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Matrix Metalloproteinase Inhibitors Disrupt Spicule Formation by Primary Mesenchyme Cells

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The primary mesenchyme cells of the sea urchin embryo construct an elaborate calcareous endoskeletal spicule beginning at gastrulation. This process begins by ingression of prospective primary mesenchyme cells into the blastocoel, after which they migrate and then fuse to form a syncytium. Skeleton deposition occurs in spaces enclosed by the cytoplasmic cables between the cell bodies. Experiments are described which probe the role of proteases in these early events of spicule formation and their role in the continued elaboration of the spicule during later stages of embryogenesis. We find that several inhibitors of metalloproteinases inhibit the continuation of spiculogenesis, an effect first reported by Roe *et al.* (*Exp. Cell Res.* 181, 542–550, 1989). A detailed study of one of these inhibitors, BB-94, shows that fusion of primary mesenchyme cells still occurs in the presence of the inhibitor and the formation of the first calcite granule is not impeded. Continued elaboration of the spicule, however, is completely stopped; addition of the inhibitor during the active elongation of the spicule stops further elongation immediately. Removal of the inhibitor allows resumption of spicule growth. The inhibition is accompanied by almost complete cessation of massive Ca ion transport via the primary mesenchyme cells to the spicule. The inhibitor does not prevent the continued synthesis of several spicule matrix proteins. Electron microscopic examination of inhibited primary mesenchyme cells shows an accumulation of characteristic vesicles in the cytoplasm. Gel zymography demonstrates that although most proteases in homogenates of primary mesenchyme cells are not sensitive to the inhibitor *in vitro,* a protease of low abundance detectable in the medium of cultured primary mesenchyme cells is inhibited by BB-94. We propose that the inhibitor is interfering with the delivery of precipitated calcium carbonate and matrix proteins to the site(s) of spicule growth. © 1998 Academic Press

INTRODUCTION

The formation of a calcareous endoskeleton by primary mesenchyme cells of the sea urchin embryo affords many opportunities to understand the cellular and molecular basis of organogenesis. In indirectly developing euechinoid sea urchins the fourth cell division generates four smaller cells called micromeres at the vegetal pole. At the fifth cell division each of these cells generates a much smaller nonskeletogenic micromere (which will populate the coelomic pouches) and four skeletogenic micromeres. These latter cells undergo three divisions to form a cohort of 32 cells on the vegetal wall of the blastula. These cells then lose their epithelial character and emigrate into the blastocoel as migrating mesenchyme cells. After several hours the primary mesenchyme cells (PMCs) adopt stereotypical locations next to the blastocoel wall in the prospective ventrolateral portion of the embryo. The PMCs then fuse to form a syncytium, the cell bodies of which are connected by long cytoplasmic cables. The calcareous endoskeleton is deposited in spaces enclosed by these PMC bodies and cables (Okazaki, 1975).

Roe *et al.* (1989) reported that metal chelators known to inhibit metalloproteinases irreversibly blocked spicule formation while most other processes of embryonic development continued. Furthermore, treatment with synthetic substrates of metalloproteinases could reversibly block spicule formation. It was suggested by Roe *et al.* (1989) that metalloproteinases might be essential for cell fusion events required for spicule formation. PMCs possess intracellular deposits of precipitated calcium (Decker and Lennarz, 1988) and spicule matrix proteins (Urry and Wilt, unpublished observations). Perhaps investigations of the cell biological basis of biomineralization in PMCs will furnish some ideas about how cells produce biominerals in other situations.

Roe *et al.* (1989) also raised the possibility that chelators could inhibit enzymes like lysyl oxidase, which are involved in collagen cross-linking. Inhibition of collagen metabolism and other components of the extracellular matrix

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is also known to affect spicule formation (Blankenship and Benson, 1984; Solursh, 1986; Wessel and McClay, 1987). There is considerable literature showing relationships between proteases, the extracellular matrix, and differentiation (reviewed by Matrisian, 1990). Metalloproteinases have been implicated in fusion of myoblasts (Yagami-Hiromasa *et al.*, 1995) and other cell types (Huovila *et al.*, 1996), morphogenesis (Chin and Werb, 1997), exocytosis (Mundy and Strittmatter, 1985; Mundy *et al.*, 1987), and processing of growth factors (Black *et al.*, 1997; Huovila *et al.*, 1996).

We set out to investigate the role of proteases in spicule formation, concentrating on the use of more specific and nontoxic inhibitors of metalloproteinases. There is considerable interest in the biomedical community to discover nontoxic protease inhibitors for clinical use. We have found that several of these inhibitors reversibly and completely block spicule elongation. A detailed study of one of these, BB-94, shows that it reversibly blocks spicule elongation, but not PMC fusion or the initial formation of a calcite granule. Study of inhibited cells suggests that the inhibited protease is functioning in the exocytosis of the spicule precursors.

MATERIALS AND METHODS

Animals and Embryos

Adult *Strongylocentrotus purpuratus* were collected in the intertidal zone off the coast of Point Arena, California. Adult *Lytechinus pictus* were obtained from Marinus Inc. (Long Beach, CA). Gametes were spawned by intracoelomic injection of 0.5 M KCl. Eggs were washed several times in 0.45 μm Millipore filtered seawater (MFSW), fertilized with a dilute suspension of sperm, and cultured with constant stirring at 15°C.

Matrix Metalloproteinase Inhibitor Treatment

Embryos were cultured in the presence of 5 μM BB-94 (a small-molecular-weight compound bearing a hydroxamic acid group, provided by British Biotechnology Ltd., Oxford, England) (Davies *et al.*, 1993; Wang *et al.*, 1994) or 15 μM thiol inhibitor (ISN-3825, provided by Peptides International, Lexington, KY) for various times after hatching. Preliminary experiments were carried out to determine which levels had biological effects without apparent toxicity, and we chose to employ concentrations near the high end of the nontoxic range. Micromeres were isolated from 16-cell-stage embryos as described by Kitajima and Okazaki (1980) and cultured in artificial seawater (ASW) (Jamarin Co., Osaka, Japan) containing 4% horse serum and 10 μg/ml gentamycin. BB-94 (5 μM) was added to the micromere cultures at 24 h. TIMP-2 (provided by Amgen, Inc., Thousand Oaks, CA; the TIMP-2 protein does not cross the blastocoel wall) was microinjected into the blastocoel of mesenchyme blastula-stage embryos (~50 pl of a 10 mg/ml solution in SW) as described previously (Ingersoll and Ettensohn, 1994). Embryos were removed from microinjection chambers and cultured in a drop of MFSW on a depression slide in a humid chamber for 24 h. Embryos were observed on an Olympus BH-2 microscope with differential interference contrast optics and photographed with Kodak T-Max 100 film.

