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THE SOLUBLE METALLOENDOPROTEASE REQUIRED IN MYOBLAST FUSION REMAINS INTRACELLULAR

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Summary. The fusion of myoblasts to myotubes requires an endogenous soluble metalloendoprotease. To determine whether this protease is released by fusing myoblasts, or stays within the cell, we examined the effects of membrane-impermeant and a membrane-permeant metalloendoprotease inhibitors. Membrane-permeant 1,10-phenanthroline, and membrane-impermeant bathophenanthroline disulfonic acid both inhibited soluble metalloendoprotease activity in homogenized myoblasts with equal potency. However, while 1,10-phenanthroline inhibited fusion, bathophenanthroline disulfonic acid had no effect. In addition, metalloendoprotease activity could not be detected in the media of fusing myoblasts, but was in the cells. These observations support the conclusion that the soluble metalloendoprotease required in fusion remains within the myoblast.

Introduction. Previous studies have demonstrated that the fusion of myoblasts to form multinucleate myotubes requires metalloendoprotease activity at the time of fusion (1,2). Myoblasts contain both soluble and membrane-associated metalloendoproteases. By demonstrating differences in inhibitor specificities between metalloendoproteases in these two compartments, we have shown previously that the soluble, and not the membrane-associated protease, is required during fusion (2). The cell-cell fusion of myoblasts is initiated at the extracellular surfaces of the plasma membrane of two cells (3,4,5). A soluble metalloendoprotease could be required in this cell-cell fusion by: a) being released by the myoblast and hydrolyzing protein on the extracellular face of the plasma membrane or b) by remaining in the cell and hydrolyzing protein in the intracellular compartment, such as cytoskeleton proteins, or the cytoplasmic domain of integral membrane proteins. If the soluble protease is released into the extracellular compartment then two predictions would be that: 1) metalloendoprotease inhibitors which cannot cross cell membranes should inhibit myoblast fusion just as effectively as inhibitors which can cross the cell membrane and 2) metalloendoprotease activity should be detected in the media of fusing myoblasts. Observations

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presented here support the conclusion that the soluble metalloendoprotease required during fusion remains within the cell during fusion.

Materials and Methods

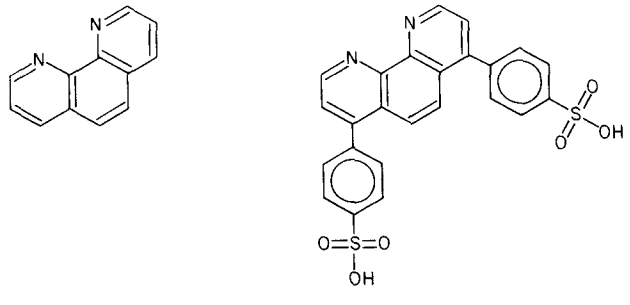
The metal chelators, 1,10-phenanthroline, which can enter cells (6), and bathophenanthroline disulfonic acid, which can not (7), were obtained from Sigma. The structure of these compounds is shown in Figure 1. Bathophenanthroline could not be employed as a control because of the cellular toxicity of solvents which dissolve this compound. 2-Aminobenzoyl-ala-gly-leu-ala-4-nitrobenzylamide was obtained from Enzyme Systems Products, Livermore, California.

Primary cultures of rat myoblasts were prepared and cultured as described previously (1). After culture in calcium deficient media for 48 hours, fusion was initiated by the introduction of 1.4 mM $CaCl_2$. 1,10 phenanthroline (in 0.5% DMSO) was added to the media at concentrations up to 1.10 mM. DMSO was shown previously to have no effect on fusion. Bathophenanthroline disulfonic acid (dissolved in buffer) was added at concentrations up to 2 mM. 1,10-Phenanthroline was added for two hours, then removed, and the cells washed in media containing 1.4 mM $CaCl_2$. Bathophenanthroline disulfonic acid was in the media for the duration of the experiment. Fusion was quantitated 24 hours later, using methods described previously (1).

Metalloendoprotease activity was assayed in the supernatant fraction of homogenized cells, as described previously (1), using the fluorescent substrate 2-aminobenzoyl-ala-gly-leu-ala-4-nitrobenzylamide.

Results and Discussion. As shown in Figure 2 the metal chelators 1,10-phenanthroline, and bathophenanthroline disulfonic acid both inhibited, in a dose dependent fashion, soluble metalloendoprotease from homogenized myoblasts. The IC_{50} for both compounds was approximately 0.2 mM.

