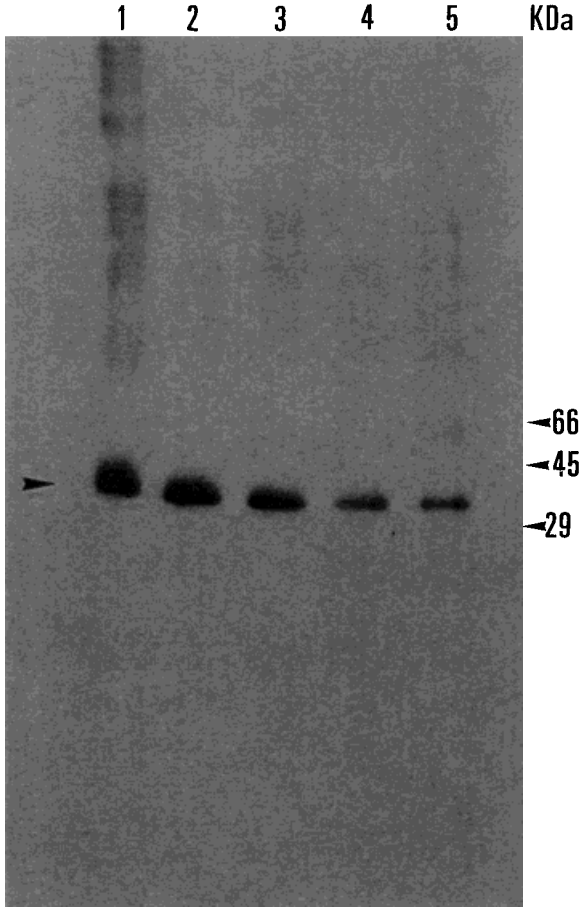

Fig. 6. Western blot analysis of eggs and various stage embryos using the anti-collagenase/gelatinase antiserum. Aliquots (15 µg) of eggs and various stage embryos were fractionated in a 3–12% (w/v) polyacrylamide gradient gel [Laemmli, 1970]: Western blot analysis was performed as described previously [Robinson, 1992]: **Lanes 1–5**, eggs, 9.5-, 32-, 53, and 70.5-h-old embryos, respectively. The relative migrations of molecular-mass markers bovine serum albumin (BSA)(66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa) are shown.

we performed high-resolution immunogold labeling analysis. A polyclonal antiserum was prepared and its specificity tested by protein gel blot analysis (Fig. 6). The antiserum was monospecific and recognized an antigen that migrated with an apparent molecular mass of 32,000 and decreased in amount during the course of development mirroring the decrease in the collagenase/gelatinase activity seen in developmental zymograms [Mayne and Robinson, 1996]. We have previously shown that, upon reduction with dithiothreitol in the presence of SDS [Laemmli, 1970], the collagenase/gelatinase migrates with a reduced apparent molecular mass of 32,000 [Mayne and Robinson, 1996]. In addition, when the purified, fully reduced collagenase/gelatinase was probed with the anti-41-kDa antiserum, a species with an apparent molecular mass of 32,000 was seen (data not shown). Immunogold labeling analysis was performed using sections of eggs and various stage embryos (Fig. 7). In the unfertilized egg, the cortical granules were heavily labeled and in addition, small granular structures also contained antigen (Fig. 7A). These latter structures had a morphology similar to yolk granules but were considerably smaller (Fig. 7B,C). Recognizable yolk granules were not labeled. The storage granules had an average diameter of 0.37 ± 0.1 µm (n = 8), compared with 0.82 ± 0.5 µm (n = 20) for yolk granules. The collagenase/gelatinase-containing granules were dispersed throughout the cytoplasm of the egg and 1-h-old embryo but were undetectable by the blastula stage. When preimmune serum was used no labeling was detected in the cortical granules or the yolk granule-like structures (Fig. 7D). In the blastula-stage embryo, the apically located extracellular matrix, the hyaline layer, was labeled