RNA Isolation and Northern Blots

RNA was isolated from 48-h embryos by the method of Chomczynski and Sacchi (1987) and analyzed on Northern blots as described by Ausubel *et al.* (1987). Blots were hybridized with random-primed DNA probe synthesized from the following cDNA clones: pNG7 for *SM30* (George *et al.*, 1991), pHS72 for *SM50* (Benson *et al.*, 1987b), pSpec1 for *spec1a* (Carpenter *et al.*, 1984), and pSpBd6 for *ubiquitin* (Nemer *et al.*, 1991) genes. Northern blots were washed at high stringency and exposed to Kodak X-OMAT film at −80°C with an intensifying screen. Blots were analyzed quantitatively on a Molecular Dynamics SF phosphorimager.

Protein Analysis

BB-94-treated and control embryos were collected at 48 h, homogenized in 5 vol of Laemmli sample buffer (Laemmli, 1970), and incubated in boiling water for 5 min. Samples were separated on 10% SDS–PAGE gels and transferred to nitrocellulose. Blots were blocked overnight in blotto (5% nonfat dry milk in 10 mM Tris-buffered saline), probed with anti-SM50 antiserum (Killian and Wilt, 1996) diluted 1:1000 in blotto for 2 h, and washed 3 × 10 min in 10 mM Tris-buffered saline containing 0.05% Tween 20 (TTBS). Blots were incubated in peroxidase-conjugated goat anti-rabbit antibody diluted 1:5000 in blotto for 1 h at room temperature, washed 3 × 10 min in TTBS, and developed using the enhanced chemiluminescence reagent (Amersham) according to the manufacturer's instructions.

To detect SM30, micromere cultures were labeled with [^{35}S]-methionine (50 μCi/ml) for 2 h. Cells were scraped from the dish and homogenized in 50 mM Tris, pH 7.5, 0.15 M NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS containing a protease inhibitor cocktail (Benson *et al.*, 1987a). After preclearing with preimmune serum and protein A–Sepharose, anti-SM30 (Killian and Wilt, 1996) and protein A–Sepharose were added to the homogenates and incubated 4 h at 4°C. The protein A–Sepharose beads were washed 5× in the homogenization buffer and once in 10 mM Tris, pH 7.5, 0.1% NP-40 and bound SM30 was eluted by boiling the beads in Laemmli sample buffer. Labeled SM30 protein was detected by SDS–PAGE and fluorography.

Cell Fusion Assay

Embryos were labeled with DiI C_{18} (Molecular Probes, Eugene, OR) by incubating hatched blastula-stage embryos in 83 μg/ml DiI, 0.28M sucrose in MFSW for 5 min followed by several washes in MFSW. Cell transplantation experiments were carried out essentially as described by Ettensohn and McClay (1986). PMCs from DiI-labeled embryos were removed with a micropipet at early mesenchyme blastula stage and one or two cells were transferred into the blastocoel of unlabeled host embryos. Host embryos were removed from microinjection chambers and allowed to develop in a drop of MFSW in the presence or absence of 5 μM BB-94 on a depression slide in a humid chamber at 15°C in the dark. After 18 h, embryos were collected and examined using the rhodamine filter set and epifluorescence optics. Fusion was evident as the transfer of dye from labeled donor PMCs to previously unlabeled host PMCs.

Labeling of Insoluble Calcium

Micromeres were cultured in the presence of 4% horse serum from 24 to 48 h during which time they were undergoing vigorous

