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Effects of Calcium and Magnesium on a 41-kDa Serine-Dependent Protease Possessing Collagen-Cleavage Activity

John J. Robinson*

Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada

Abstract We report here the continued characterization of a 41-kDa protease expressed in the early stage of the sea urchin embryo. This protease was previously shown to possess both a gelatin-cleavage activity and an echinoderm-specific collagen-cleavage activity. In the experiments reported here, we have explored the biochemical nature of this proteolytic activity. Pepstatin A (an acidic protease inhibitor), 1,10-phenanthroline (a metalloprotease inhibitor), and E-64 (a thiol protease inhibitor) were without effect on the gelatin-cleavage activity of the 41-kDa species. Using a gelatin substrate gel zymographic assay, the serine protease inhibitors phenylmethylsulfonyl fluoride and benzamide appeared to partially inhibit gelatin-cleavage activity. This result was confirmed in a quantitative gelatin-cleavage assay using the water soluble, serine protease inhibitor [4-(2-aminoethyl)benzenesulfonylfluoride]. The biochemical character of this protease was further explored by examining the effects of calcium and magnesium, the major divalent cations present in sea water, on the gelatin-cleavage activity. Calcium and magnesium competed for binding to the 41-kDa collagenase/gelatinase, and prebound calcium was displaced by magnesium. Cleavage activity was inhibited by magnesium, and calcium protected the protease against this inhibition. These results identify calcium and magnesium as antagonistic agents that may regulate the proteolytic activity of the 41-kDa species. J. Cell. Biochem. 80: 139–145, 2000.

Key words: collagenase/gelatinase; proteolysis; sea urchin

Many cellular properties such as shape, migration, proliferation, and differentiation are influenced by the extracellular environment [Adams and Watts, 1993]. In particular, the extracellular matrix and the ectodomains of transmembrane proteins can mediate interactions between the cell surface and the pericellular space. In addition to binding growth factors and mediating interactions with cell-surface receptors, the extracellular matrix can also influence cellular properties through a direct interaction with the cell surface. Recognition of the important functional roles played by the extracellular matrix has resulted in a considerable body of research directed towards understanding the regulation of extracellular matrix composition. A key element in regulating the protein composition of the pericellular space involves cleavage reactions mediated by metallo- and serine proteases [Matrisian, 1992; Bode et al., 1995, 1996; Werb, 1997]. These proteases utilize cell-surface proteins and components of the extracellular matrix as substrates [Werb and Yan, 1998]. Proteolytic processing of the extracellular matrix and the ectodomains of transmembrane proteins has profound effects on cell behavior and can result in a number of physiologic and pathologic cellular phenotypes. Understanding the biochemical basis for these activities will lead to the development of selective inhibitors for use in treatment protocols. Consequently, researchers are engaged in an intense effort to define the properties of the proteases that control the protein composition of the pericellular space.

The sea urchin embryo assembles two extracellular matrices, the hyaline layer on the api-

Abbreviations used: AEBSF, [4-(2-aminoethyl)benzenesulfonylfluoride]; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonylfluoride.

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*Correspondence to: John J. Robinson, Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X9. E-mail: johnro@morgan.ucs.mun.ca

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cal surface and the basal lamina on the basal surface. These matrices are in intimate contact with many different cell types in the embryo and are essential for normal development [Wessel et al., 1989; Wessel et al., 1991; Ramachandran et al., 1993]. We and others have identified collagen- and gelatin-cleavage activities in the sea urchin embryo [Quigley et al., 1993; Karakiulakis et al., 1993; Vafa and Nishioka, 1995; Mayne and Robinson, 1996; Robinson, 1997b]. In particular, we have purified and characterized a 41-kDa species that possesses both collagen- and gelatin-cleavage activities. We found that proteolytic activity was dependent upon Ca^{2+}, and using both immunogold labeling and in situ immunofluorescence analyses we have shown that this species is localized to both the hyaline layer and basal lamina of the sea urchin embryo [Mayne and Robinson, 1998; and unpublished data].

