Binding of Protease Nexin-1 to the Fibroblast Surface Alters Its Target Proteinase Specificity*

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Protease nexin-1 is a protein proteinase inhibitor that is secreted by a variety of cultured cells and rapidly forms complexes with thrombin, urokinase, and plasmin; the complexes then bind back to the cells and are internalized and degraded. In fibroblast cultures, protease nexin-1 is localized to the extracellular matrix. Here we report that protease nexin-1, which is bound to the surface of fibroblasts, forms complexes with thrombin, but not urokinase or plasmin. Experiments were conducted to determine directly if protease nexin-1 binding to the fibroblast surface alters its proteinase specificity. To do this, cell surface protease nexin-1 was inhibited using anti-protease nexin-1 monoclonal antibodies that stoichiometrically block its ability to form complexes with target proteinases. Then, purified protease nexin-1 was added to these cells; the cell-bound molecule formed complexes with thrombin, but not urokinase or plasmin. Similar experiments showed that protease nexin-1 bound to preparations of fibroblast extracellular matrix also formed complexes with thrombin, but not urokinase or plasmin. Components of the extracellular matrix other than heparin-like glycosaminoglycans are required for this regulation since heparin did not block the formation of complexes between protease nexin-1 and urokinase or plasmin. These results suggest that protease nexin-1 is primarily a thrombin inhibitor in interstitial fluids where much of it would be bound to cell surfaces.

Protease nexin-1 is an M, 43,000 protein proteinase inhibitor which controls several serine proteinases in the extracellular environment (1-5). It was first observed in serum-free culture medium from human fibroblasts (1), but is secreted by several types of cultured cells (3). Protease nexin-1 rapidly inhibits thrombin, urokinase, plasmin, and trypsin by forming stoichiometric complexes with their catalytic site serine residues (4). The protease nexin-1-proteinase complexes bind to the cells which secrete protease nexin-1, and via the protease nexin-1 moiety of the complex, are rapidly internalized and degraded (2, 6). Thus, protease nexin-1 provides a mechanism to control these serine proteinases at and near the cell surface.

Studies on likely biological roles of protease nexin-1 have shown that it modulates thrombin-stimulated cell division (7), and that added protease nexin-1 inhibits the degradation of smooth muscle ECM that is brought about by fibrosarcoma cells (8). Experiments employing monoclonal antibodies to protease nexin-1 that stoichiometrically block its ability to inhibit target proteinases (9) have indicated that a platelet form of protease nexin-1 modulates the number of thrombin binding sites on platelets (20). Finally, protease nexin-1 has the same amino acid sequence as a proteinase inhibitor produced by glioma cells (10, 11) that stimulates neurite outgrowth from cultured neuroblastoma cells (12) and primary sympathetic neurons (13).

Recent studies indicate that the cell surface and ECM are key sites of protease nexin-1 action. Immunofluorescence studies and enzyme-linked immunosorbent assays showed that protease nexin-1 is bound to the ECM of human fibroblasts (14). Moreover, fixed human fibroblasts, as well as ECM prepared from unfixed cells, accelerate the inactivation of thrombin by protease nexin-1 (15). This acceleration is mostly due to heparan sulfate (16).

Taken together, the above studies on cultured cells emphasize the likely importance of cell-bound protease nexin-1. Its importance seems even more likely in interstitial spaces where the amount of available cell surface is greater than in cell culture. In view of this, we examined the effect of cellular binding on the proteinase specificity of protease nexin-1.

EXPERIMENTAL PROCEDURES

Materials—Human neonatal foreskin fibroblasts were isolated and cultured as previously described (17). Protease nexin-1 was purified to apparent homogeneity from serum-free medium conditioned by human foreskin fibroblasts using an immobilized anti-protease nexin-1 monoclonal antibody as previously described (18). Human α-thrombin was kindly provided by Dr. John W. Fenton II, New York State Department of Health, Albany, NY (19). Human urokinase (M, 33,000 form) and human plasmin were from Calbiochem. Anti-protease nexin-1 monoclonal antibodies were prepared and characterized as previously described (9).

Iodination of Proteinases—Thrombin, urokinase, and plasmin were iodinated by the chloroglycouril method using IODO-GEN and Na125I (20). The specific activities were 23,100 cpm/ng, 9,700 cpm/ng and 1,600 cpm/ng for thrombin, urokinase, and plasmin, respectively. Plasmin was intentionally iodinated to a relatively low specific activity to prevent inactivation; autoradiogram exposure times were increased accordingly. The 125I-proteinases were active, since virtually all of each 125I-proteinase was incorporated into protease nexin-1-125I-proteinase complexes after incubation with an excess of protease nexin-1 (data not presented). In the experiments presented, protease nexin-1 was not present in excess, so only part of the 125I-proteinase complexes after incubation with an excess of protease nexin-1 (data not presented).
Radioactivity was incorporated into protease nexin-1-\(^{125}\)I-proteinase complexes.