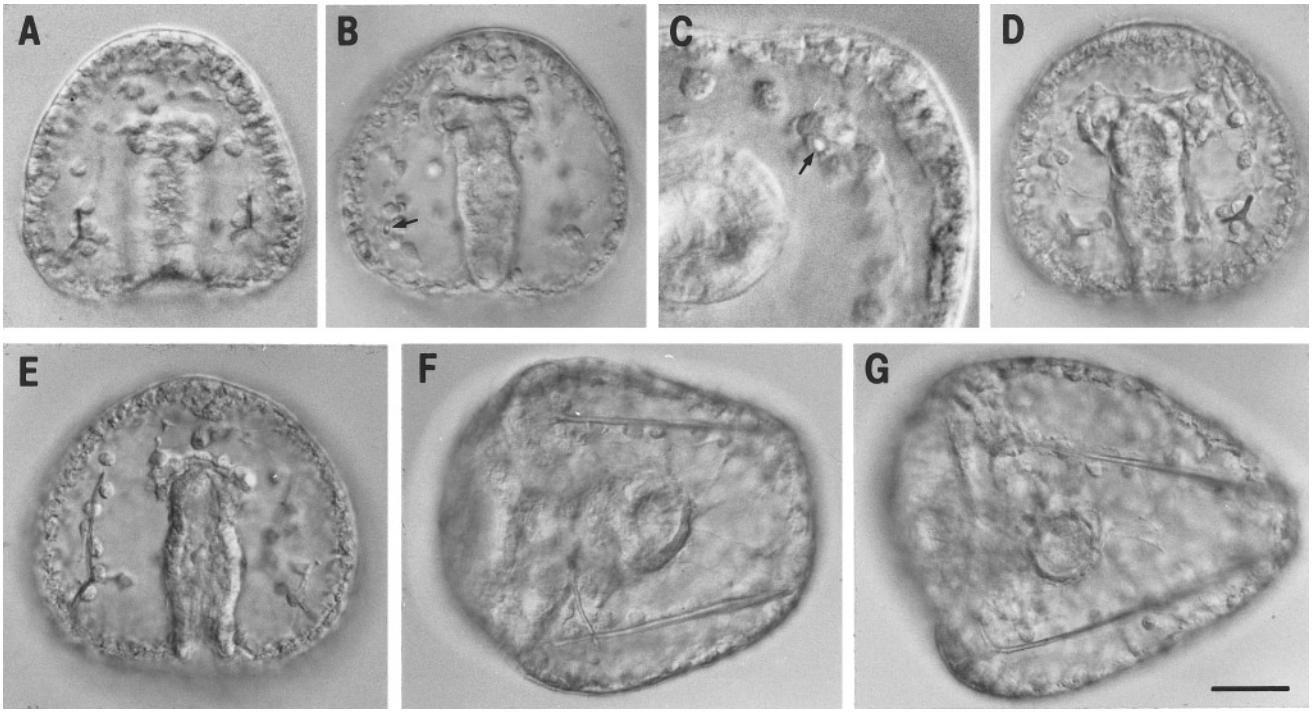


FIG. 1. The effect of matrix metalloproteinase inhibitors on sea urchin development. Embryos were treated with inhibitors beginning at 24 h and allowed to develop to 48 h. (A) TIMP-2 (~1 ng) was injected into the blastocoel. (B, C) BB-94 was added to ASW (5 μM). (D) BB-94 was added to ASW at 36 h and embryos were allowed to develop to 48 h. (E) Untreated control embryos at 48 h. (F) Embryo exposed to BB-94 from 24 to 48 h, washed, and then allowed to develop in ASW from 48 to 72 h. (G) Control embryo at 72 h. Scale bar, 30 μM for A, B, and D–G and 60 μM for C.

spicule elongation. Insoluble calcium labeling was performed by a method adapted from that of Hwang and Lennarz (1993). Serum-containing medium was removed and replaced with ASW containing 10 μCi/ml $^{45}CaCl_2$. At the end of the labeling period, the ^{45}Ca was removed and the cells were washed into complete ASW and either chased with ASW or collected. The cells were dislodged with a plastic policeman, collected by centrifugation (800*g*, 10 min), and washed 2× in ASW. The cell pellets were homogenized in 2% sodium cholate, 5 mM $CaCl_2$, 20 mM Tris, pH 7.5, and insoluble material was pelleted in a microfuge. The supernatant was removed and the remaining pellet of cholate-insoluble material (mostly spicules) was demineralized in 1 M HCl. A portion of each sample was removed for protein determination using BCA reagent (Pierce). Econo-safe liquid scintillant (Research Products International) was added to all samples and they were counted in a Beckman scintillation counter.

Zymography

Micromeres were cultured in the presence of 4% horse serum from 24 to 36 h after fertilization followed by culture in ASW for an additional 18 h. PMC-conditioned ASW was collected, cells were removed by centrifugation (1000*g*, 10 min), and the medium was dialyzed against dH_2O and lyophilized. Samples were analyzed by gelatin zymography as described in Brenner *et al.* (1989). Samples were dissolved in Laemmeli sample buffer without reducing agents and separated on 7.5% SDS–PAGE gels containing 1 mg/ml gelatin. After electrophoresis, gels were washed in 2.5% Triton X-100 for 20 min and incubated overnight in 50 mM Tris, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$ at 37°C in the presence or absence of protease inhibitors. Gels were stained with Coomassie blue, destained, and dried.

Electron Microscopy

Micromeres were cultured on Therminox coverslips in the presence or absence of 5 μM BB-94. Cells were fixed at 55 h in glutaraldehyde, followed by osmium ferricyanide as described by McDonald (1984). Cells were serially sectioned, stained with uranyl acetate and lead citrate, and examined on a JEOL electron microscope.

RESULTS

Reversible Inhibition of Spiculogenesis by Protease Inhibitors

As an initial attempt to investigate the role of matrix metalloproteinases in sea urchin development, we employed the use of specific inhibitors of matrix metalloproteinases (MMPs). We treated embryos at different stages of development with a variety of MMP inhibitors and assayed their effect on development. Microinjection of the TIMP-2 protein into the blastocoel at mesenchyme blastula stage

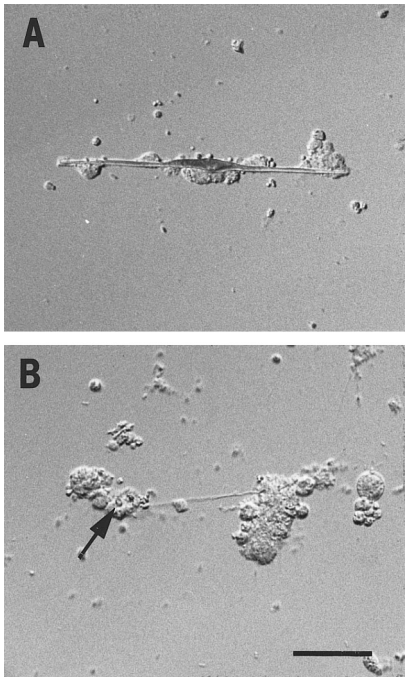


FIG. 2. The effect of BB-94 on spicule formation in PMC cultures. Micromeres from 16-cell-stage embryos were cultured in 4% horse-serum-containing medium. These cells differentiate into PMCs and make elongated spicule rods (A). Cells cultured in the presence of 5 μM BB-94 make only small granules of spicule material in cell clusters (B, arrow). A filopodial cable between cell clusters seen in B does not contain spicule material. Scale bar, 30 μM.

caused an inhibition of spicule elongation. Spiculogenesis was initiated and small triradiate spicules were formed, but in all the injected embryos the spicules did not elongate from the ventrolateral clusters (Fig. 1A). Gastrulation, endoderm differentiation, pigment cell development, and other aspects of morphogenesis were unaffected. Injection of TIMP-2 into the blastocoel before PMC ingression did not inhibit ingression. Injection of seawater or control proteins had no effect on morphogenesis (data not shown). Injection of TIMP-2 into the space between the embryo and the fertilization envelope inhibited the collagenase-like hatching enzyme and blocked hatching. Therefore, the inhibitor is active in seawater and is able to inhibit sea urchin MMPs.