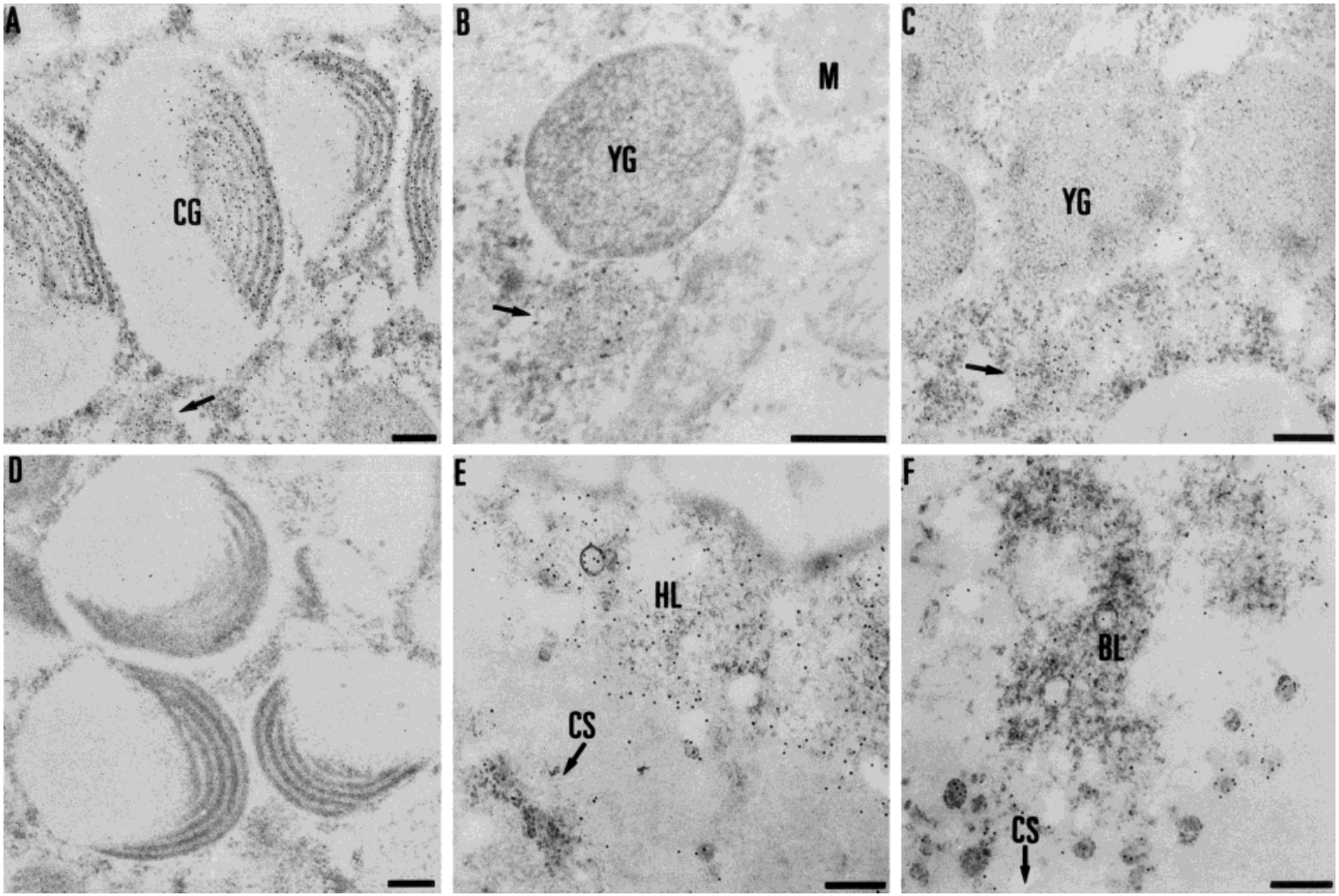

Fig. 7. Immunogold labeling of eggs and various stage embryos. **A–C:** Sections of unfertilized eggs probed with the anti-collagenase/gelatinase antiserum. **D:** Section of an unfertilized egg probed with preimmune serum. **E,F:** Apical and basal surfaces, respectively, of sections of blastula-stage embryos probed with the anti-collagenase/gelatinase antiserum. **G,H:** Apical and basal surfaces, respectively, of gastrula-stage embryos probed with the anti-collagenase/gelatinase antiserum. Antiserum was used at a dilution of 1:200 (v/v). Scale bar = 0.25 μm. CG, cortical granule; YG, yolk granule; M, mitochondrion; HL, hyaline layer; CS, cell surface; BL, blastocoel. Figure 7, panels G and H appear on the following page.