In the work reported here we have determined the biochemical nature of the 41-kDa gelatin-cleavage activity. In addition, we have identified antagonistic effects for Ca^{2+} and Mg^{2+} on this proteolytic activity. The contrasting effects of these cations could serve to moderate the cleavage activity of the 41-kDa species on the surface of the embryo.

MATERIALS AND METHODS

Growth of Embryos

Strongylocentrotus purpuratus (Sp) were purchased from Seacology, Vancouver, Canada and gametes were obtained by intracoelomic injection of 0.5 M KCl.

Purification of the 41-kDa Collagenase/Gelatinase

The 41-kDa species was purified from unfertilized eggs as described previously [Mayne and Robinson, 1996].

Gelatin Substrate Gel Zymography

Substrate gel zymography was performed essentially as described previously [Heussen and Dowdle, 1980]. Sodium dodecyl sulfate (SDS)-containing gels were prepared by copolymerizing acrylamide and gelatin at a final concentration of 0.1% (w/v). Samples of the purified gelatinase were dispersed for 30 min at room temperature in Laemmli solubilizing solution from which both dithiothreitol and bromophenol blue had been omitted [Laemmli, 1970]. Electrophoresis was performed at 10 mA and 4°C for 4.5 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.

Displacement Assay

Aliquots of the purified 41-kDa collagenase/gelatinase were dot-blotted onto nitrocellulose, and the membranes were equilibrated for 70 min at room temperature in a solution containing 10 mM imidazole-HCl, pH 6.8, and 60 mM KCl. Pieces of nitrocellulose, half containing bound protein and half with no bound protein as a control for backround binding of ^{45}Ca, were incubated for 15 min at room temperature in equilibration buffer containing $^{45}CaCl_2$ (5 mCi/L). Following incubation, the membranes were washed for 5 min at room temperature in 1 mM Tris-HCl, pH 7.5, or in the same buffer containing various concentrations of $MgCl_2$. Membranes were then air dried and counted in 10 mL of Scinti Verse E (Fisher) in a Beckman model LS9000 liquid scintillation counter. A correction for backround binding was made by subtraction of the counts bound to the blank membranes.

Quantitative Gelatinase Assay

Gelatinase activity was assayed by quantitating the cleavage of gelatin into trichloroacetic acid (TCA)-soluble peptides [Mayne and Robinson, 1996]. Aliquots of the gelatinase were incubated in a total volume of 100 μL containing 50 mM Tris-HCl, pH 8.0, 0.1% (w/v) gelatin, and various concentrations of $CaCl_2$ and/or $MgCl_2$ at 37°C. A control was set up to ensure that gelatin cleavage was dependent on the gelatinase. At the end of the incubations, the samples were precipitated with an equal volume of ice-cold 20% (w/v) TCA at 0°C for 20 min. A control for determining the total amount of protein present before hydrolysis by the gelatinase contained all the reagents above and the TCA was added at zero time. Samples were centrifuged at 16,000*g* for 10 min and the protein pellet was quantitated [Lowry et al., 1951]. One unit of gelatinase activity is defined as 0.1 μg gelatin cleaved/min. All assays were