Treatment of embryos with the small molecule inhibitors BB-94 or thiol inhibitor, added to the seawater, had very similar effects on morphogenesis. Treatment with these inhibitors at the mesenchyme blastula stage resulted in an inhibition of spicule formation in all embryos (Figs. 1B and 1C). Spicule initiation did occur in these embryos as evidenced by the small granules of spicule material present in the ventrolateral clusters. However, these granules did not elongate to form triradiate spicules. These inhibitors had no other pronounced effects on morphogenesis. Gastrulation, PMC ingression, and pigment cell differentiation all occurred normally. Embryos cultured continuously in the presence of these inhibitors continue to develop rather normally except for spicule elongation, and they eventually developed into a larva with short, crumpled anal arms and oral hoods. This suggests that some of the prominent shape changes undergone by the embryo during pluteus larva formation are not entirely due to the spicule. Initiation of the

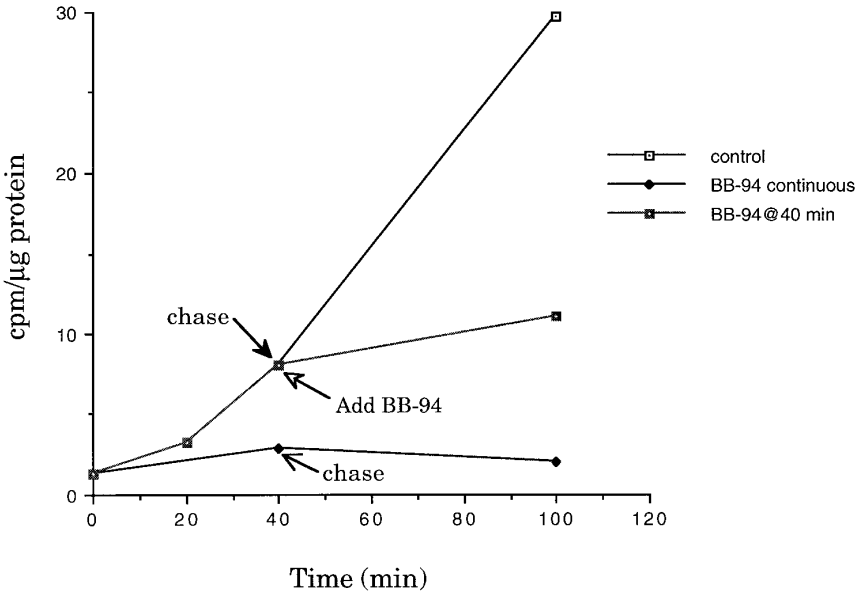


FIG. 3. The effect of BB-94 on calcium incorporation into the spicule. Cultured PMCs were incubated in the presence of ^{45}Ca for 40 min in the presence (BB-94 continuous, ♦) or absence (control□ and BB-94 chase■) of BB-94. The cells were rinsed into complete ASW and chased an additional 60 min in the presence (BB-94 continuous, ♦; BB-94@40 min ■) or absence (control, □) of BB-94. Samples were collected at various times and the amount of ^{45}Ca incorporated into insoluble spicule material was determined.

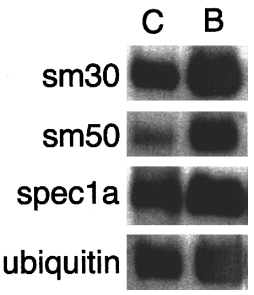


FIG. 4. Northern blot analysis of spicule matrix protein genes. Total RNA from 48-h control embryos (C) and embryos treated with BB-94 from 24 to 48 h (B) was analyzed on Northern blots and probed for the spicule matrix proteins *SM50* and *SM30* as well as the aboral ectoderm-specific gene *spec1a*. Five such experiments were carried out. *SM50* and possibly *SM30* mRNA levels appear to be elevated relative to the ubiquitin-loading control, while the aboral ectoderm-specific *spec1a* appears at about control levels.

formation of anal arms and the oral hood are apparently inherent in the ectoderm, and the spicules seem to elongate and support these newly formed structures. Addition of the inhibitors at fertilization had no additional effects on morphogenesis except that hatching was inhibited. Thus, these inhibitors are active in seawater for up to 18 h and they do not interfere with any other events in early morphogenesis. Both BB-94 and the thiol inhibitor have identical effects on spicule formation in nearly all the treated embryos and gave nearly identical results in all subsequent assays. For the sake of simplicity, only the results for BB-94 will be presented hereafter.

BB-94 is also effective at inhibiting continued spicule formation once it has begun. Addition of BB-94 during spicule formation resulted in a rapid inhibition of spicule elongation (Fig. 1D). No measurable growth of spicules was observed subsequent to addition of BB-94, as judged by measurements with an ocular micrometer. Furthermore, if BB-94 and calcein are introduced simultaneously, no calcein fluorescence is observed in the spicules. Calcein is a fluorophore that becomes brightly fluorescent when precipitated or cocrystallized with calcium salts and hence is an excellent marker for spicule formation (Ilan *et al.*, 1996; Guss and Ettensohn, 1997). This effect of BB-94 is stage independent and rapid inhibition of spicule elongation occurs at any stage of development.

BB-94 inhibition of spicule elongation is reversible. Removal of BB-94 from the seawater allows the PMCs to recover their ability to produce elongated spicules (Fig. 1F). The patterning and branching of the spicule are not always completely normal during recovery from BB-94 inhibition. However, embryos always recover their ability to produce more elongated spicules within 1 h of BB-94 removal as judged by direct measurement with a micrometer or calcein incorporation (data not shown).

These inhibitors could be inhibiting PMC proteases or a proteases produced by other cell types that are necessary for PMC spiculogenesis. We tested whether BB-94 acts directly on PMCs by culturing micromeres in the presence of BB-94. In control cultures, many long spicule rods were produced (Fig. 2A). However, in cells treated with BB-94, only small calcite granules of spicule material were produced in clusters of cells (Fig. 2B, arrow). This demonstrates that BB-94 acts directly on PMCs and suggests that it is probably inhibiting a MMP that is produced by PMCs and that plays an important role in spicule elongation.

Cessation of spiculogenesis would be expected to coincide with perturbation of the massive Ca ion influx and precipitation that these cells are known to carry out (Nakano *et al.*, 1963). We examined the flux of calcium through the PMCs and into the spicule using ^{45}Ca. Cultured PMCs in the most active phase of spicule elongation were labeled with $^{45}CaCl_2$ for 40 min in the presence or absence of BB-94; then the cultured cells were washed and incubated in seawater without radioactive calcium, in either the presence or absence of BB-94. Samples were removed for analysis at various time points and the amount of radioactivity incorporated into spicule material was determined. In control cultures (labeled for 40 min and then washed and cultured 60 min more), there is a linear accumulation of ^{45}Ca in the spicule over time (Fig. 3). There is a large increase in ^{45}Ca incorporated during the chase period, indicating that PMCs sequester a large intracellular pool of Ca for use in spiculogenesis. If cultures are treated with BB-94 throughout both the labeling and chase periods, almost no detect-