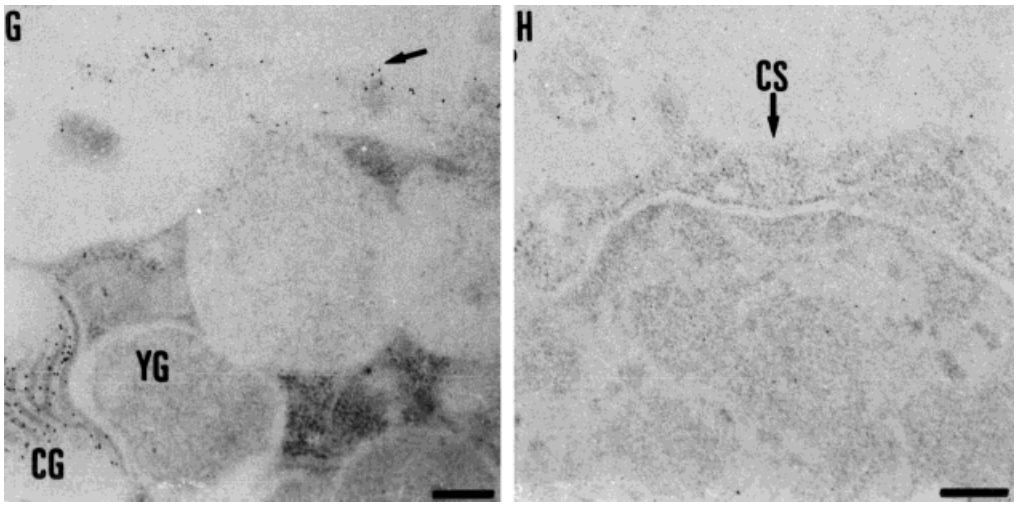

Figure 7. (Continued.)

(Fig. 7E), as was the blastocoel (Fig. 7F). No label could be detected intracellularly. At the gastrula stage the apical surface labeling was greatly diminished (Fig. 7G), while no labeling was evident on the basal surface (Fig. 7H). The gastrula stage section in Figure 7G retained a cortical granule that had failed to fuse with the egg plasma membrane at fertilization; this granule retained the collagenase/gelatinase antigen. Clearly, these results identify the cortical granules and, as yet uncharacterized, small granules within the egg as the storage compartments for the collagenase/gelatinase, a protein exported to both the apical and basal surfaces of the embryo.

DISCUSSION

The matrix metalloproteinases (MMPs) play important roles in embryonic development and organogenesis, in normal physiological processes such as wound healing, angiogenesis, and pregnancy, as well as in pathological processes such as arthritis and tumor cell metastasis. These effects are mediated through either controlled or uncontrolled degradation of the ECM. Studies with vertebrate systems have identified a variety of native or denatured extracellular matrix components as substrates for MMP activities. We have begun a study of MMP activities in the sea urchin egg and embryo. Using gelatin substrate gel zymography, we identified a 41-kDa species as the major gelatinase activity present in the egg and early embryo [Mayne and Robinson, 1996]. This activity was inhibited by Zn^{2+} and EDTA and reactivated by Ca^{2+} at a concentration of 10 mM. A variety of divalent metal ions, at millimolar concentrations, were variously effective at reactivating the EDTA-inhibited enzyme, suggesting a broad but weak metal ion binding capacity. Further work has shown that the gelatinase also possesses an echinoderm specific collagenase activity [Robinson, 1997b].

The results reported in this paper extend our previous studies and define the details of both Ca^{2+}- binding to and reactivation of the collagenase/gelatinase. Our results document the behavior of a protein that functions in an environment containing 10 mM Ca^{2+}. While much effort has been directed toward understanding the behavior of cytosolic Ca^{2+}-binding proteins relatively little is known about the interactions of proteins with Ca^{2+} in the extracellular environment. In mammals, the extracellular environment contains Ca^{2+} concentrations as high as 3 mM [Carafoli, 1987]; this allows low-affinity binding sites to interact with Ca^{2+}. In particular, proteins rich in acidic amino acid residues can bind Ca^{2+} with low affinity [Maurer et al., 1996]; in addition, oxygen atoms from carboxyamide and alcohol side chains as well as the carbonyl oxygens of the polypeptide backbone also serve as weak Ca^{2+} binding sites [Williams, 1977]. The extracellular environment of the sea urchin embryo contains 10 mM Ca^{2+} and affords the collagenase/gelatinase weak interactions with Ca^{2+}, which could not occur intracellularly. The apparent dissociation constant for reactivation (3.7 mM) suggests the possibility that the collagenase/gelatinase activ-