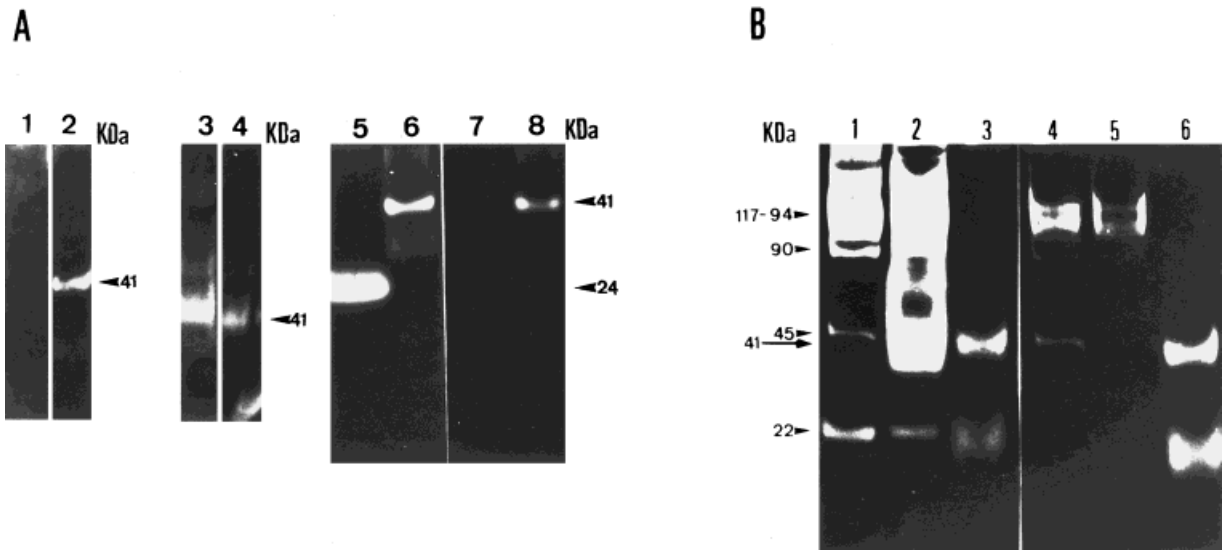

Fig. 1. The effects of various protease inhibitors on the gelatin-cleavage activity of the 41-kDa species. **A:** Following fractionation in a gelatin substrate gel, the strips were processed in the absence of inhibitors (**lanes 2, 3, 5, and 6**) or in the presence of 5 mM EDTA (**lane 1**), 50 μg/ml PMSF (**lane 4**), or 10 mM benzamide (**lanes 7 and 8**). The purified 41-kDa species was fractionated in lanes 1, 2, 3, 4, 6, and 8 and bovine trypsin was fractionated in lanes 5 and 7. **B:** Following fractionation in a gelatin substrate gel, the strips were processed in the absence (**lanes 1–3**) or in the presence (**lanes 4–6**) of 2 mM 1,10-phenanthroline. Zinc-dependent metalloproteases, associated with the extraembryonic hyaline layer, were fractionated in lanes 1, 2, 4, and 5 and the purified 41-kDa species was fractionated in lanes 3 and 6.

performed within the linear range of gelatinase activity versus time and gelatinase activity versus gelatinase concentration.

When quantitative assays were performed in the presence of protease inhibitors, the 41-kDa collagenase/gelatinase was preincubated with the inhibitor for 30 min at 37°C before addition of the substrate.

RESULTS

We have previously purified and partially characterized the 41-kDa collagenase/gelatinase [Mayne and Robinson, 1996; Mayne and Robinson, 1999]. Proteolytic activity was dependent upon Ca^{2+} and showed specificity for echinoderm collagen and gelatin with no detectable cleavage activity towards bovine serum albumin or hemoglobin. In an additional report, we demonstrated the localization of the collagenase/gelatinase to the extraembryonic matrices of the sea urchin embryo [Mayne and Robinson, 1998]. In the experiments reported here, we extend these previous studies by utilizing both qualitative and quantitative gelatin-cleavage assays to determine the biochemical nature of this proteolytic activity. We initially used gelatin substrate gel zymography to examine the effects of EDTA, PMSF, benzamidine, and 1,10-phenanthroline on the 41-kDa cleavage activity (Fig. 1). As expected, activity was completely abolished in the presence of 5 mM EDTA (Fig. 1A, lane 1), whereas the irreversible serine protease inhibitor PMSF appeared to cause partial inhibition (lanes 3 and 4). To further test the requirement for a reactive serine residue, we utilized the reversible inhibitor benzamidine and included trypsin, a known serine protease, as an internal control. In the absence of inhibitor, the gelatin-cleavage activity of both trypsin (lane 5) and the 41-kDa species (lane 6) was evident. However, in the presence of 10 mM benzamidine, tryptic cleavage activity was completely abolished and the 41-kDa activity again appeared to be partially inhibited (lanes 7 and 8, respectively). A requirement for Zn^{2+} was probed using the largely Zn^{2+}-specific chelator 1,10-phenanthroline (Fig. 1B). As a control, we fractionated several Zn^{2+}-dependent gelatinase activities, isolated from sea urchin hyaline layers (lanes 1, 2, 4, and 5) alongside the 41-kDa collagenase/gelatinase (lanes 3 and 6). We have previously shown that the cleavage activities in lanes 1 and 2 were Zn^{2+}-dependent [Flood et al., 2000] and, as expected, these activities were substantially reduced in