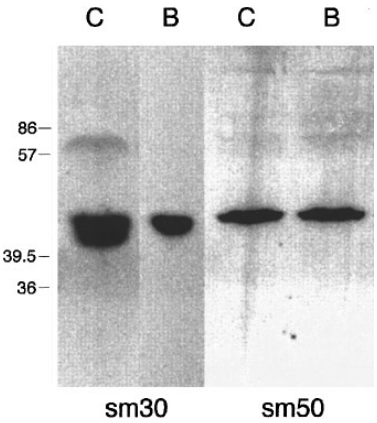
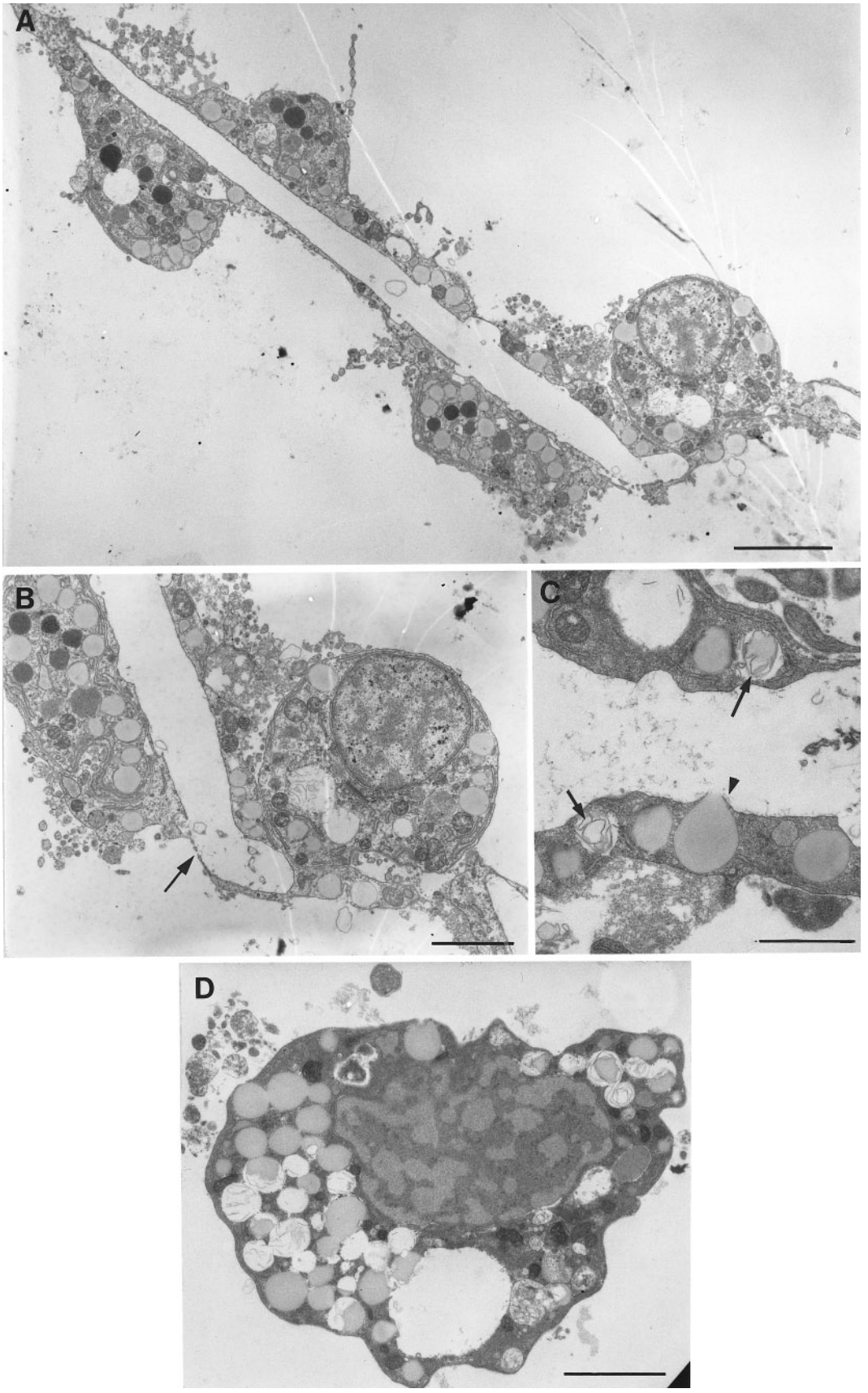


FIG. 5. Determination of spicule matrix protein levels in BB-94-treated embryos. SM30 was detected as an immunoprecipitated [^{35}S]methionine-labeled protein from micromere cultures. In control cultures (C), the normal SM30 doublet is detected, at this exposure level, as a broad band, while in BB-94-treated cultures (B) only the upper band of the SM30 doublet is present. The level of radioactive SM30 protein in the BB-94-treated samples appears to be somewhat reduced compared to controls. The signal present at approximately 65 kDa in control sample is contaminating background precipitate that is variably present in analyses of this type. SM50 was analyzed on conventional Western blots probed with anti-SM50. In this case, the levels of SM50 appear identical between control (C) and BB-94-treated (B) embryos.



able ^{45}Ca is incorporated into the spicule. Cells loaded with ^{45}Ca in the absence of BB-94 for 40 min and then treated with BB-94 during the 60-min chase in the absence of ^{45}Ca show little additional accumulation of radioactivity in the spicule after BB-94 addition. This is consistent with earlier experiments demonstrating that BB-94 causes a very rapid inhibition of spicule elongation. Hence, a possible effect of this inhibitor may be to rapidly block properly directed calcium carbonate delivery from the cell into the appropriate domain of the elongating spicule.

The Effect of BB-94 on Spicule Matrix Protein Accumulation

Since the MMP inhibitors have such a dramatic effect on spicule elongation, we were interested in determining what effects they have on the synthesis or accumulation of spicule matrix proteins and their corresponding mRNAs. Total RNA from control and BB-94-treated embryos was analyzed on Northern blots and probed with cDNA probes for the spicule matrix protein genes *SM50* and *SM30* as well as the aboral ectoderm specific gene *spec 1a*. *SM30* and *SM50* message levels appear to be elevated relative to the ubiquitin loading control, while the aboral ectoderm-specific *spec1a* appears at about control levels (Fig. 4). Quantitation of the signal on a phosphorimager shows an increase in the level of *SM30* by 1.63 ± 0.57-fold and an increase in *SM50* message level by 1.9 ± 0.24-fold, while the *spec 1a* message was present at 1.29 ± 0.29-fold of control. These numbers are the averages of five independent experiments. Therefore, treatment of embryos with BB-94 results in a modest increase in the levels of *SM50* and perhaps *SM30* messages. Analysis of *SM30* and *SM50* RNA on polyribosome gradients from BB-94-treated embryos indicated that all of these mRNAs are loaded on polysomes and are presumably translated (data not shown). This could conceivably lead to a modest accumulation of SM30/50 matrix protein if the translation rate and stability of the proteins were unaffected.