1,10-Phenanthroline inhibits myoblast fusion by inhibiting a metal-dependent endoprotease, as we have shown previously (1,2). As shown in



1,10-Phenanthroline Bathophenanthroline Disulfonic Acid

Figure 1. Structure of 1,10-phenanthroline and bathophenanthroline disulfonic acid.

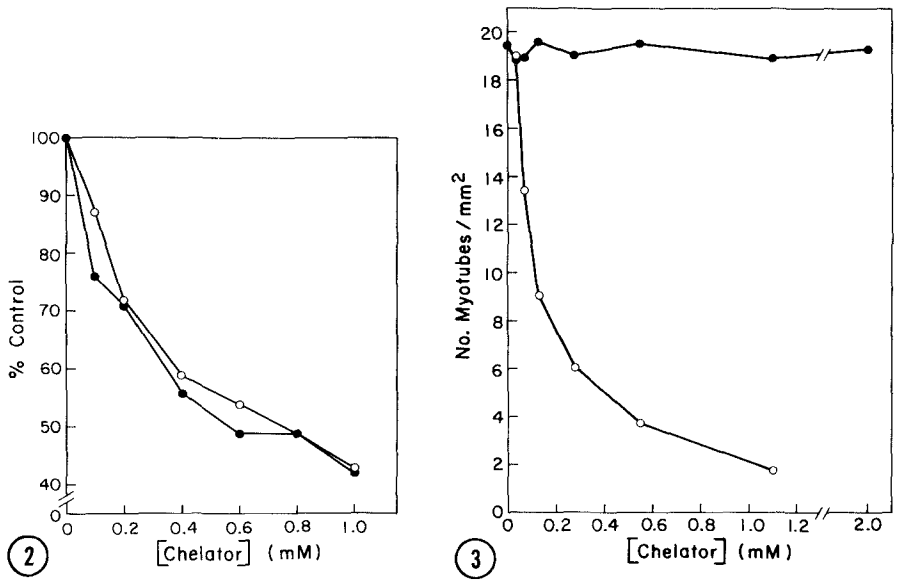


Figure 2. Inhibition of soluble myoblast metalloendoprotease activity by increasing concentrations of 1,10-phenanthroline (○) and bathophenanthroline disulfonic acid (●). Metalloendoprotease is assayed by spectrofluorometric measurement of hydrolysis of 2-aminobenzoyl-ala-gly-leu-ala-4-nitrobenzylamide.

Figure 3. Effects of 1,10-phenanthroline (○) and bathophenanthroline disulfonic acid (●) on myoblast fusion. Methods described in text.

Figure 3, the addition of 1,10-phenanthroline to the media prevented the fusion of myoblasts while membrane impermeant bathophenanthroline disulfonic acid over the same range of concentrations had no effect on fusion. Figure 4 demonstrates the morphology of myoblasts incubated with both compounds.

The fusion of myoblasts to multinucleate myotubes requires that the cells first withdraw from the cell cycle to begin the process of differentiation (3,4). Studies by us have demonstrated that metalloendoprotease activity is required at the time of fusion, since a number of metalloendoprotease inhibitors will prevent fusion. Further studies have shown that myoblasts have a membrane-associated metalloendoprotease and soluble metalloendoprotease. These metal dependent endoproteases have slightly differing inhibitor specificities. By examining the effects of these inhibitors on myoblast fusion, we previously demonstrated that a soluble, and not membrane associated, metalloendoprotease was required in fusion (2).

A soluble protease could be necessary for cell-cell fusion by either a) being released by the cell where it could hydrolyze extracellular face protein, or b) by remaining in the cell to hydrolyze intracellular protein, such as cytoskeleton, or the cytoplasmic domain of integral