TABLE I. Quantitation of the 41-kDa Gelatin-Cleavage Activity in the Presence of Protease Inhibitors

Inhibitor	Percent activity remaining[a]
AEBSF[b] (0.6 mM)	49.1 ± 5.5
E-64 (12 μM)	101 ± 3.6
1,10-Phenanthroline (2 mM)	117 ± 2.0
Pepstatin A (1 μM)	98 ± 4.8

[a]Values represent mean ± SEM, n = 3.
[b]AEBSF, [4-(2-aminoethyl) benzenesulfonylfluoride].

TABLE II. Displacement of Prebound Ca^{2+} by Increasing Concentrations of $MgCl_2$[a]

$MgCl_2$ concentration (μM)	Ca^2 remaining bound (%)
0	100
5	65.1
10	50.2
20	33.7
30	10.5

[a]The calcium remaining bound in the absence of exogenously added $MgCl_2$ was normalized to 100%.

the presence of 1,10-phenanthroline (lanes 4 and 5). Interestingly, 1,10-phenanthroline appeared to activate the 41-kDa collagenase/gelatinase (compare lanes 3 and 6). This result suggests that the trace amounts of Zn^{2+} present in our buffers may be inhibitory to the 41-kDa activity. The smaller molecular mass species seen in lanes 3 and 6 is a reduced form of the 41-kDa species [see Mayne and Robinson, 1996]. These qualitative results are suggestive of a requirement for a reactive serine residue in the 41-kDa collagenase/gelatinase.

To further test the requirement for a reactive serine residue, we utilized a quantitative gelatin-cleavage assay in the presence of inhibitors of the various classes of proteases (Table I). Preincubation of the 41-kDa collagenase/gelatinase with the water soluble, serine protease inhibitor [4-(2-aminoethyl)benzenesulfonylfluoride] (AEBSF), resulted in 50.9% inhibition. In parallel experiments, AEBSF caused 89.5% inhibition of the tryptic gelatin-cleavage activity. The thiol protease inhibitor E-64, the Zn^{2+}-metalloprotease inhibitor 1,10-phenanthroline, and the acidic protease inhibitor pepstatin A all failed to inhibit the gelatin-cleavage activity. The likelihood that the 41-kDa species is an aspartate-dependent protease is further diminished by a measured pH optimum of 8.5 [Mayne and Robinson, unpublished result].

To further explore the biochemical nature of the 41-kDa cleavage activity, we investigated the effects of Ca^{2+} and Mg^{2+} on gelatinase activity. This approach seemed biologically relevant because sea water contains 10 mM Ca^{2+} and 50 mM Mg^{2+}. In an initial experiment, we measured the ability of Mg^{2+} to displace prebound Ca^{2+} from the purified 41-kDa species (Table II). As the concentration of exogenously added Mg^{2+} was increased from 0 to 30 μM, the percentage of Ca^{2+} remaining bound to the collagenase/gelatinase decreased from 100 (normalized) to 10.5. In a complementary set of experiments, we examined the ability of Mg^{2+} to directly compete with Ca^{2+} for binding to the 41-kDa collagenase/gelatinase. In the presence of 10 mM Ca^{2+}, increasing the concentration of exogenously added Mg^{2+} from 0 to 30 mM decreased Ca^{2+} binding from 100% (normalized) to 26.2% (data not shown). These results clearly demonstrate that both cations can bind to the 41-kDa collagenase/gelatinase.