We examined the levels of spicule matrix proteins in normal and BB-94-treated embryos by Western blots and immunoprecipitation. The levels of SM50 protein were analyzed on conventional Western blots. Equal amounts of protein from control and BB-94-treated embryos were separated by SDS–PAGE and the levels of SM50 were determined by probing the blots with the anti-SM50 antibody (Fig. 5). These results demonstrate that SM50 is present at nearly the same level in control and BB-94-treated embryos. Since there is a modest but significant increase in the level of SM50 mRNA in BB-94-treated embryos, but normal levels of the SM50 protein, there may be some posttranslational regulation of the levels of this protein in the cells.

SM30 protein from whole embryos could not be detected on Western blots; therefore, SM30 was detected as an [^{35}S]-methionine-labeled protein immunoprecipitated from micromere cultures and analyzed by electrophoresis and fluorography. The levels of SM30 appear to be slightly reduced in BB-94-treated cultures. Immunoprecipitations from BB-94-treated cultures show a single band at 46 kDa. This is characteristic of the intracellular isoform of SM30 (Hamilton and Wilt, unpublished data). Immunoprecipitation of SM30 from control cultures shows both the 46- and 43-kDa isoforms of SM30 which are produced when spicules are being formed (Killian and Wilt, 1996). This result suggests that some processing step in SM30 genesis is inhibited by BB-94, either directly or indirectly. Staining of BB-94-inhibited embryos with antibodies to SM30/50 did not reveal any obvious alterations in staining patterns compared to control embryos (data not shown).

Examination of BB-94-Treated PMCs by Electron Microscopy

We examined control and BB-94-treated PMCs in culture by electron microscopy to determine if there are any alterations in the ultrastructure of the PMCs in the presence of the inhibitor. Control PMCs formed long spicules in culture (Fig. 6A). The spicule itself demineralizes during fixation leaving a predominantly empty space where the spicule once was. One characteristic of the ultrastructure of PMCs that we noticed was the relative abundance of medium density grey veiscles in the cell. These vesicles are present in normal PMCs in more moderate numbers (Fig. 6A) and are nearly absent in PMCs cultured in the absence of serum and therefore not making spicules (data not shown). We have observed these vesicles in the apparent process of secreting their contents into the spicule space (Fig. 6C, arrowhead). We also observed vesicles with a lamellar structure, which we interpret to be postsecretory medium density grey vesicles (Fig. 6C, arrows). One possibility is that these are the vesicles that store and secrete the spicule matrix proteins and/or precipitated calcium. In BB-94-treated cells, these medium density vesicles are relatively numerous and other vesicular types (very dense, endocytic, etc.) are relatively less numerous. Some of the medium dense vesicles can be seen to be composed partially of lamellae, partially

FIG. 6. Electron microscopic visualization of the effects of BB-94. PMCs in culture were examined by electron microscopy at 55 h after fertilization. At this time, the PMCs have synthesized long spicule rods (A). Higher magnification of the tip of one of these spicules shows the small holes present in the syncytial cable (B, arrow). PMCs have a large number of characteristic medium grey vesicles that appear to be fusing with the spicule sheath and depositing their contents into the spicule space (C, arrowhead). After these vesicles apparently secrete their contents, a lamellar network remains (C, arrows). PMCs cultured in the presence of BB-94 accumulate a large number of these vesicles in their cytoplasm, some of which appear to be in the process of degradation (D). Scale bar, 5.2 μM for A, 2.6 μM for B and D, and 1.5 μM for C.

of grey background (Fig. 6D), which we suggest is a pathological appearance caused by BB-94 treatment. Of course, these static images do not themselves prove how spicule formation occurs nor how the inhibitor blocks the process, but they are consistent with the idea that BB-94 may somehow block the exocytosis of these vesicles into the spicule space.

The Effect of BB-94 on PMC Fusion

It has been previously suggested that metalloproteinase inhibitors might block spicule formation in PMCs by inhibiting cell fusion and syncytium formation (Roe *et al.*, 1989). We tested whether BB-94 inhibits cell fusion by PMCs, *in vivo*, using a dye transfer assay. In this assay, one or two PMCs labeled with the lipophilic dye DiI were transplanted into the blastocoel of an unlabeled mesenchyme blastula-stage embryo. These embryos were allowed to develop in the presence or absence of BB-94. The labeled donor cells mixed with the endogenous unlabeled cells and became incorporated into the normal PMC ring. If fusion occurs, dye will be transferred from the labeled donor cell membranes to the host cell membranes, and the latter should become fluorescently labeled. Figures 7A and 7B show a control embryo with a labeled PMC (Fig. 7A, arrow) 18 h after transplantation at about 48 h of development. The brightly labeled donor PMC has transferred some dye to neighboring cells in the syncytium. In the presence of BB-94 (Figs. 7C and 7D) cell fusion and dye transfer occur normally. In this case, the dye from the labeled donor cells (Fig. 7C, arrow) has spread throughout the PMC ring. The donor cell is out of focus, creating an apparent large, diffuse image, in order to show distant PMCs in focus to which dye has been transferred. Intentionally transplanting non-PMCs (secondary mesenchyme cells) that had been DiI labeled resulted in no transfer of dye to host PMCs (data not shown). Therefore, PMC fusion and syncytium formation appear to occur normally in the presence of BB-94 and the inhibition of cell fusion is not the mechanism by which BB-94 inhibits spicule formation.