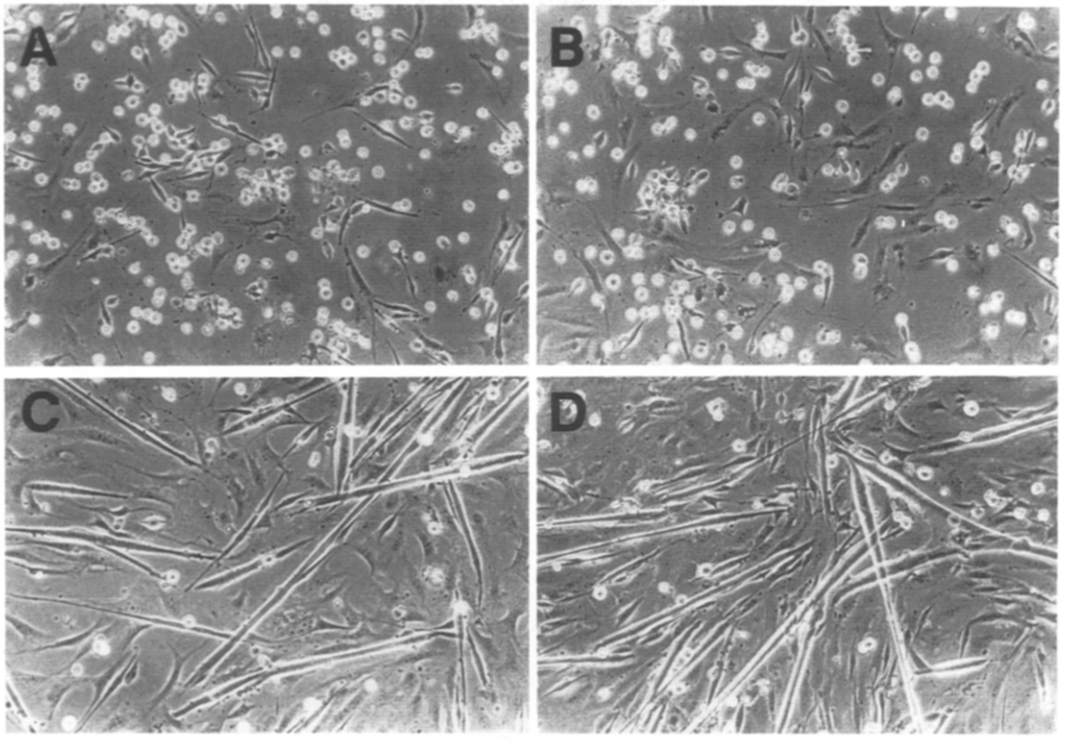


Figure 4. Effects of 1,10-phenanthroline (1.10 mM) (B); and bathophenanthroline disulfonic acid (1.10 mM) (D) on myoblast fusion 24 hours after the introduction of $CaCl_2$ into the media. Myoblasts grown in calcium deficient media (A), and myoblasts in $CaCl_2$ (1.40 mM) for 24 hours (C).

transmembrane protein. A variety of cells, for example neutrophils (8), synovial cells (9,10), and cartilage (11) release metalloendoproteases into the extracellular space. These proteases have been implicated in cell migration and tissue modelling. We therefore sought to determine whether the soluble metalloendoprotease required in myoblast fusion was released into the extracellular space by these cells. Although this protease could be easily assayed in myoblasts (1,2) using the sensitive synthetic protease substrate 2-aminobenzoyl-ala-gly-leu-ala-4-nitro-benzylamine, we could not detect metalloendoprotease activity in the media of fusing myoblasts (data not shown). Failure to detect metalloendoprotease activity in the media could have several explanations: 1) the protease is not released, 2) the protease is released, but at levels too low to detect, or 3) the protease is released and is then rapidly inactiviated by endogenous specific or nonspecific inhibitors.

One strategy to answer the question of cellular release of this protease would be to determine whether a metalloendoprotease inhibitor which inhibits the isolated protease, but does not enter the cell, has any

effect on fusion. Both 1,10-phenanthroline and bathophenanthroline chelate metals, and inhibited soluble metalloendoprotease from homogenized myoblasts. 1,10-Phenanthroline enters cells and inhibits myoblast fusion. Bathophenanthroline sulfonic acid, which does not cross the cell membrane because of its charged groups, had no effect on fusion.

A soluble, presumably cytoplasmic, metalloendoprotease could be required in fusion to hydrolyze protein on the cytoplasmic face of the plasma membrane. The role of cytoskeleton in myoblast fusion has not been investigated, but rearrangement of such protein could be a prerequisite to membrane-membrane fusion. Membrane fusion, for example, during exocytosis in adrenal chromaffin cells appears to require cytoskeleton protein (12) and also requires an endogenous metalloendoprotease (13). Based on the observations presented here, we are now examining myoblast cytoskeletal proteins during fusion as potential substrates for the metalloendoprotease.

Acknowledgement

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