To determine the biologic consequences of these interactions, we utilized gelatin substrate gel zymography to examine the effects of Mg^{2+} on the 41-kDa gelatin-cleavage activity (Fig. 2A). Activity was detected in the presence of 10 mM Ca^{2+} (lane 1) with somewhat decreased activity seen in the absence of exogenously added Ca^{2+} (lane 2). No activity was detected in the absence of Ca^{2+} and the presence of 5 mM (lane 3), 10 mM (lane 4), or 30 mM (lane 5) Mg^{2+}. These results clearly identify Mg^{2+} as an inhibitor of the gelatinase activity. To further explore the relationships between Ca^{2+}, Mg^{2+}, and the 41-kDa gelatinase activity we examined the effect on gelatin-cleavage activity of increasing concentrations of Mg^{2+} in the presence of a fixed concentration of Ca^{2+} (Fig. 2B). As expected, activity was detected in the absence of Mg^{2+} and the presence of 10 mM Ca^{2+} (lane 1). In the presence of 10 mM Ca^{2+} and increasing concentrations of Mg^{2+}, we detected reduced gelatin-cleavage activity: 1 mM (lane 2), 10 mM (lane 3), 30 mM (lane 4), and 50 mM (lane 5) Mg^{2+}. However, the loss of activity in the presence of Ca^{2+} and Mg^{2+} appeared to be less than that seen in the presence of Mg^{2+} alone. This result, coupled with the demonstration that Mg^{2+} can

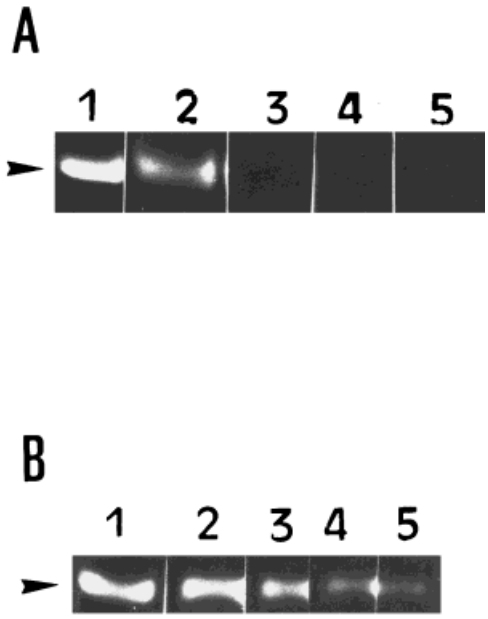

Fig. 2. Gelatin substrate gel zymographic analysis of the 41-kDa collagenase/gelatinase in the presence of Ca^{2+} and Mg^{2+}. **A:** Gelatin substrate gel zymographic analysis of the purified 41-kDa collagenase/gelatinase in the presence of Ca^{2+} or Mg^{2+}. Gel strips were processed in the presence of 10 mM $CaCl_2$ (**lane 1**), in the absence of exogenously added $CaCl_2$ (**lane 2**), or in the absence of Ca^{2+} and the presence of 5 mM (**lane 3**), 10 mM (**lane 4**), or 30 mM (**lane 5**) $MgCl_2$. **B:** Gelatin substrate gel zymographic analysis of the purified 41-kDa collagenase/gelatinase in the presence of Ca^{2+} and Mg^{2+}. Gel strips were processed in the presence of 10 mM $CaCl_2$ (**lane 1**) or in the presence of 10 mM $CaCl_2$ and increasing concentrations of $MgCl_2$: 1 mM (**lane 2**), 10 mM (**lane 3**), 30 mM (**lane 4**), or 50 mM $MgCl_2$ (**lane 5**).