Identification of a PMC-Specific, BB-94-Sensitive Protease

We used gelatin zymography to identify BB-94-sensitive proteases produced by PMCs. Micromeres were cultured in the presence of horse serum from 24 to 36 h postfertilization, followed by culture in ASW without serum for an additional 18 h. We analyzed this PMC-conditioned ASW on gelatin zymography gels and tested for the presence of BB-94-sensitive, gelatin-degrading proteases. One PMC-specific 51-kDa protease was found that was inhibited by BB-94 (Fig. 8, arrow). Other BB-94-inhibited proteases present in the lane are contaminating horse serum MMPs. The other lower molecular weight proteases detected on these gels are PMC-specific proteases of an unknown class. We tested the inhibitor sensitivity of this 51-kDa protease against different protease inhibitors. This protease is sensitive to all MMP inhibitors tested (BB-94, thiol inhibitor, *o*-phenanthroline, and EDTA) but is not sensitive to the serine protease inhibitors PMSF and benzamidine at concentrations that effectively inhibited proteases of the appropriate class. Thus, we believe that we have identified a candidate metalloprotease that may play a crucial role in spicule formation by PMCs. It is a 51-kDa protease that is inhibited by MMP inhibitors, but not by inhibitors of other classes of enzymes. Activation of these enzymes with organomercurial compounds had no effect on their activity (data not shown).

DISCUSSION

We have provided evidence that a metalloprotease inhibitor arrests development of the endoskeleton and extended earlier findings that implicated metalloproteases in spiculogenesis. The inhibitors are nontoxic and reversible and affect only growth of the spicule, not its initiation. While many modes of action of the inhibitor are possible, there is considerable circumstantial evidence indicating that secretion of the spicule components is affected by the inhibitor. The inhibitor should provide a valuable tool for the study of biomineralization in sea urchin embryos. BB-94 has been successfully employed to arrest human colon tumor growth (Wang *et al.*, 1994) and murine ovarian carcinoma (Davies *et al.*, 1993). Presumably its antitumor activity is due to effects on metalloproteases that affect the extracellular matrix. Chin and Werb (1997) have recently shown that several different specific metalloprotease inhibitors, including TIMP-2 used by us, profoundly derange morphogenesis and cell migrations in epithelia, muscle, and cartilage of the mandibular arch in mice. Proteases are involved in so many aspects of cell and tissue activity in development that perhaps it is not surprising there are so many different consequences of applications of inhibitors in different biological contexts. The specificity of the effect of BB-94 found here is, nonetheless, striking.

Characteristics of the Inhibition

BB-94, thiol inhibitor, and TIMP-2 all inhibit metalloproteinases at the active site, albeit by different chemical mechanisms. All three inhibitors produced the same highly specific and reversible inhibition of spicule elongation. Studies utilizing BB-94 showed the inhibition had no effect on cell viability or embryonic development, except for spicule elongation. Nucleation of the first calcite crystal in the ventrolateral PMC cluster was not impeded by any of the inhibitors, implying that calcite nucleation and subsequent spicule elongation have somewhat different mechanisms. The specificity and reversibility of BB-94 contrasts with the irreversible and less specific inhibition of spiculogenesis using metal-chelating agents like *o*-phenanthroline (Roe *et al.*, 1989). It is clear that BB-94 does not interfere with cell fusion events necessary to form the syncytium. We believe that it is likely that BB-94 and probably the other inhibitors

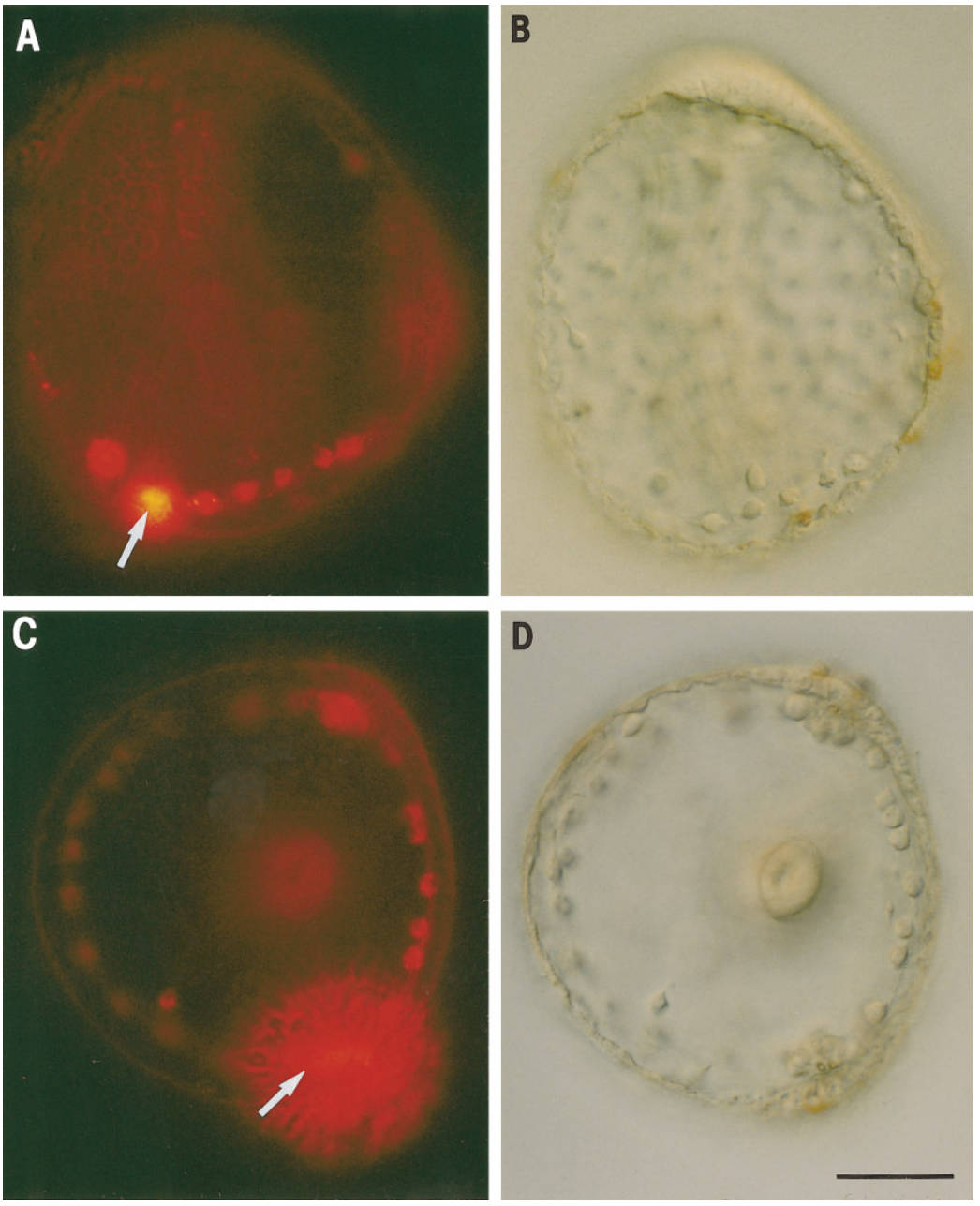


FIG. 7. The effect of BB-94 on PMC fusion. In the control embryo shown in this figure (A, B), a single labeled PMC (A, arrow) has transferred dye to neighboring PMCs in the ring. In embryos cultured in BB-94 after the transplant (C, D), cell fusion occurs normally. Two donor PMCs which are out of the plane of focus (C, arrow) have joined the PMC syncytium and dye has spread throughout the PMC ring. Scale bar, 30 μM.

we have studied affect an important component of the biomineralization pathway needed for spicule elongation.