ity might be regulated at the cell surface, affording Ca^{2+} a dynamic, rather than static, activator role. The existence of microenvironments at the surface of the sea urchin embryo, perhaps regulated by other Ca^{2+}- binding/storage proteins such as hyalin, could provide for a regulatory role for Ca^{2+}. Hyalin, a major protein component of the apically located hyaline layer, is a large polyanion with weak binding affinity (kd = 0.12 mM) for up to 400 calcium ions [Robinson and Brennan, 1991]. Since hyalin forms a continuous layer on the surface of the embryo [McClay and Fink, 1982], it may serve as a storage reservoir for Ca^{2+} and thereby regulate the interactions of other cell surface proteins with this cation. In this context, it is particularly interesting that the dissociation of hyalin from the surface of 1-h-old embryos also resulted in the release of the collagenase/gelatinase from the surface of these embryos (Fig. 5, lane 1). The biphasic nature of the Ca^{2+}-collagenase/gelatinase interactions suggests two classes of weak binding sites. Saturation of the first set of sites (kd = 0.5 mM) correlated with both the binding of 80 moles Ca^{2+} per mole of 41-kDa subunit and renaturation of the collagenase/gelatinase. This massive Ca^{2+} binding did not appear to induce structural changes in the protein, suggesting the possibility that Ca^{2+} may be facilitating the binding of substrate perhaps through a neutralization of repulsive charges on the enzyme and substrate and/or through a salt bridging action. Clearly, more work is required to understand both the nature and consequences of Ca^{2+}–protein interactions in the extracellular environment where substantial numbers of calcium ions can bind at low-affinity sites.

An extracellular localization is one of several criteria that must be satisfied before designating a protein as an MMP. In the study reported in this paper, we have detected the collagenase/gelatinase in both the hyaline layer and basal lamina. Both ECMs have been studied in some detail and appear to contain components similar to the collagens, proteoglycans and structural glycoproteins found in vertebrate ECMs [Wessel et al., 1984; Wessel and McClay, 1987; Brennan and Robinson, 1994]. A unique feature of the 160-kDa species is that it possesses both collagenase and gelatinase activities and is therefore equipped with the ability to completely degrade native collagen. We have previously shown that the collagenase/gelatinase possesses cleavage activity toward both vertebrate and invertebrate gelatins but has a collagenase activity restricted to echinoderm collagen [Robinson, 1997b], while no cleavage activity was detected when bovine serum albumin or bovine hemoglobin were tested as substrates [Mayne and Robinson, 1996]. It seems likely that the physiological substrates for this species are collagen and gelatin molecules in both the hyaline layer and basal lamina extracellular matrices.

The localization of the collagenase/gelatinase to the cortical granule compartment in the unfertilized egg is not unexpected for a protein exported to the apical surface of the embryo. However, recently several additional but poorly characterized egg storage compartments have been identified for proteins destined for export. Indirect immunofluorescence analyses have revealed dispersed, punctate patterns of staining for antigens exported to the basal lamina [Wessel et al., 1984; Alliegro and McClay, 1988; Brennan and Robinson, 1994]. Matese et al. [1997] have identified five compartments for proteins exported to the hyaline layer and/or basal lamina: cortical granules, basal lamina vesicles, apical vesicles, echinonectin vesicles, and a fifth compartment. Wessel and Berg [1995] identified membrane-bound vesicles, proximal to the Golgi, as the storage compartment for ECM 3, a protein destined for export to the basal lamina. Using high-resolution immunogold labeling, we have detected the collagenase/gelatinase in yolk granule-like structures in the unfertilized egg; these structures have a morphology similar to that of yolk granules but are considerably smaller. A number of reports have described a role for yolk granules or yolk granule-like structures as storage compartments for proteins destined for export from eggs. Mayne and Robinson [1998] identified yolk granules as the storage compartment for HLC-32, a protein exported to both the hyaline layer and basal lamina. Gratwohl et al. [1991] have demonstrated that toposome, a protein destined for export to the hyaline layer, is localized to both cortical and yolk granules in the unfertilized egg. Fuhrman et al. [1992] have found that another hyaline layer protein, echinonectin, is localized to membrane-bound vesicles in the unfertilized egg. Similarly, in both *Xenopus* [Outenreath et al., 1988] and *C. elegans* [Creutz et al., 1996], exported proteins have been found in yolk granule-like structures in the unfertilized egg. Clearly, the sea urchin

egg is a biologically sophisticated cell that can direct proteins to several different compartments, store these proteins, and export them after fertilization to the hyaline layer and/or basal lamina in a time-dependent manner. A great deal of work remains to be done to elucidate the signals required for directing proteins to these various compartments, as well as those that direct these different classes of vesicles to the apical surface of the egg or the apical and/or basal surface of presumptive ectoderm cells. The results reported in this paper suggest that the collagenase/gelatinase can be directed to two distinct storage compartments within the egg. The identity of the signal(s) responsible for trafficking to these compartments remains to be elucidated.

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