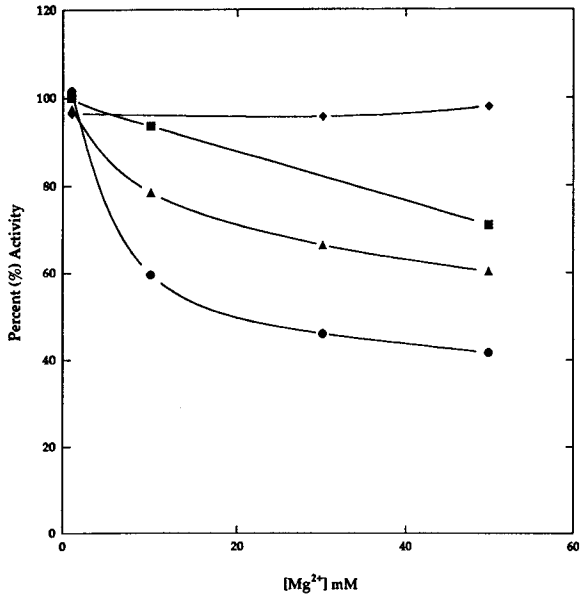

Fig. 3. Quantitative assay of the 41-kDa gelatinase activity in the presence of Ca^{2+} and Mg^{2+}. Gelatinase activity was determined in the presence of increasing concentrations of $MgCl_2$ and 0 (●), 1 mM (▲), 10 mM (■), or 20 mM (◆) $CaCl_2$. Percent activity was determined relative to an aliquot of the gelatinase that was assayed in the presence of 10 mM $CaCl_2$ and normalized to 100% activity.

compete with Ca^{2+} for binding to the 41-kDa collagenase/gelatinase, suggests that the inhibition by Mg^{2+} may be due to the binding of this cation to low-affinity Ca^{2+}-binding sites.

We used quantitative gelatinase assays to further explore the effects of Ca^{2+} and Mg^{2+} on the 41-kDa gelatin-cleavage activity (Fig. 3). In the absence of exogenously added Ca^{2+}, increasing concentrations of Mg^{2+} resulted in a decrease in gelatin-cleavage activity; at a concentration of 50 mM, the concentration of Mg^{2+} present in sea water, approximately 60% of the gelatinase activity was lost. The effect of Mg^{2+} was however modulated by Ca^{2+}; the inhibitory effect of Mg^{2+} was substantially diminished in the presence of increasing concentrations of Ca^{2+} (1, 10, and 20 mM). At 20 mM Ca^{2+}, the 41-kDa collagenase/gelatinase was completely protected against the inhibitory effects of Mg^{2+}. Interestingly, at the concentrations of Ca^{2+} and Mg^{2+} present in sea water, 10 mM and 50 mM respectively, the 41-kDa species was partially inhibited. This result suggests the possibility of a dynamic role for Ca^{2+} and Mg^{2+} in modulating the activity of the 41-kDa collagenase/gelatinase on the surface of the embryo.

DISCUSSION

The pericellular space is composed of the extracellular matrix and the ectodomains of transmembrane proteins. These protein species are intimately involved in a range of biologic processes that control many facets of cell behaviour. The extracellular matrix can modulate signal transduction events both directly and indirectly while ectodomains can serve as receptors for signaling molecules such as growth factors and, in some instances, as a source of diffusible, bioactive signaling molecules generated by proteolytic cleavage [Werb and Yan, 1998]. Maintaining the correct mix and form of proteins in the pericellular environment is dependent, in part, upon proteolytic activities. These proteases belong to four families: the matrix metalloproteases, the adama-

lysins, the astacins, and serine proteases [Werb, 1997]. The first three families are metalloproteases characterized by a requirement for Zn^{2+} at the active site. Some members of these families also require Ca^{2+} for activity.