There are a number of reports on identification and characterization of metalloproteinases in sea urchin embryos (Quigley *et al.*, 1993; Vafa and Nishioka, 1995; Mayne and Robinson, 1996). We searched for a metalloproteinase in homogenates of embryos and of cultured PMCs that was sensitive to BB-94 without success. It is possible that the enzyme is primarily intracellular but labile or very sensitive to degradation in cellular homogenates, even though protease inhibitors were present in homogenization medium. However, we did find an activity inhibited by BB-94 in the culture medium of PMC cultures. This may reflect some aspect of the protease action, e.g., it is released during exo-

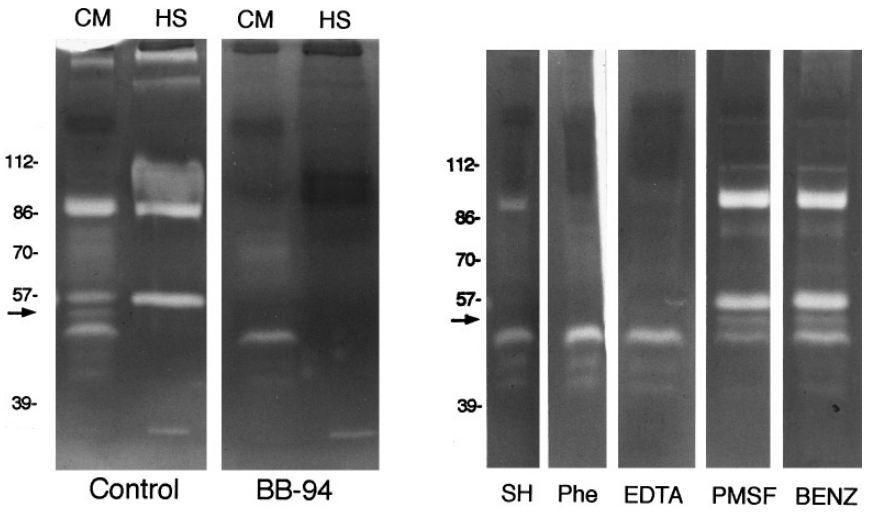


FIG. 8. Zymographic analysis of PMC metalloproteases. PMC-conditioned medium (CM) and horse serum (HS) were analyzed on gelatin zymography gels in the presence or absence of 5 μM BB-94 (left). A BB-94-sensitive protease at approximately 51 kDa was evident in PMC-conditioned medium, but not in horse serum (arrow). Analysis of PMC-conditioned medium on gelatin zymography gels in the presence of a variety of protease inhibitors indicates that the 51-kDa protease is a matrix metalloproteinase (right). SH, thiol inhibitor; Phe, 1,10-phenanthroline; EDTA, ethylenediaminetetracetic acid; PMSF, phenylmethylsulfonyl fluoride, Benz, benzamidine.

cytosis, or it participates in posttranslational modification of matrix proteins in the location where spiculogenesis occurs. There is precedent for involvement of metalloproteinases in exocytosis (Mundy and Strittmatter, 1985; Mundy *et al.*, 1987), for remodeling of the matrix of the blastocoel of the sea urchin embryo (Vafa *et al.*, 1996), or for removal of proteoglycans that inhibit mineralization in extracellular vesicles in chondrocyte cultures (Schmitz *et al.*, 1996). Further experiments will be required to resolve the exact role of metalloproteinases in spicule elongation.

We found no effect on transcription of an aboral ectoderm-specific gene, *spec-1a,* which is consistent with the BB94 treatment being selective and nontoxic. We were surprised to find a modest stimulation of *SM50* transcript accumulation. This stimulation was not reflected in greater accumulation of spicule matrix proteins nor did it alter their cellular distribution. This may indicate some translational or posttranslational regulation of spicule matrix protein accumulation in inhibited PMCs. It may also simply reflect that the techniques employed detect relatively small differences in the amount of mRNA more easily than small differences in accumulation of spicule matrix protein.

How Does the Inhibitor Act?

How does the inhibition of a protease interfere with spicule formation? Since the fundamental mechanism(s) of biomineralization, in this and other instances, is largely unknown (Lowenstam and Weiner, 1989; Simkiss and Wilbur, 1989), we can only speculate. There are several possibilities for a somewhat indirect mode of action. Among these are (1) inhibition of the protease might interfere with extracellular matrix remodeling, which in some unknown way is essential for spiculogenesis; (2) protease action might in some way be required for carbonic anhydrase activity which might be needed for calcium carbonate precipitation; and (3) the protease might be needed for intracellular trafficking and/or vesicle fusion during exocytosis of essential components for spicule elongation. These are not mutually exclusive possibilities, nor is this list exhaustive; it is merely intended to illustrate the range of possibilities. Notwithstanding, we consider secondary effects on the extracellular matrix as an unlikely explanation since BB-94 is active during all phases of spicule elongation. Requirements for blastocoel extracellular matrix integrity seem only to be required at early stages; administration of β-amino proprionitrile, an agent that profoundly disrupts the blastocoel matrix and inhibits initiation of spicule formation, has little effect if administered during spicule elongation (Wessel and McClay, 1987; Wessel *et al.*, 1991). We looked for effects of BB-94 exposure on carbonic anhydrase activity in embryo homogenates and found no effect. The data considered as a whole are consistent with the idea that BB-94 affects delivery of components from the cell to the elongating spicule, and we favor that possibility. The involvement of proteases in vesicle fusion and exocytosis has been shown for other systems. It should be interesting to isolate the BB-94-sensitive protease and develop tools to study its function in spicule growth directly.

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