The results reported here identify the 41-kDa collagenase/gelatinase as a member of the serine-dependent class of proteases. Inhibition of gelatin-cleavage activity was noted in the presence of either reversible or irreversible serine protease inhibitors. However, in contrast to the near complete inhibition of trypsin, the archetypical serine protease, only partial inhibition of the 41-kDa species occurred. In addition, the approximately 50% inhibition seen in our quantitative assay required preincubation of the collagenase/gelatinase with the inhibitor before addition of the substrate. These results suggest that the reactive serine residue is at least partially sequested from the bulk phase medium. Previous work from this laboratory has shown that the 41-kDa species is not a nonspecific protease but rather has a collagen-cleavage activity specific for sea urchin collagen as well as a broader based gelatin-cleavage activity [Mayne and Robinson, 1996; Robinson 1997a; Mayne and Robinson, 1999]. We have previously identified several noncollagenous components of the sea urchin extraembryonic matrix [Robinson, 1990]. When these proteins were incubated with the 41-kDa species, we failed to detect any cleavage [Robinson, unpublished data]. We therefore believe that the collagen and gelatin components of the sea urchin extraembryonic matrices serve as physiologic substrates for the 41-kDa cleavage activity. To our knowledge all eucaryotic collagenases previously described possess an active site Zn^{2+} and belong to the metalloprotease class of endopeptidases. Although many cell-surface located serine proteases have been described, these activities appear to be responsible for cleaving the ectodomains of transmembrane proteins, as well as selected components of the extracellular matrix, and none have been shown to possess collagen-cleavage activity [Subramanian et al., 1997; Logeat et al., 1998; Werb, 1997]. The 41-kDa collagenase described here may be unique in belonging to the serine-dependent class of proteases. The structural basis for the unique serine-dependent nature of the 41-kDa cleavage activity is unknown at present. Collagen-cleavage activity may be facilitated by structural features of the 41-kDa species itself or that of echinoderm collagen.

We have previously shown that Ca^{2+} reactivates the EGTA-inhibited enzyme with an apparent kd of 3.7 mM [Mayne and Robinson, 1998]. In addition, we found that Ca^{2+} bound to the 41-kDa collagenase/gelatinase with an intrinsic dissociation constant of 0.5 mM. These results suggested that the 41-kDa species possessed low-affinity, Ca^{2+}-binding sites. The additional metal ion binding data reported here suggest that Ca^{2+} and Mg^{2+} can compete for binding to these low-affinity sites. Coupled with the localization of the 41-kDa collagenase/gelatinase to the extraembryonic matrix, the antagonistic effects of Ca^{2+} and Mg^{2+} on the 41-kDa activity, at concentrations reflective of those present in sea water, pose an interesting possibility concerning the level of "on site" cleavage activity. In the presence of bulk phase sea water, cleavage activity would be approximately 65% of maximum (Fig. 3). However, if components of the extraembryonic matrix could be sequestered from bulk phase sea water, then the level of 41-kDa cleavage activity would be different from that expected in the presence of 10 mM Ca^{2+} and 50 mM Mg^{2+}. In this regard, we have previously shown that hyalin, a major protein component of the apically located extraembryonic matrix (the hyaline layer) binds 400 moles of Ca^{2+} per mole protein at low-affinity sites [Robinson and Brennan, 1991]. The prevalence and large Ca^{2+}-binding capacity of hyalin may enable this protein to serve as a Ca^{2+} sink or reservoir within the hyaline layer, which could alter the local concentration of Ca^{2+} seen by other components of the layer. Proteoglycan components of extracellular matrices are known to bind growth factors and alter the local concentrations of these ligands in the vicinity of their receptors [Jessell and Melton, 1992]. Similarly, hyalin could contribute towards creating an ionic environment within the extraembryonic matrix that is significantly different from that found in the surrounding sea water. This possibility will need to be explored experimentally.

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