Reactivity of Cell-bound Protease Nexin-1 with \(^{125}\)I-Proteinases—Proteinases were seeded in 35-mm diameter culture dishes in Dulbecco's modified Eagle's medium containing 10% calf serum (Gibco) and grown to confluence. The confluent cultures were then incubated for 48 h in serum-free Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin (Sigma) and subsequently rinsed five times with phosphate-buffered saline. In some experiments, the rinsed cultures were treated with 0.1 mM phenylarsenoxide to prevent endocytosis and then incubated for 15 min at 37 °C with the medium removed, and the cells were rinsed and solubilized in Laemmli sample buffer (23). \(^{125}\)I-Labeled proteins in the medium and cell samples were analyzed separately by SDS-PAGE using 7.5% acrylamide gels according to Laemmli (23). \(^{125}\)I-Labeled proteinases were removed, and the cells were rinsed and solubilized in 250 μl of Laemmli sample buffer (23). The solubilized cells were analyzed by SDS-PAGE, and the \(^{125}\)I-proteins were visualized by autoradiography as described under "Experimental Procedures." The antibody solution was removed, and the cells were then incubated for 15 min at 25 °C with \(^{125}\)I-plasmin at 500 ng/ml (lane 1), 100 ng/ml (lane 2), 75 ng/ml (lane 3), 50 ng/ml (lane 4), or 25 ng/ml (lane 5). Panel C, rinsed cells were incubated for 15 min at 25 °C with \(^{125}\)I-urokinase at 500 ng/ml (lane 1) or 1 μg/ml (lane 2). After these incubations, the \(^{125}\)I-proteinases were removed, and the cells were rinsed and then solubilized in 250 μl of Laemmli sample buffer (25). The solubilized cells were analyzed by SDS-PAGE, and the \(^{125}\)I-proteins were visualized by autoradiography as described under "Experimental Procedures."
Alteration of Protease Nexin-1 Specificity

of purified protease nexin-1 was added to the cells (Figs. 3 and 4, lanes 1–5). Then, 125I-thrombin (Fig. 3, lanes 0 to 5) or 125I-urokinase (Fig. 4, lanes 0 to 5) was added to the cells to determine the reactivity of cell-bound protease nexin-1 as described above. Fig. 3 shows that the added protease nexin-1 bound to the cells in a dose-dependent manner and that it formed complexes with 125I-thrombin that co-migrated with complexes formed from 125I-thrombin and protease nexin-1 from the fibroblast serum-free conditioned medium. In a parallel experiment with 125I-urokinase, however, the added protease nexin-1 that bound to the cell surface did not form protease nexin-1-125I-urokinase complexes; instead, 125I-urokinase formed complexes with a cell surface component, noted above, that was larger than the protease nexin-1-125I-urokinase complexes formed from 125I-urokinase and protease nexin-1 from the fibroblast serum-free conditioned medium (Fig. 4). Similar experiments also showed that added protease nexin-1 that bound to the cell surface did not form complexes with 125I-plasmin (data not presented). It should be emphasized that the experiments just described were conducted using broad concentration ranges of 125I-urokinase and 125I-plasmin, as well as various incubation conditions. Together, these experiments showed that added protease nexin-1 bound to the fibroblast surface and that upon binding it formed complexes with 125I-thrombin but not 125I-urokinase or 125I-plasmin.

**Fig. 3.** Effect of binding purified protease nexin-1 to the cell surface on its reactivity with 125I-thrombin. Confluent fibroblast cultures were incubated in serum-free medium and rinsed as described under "Experimental Procedures." For lanes 0 to 5, rinsed cells were incubated for 60 min at 37°C with 100 μg/ml of an anti-protease nexin-1 monoclonal antibody that blocks the ability of protease nexin-1 to inhibit its target proteinases (9). After removal of the antibody solution, the cells were incubated for 60 min at 37°C with either no protease nexin-1 (lane 0) or protease nexin-1 at 250 ng/ml (lane 1), 500 ng/ml (lane 2), 750 ng/ml (lane 3), 1 μg/ml (lane 4), or 2 μg/ml (lane 5). After removal of the protease nexin-1 solution, the cells were rinsed and then incubated for 30 min at 37°C with 150 ng/ml 125I-thrombin. After removing the 125I-thrombin, the cells were rinsed and solubilized and cell-associated 125I-proteins were visualized by autoradiography following SDS-PAGE. Lane A, 125I-thrombin; lane B, 125I-thrombin plus protease nexin-1 from fibroblast serum-free medium. The arrow denotes protease nexin-1-125I-thrombin complexes.

**Fig. 4.** Effect of binding purified protease nexin-1 to the cell surface on its reactivity with 125I-urokinase. Confluent fibroblast cultures were incubated in serum-free medium and rinsed as described under "Experimental Procedures." For lanes 0 to 5, rinsed cells were incubated for 60 min at 37°C with 100 μg/ml of an anti-protease nexin-1 monoclonal antibody that blocks the ability of protease nexin-1 to inhibit its target proteinases (9). After removal of the antibody solution, the cells were incubated for 60 min at 37°C with either no protease nexin-1 (lane 0) or protease nexin-1 at 250 ng/ml (lane 1), 500 ng/ml (lane 2), 750 ng/ml (lane 3), 1 μg/ml (lane 4), or 2 μg/ml (lane 5). After removal of the protease nexin-1 solution, the cells were rinsed and then incubated for 30 min at 37°C with 500 ng/ml 125I-urokinase. After removing the 125I-urokinase, the cells were rinsed and solubilized and cell-associated 125I-proteins were visualized by autoradiography after SDS-PAGE. Lane A, 125I-urokinase; lane B, 125I-urokinase plus protease nexin-1 from fibroblast serum-free medium. The arrow denotes protease nexin-1-125I-urokinase complexes.

**Fig. 5.** Effect of binding purified protease nexin-1 to ECM on its reactivity with 125I-proteinases. ECM was prepared from confluent fibroblasts as described under "Experimental Procedures." For lanes 0 to 4, the dishes containing ECM were incubated for 60 min at 37°C with either no protease nexin-1 (lane 0) or protease nexin-1 at 250 ng/ml (lane 1), 500 ng/ml (lane 2), 750 ng/ml (lane 3), or 1 μg/ml (lane 4). After removal of the protease nexin-1 solution, the dishes were rinsed and then incubated for 30 min with 150 ng/ml 125I-thrombin (panel A), 500 ng/ml 125I-urokinase (panel B), or 1.0/ μg/ml 125I-plasmin (panel C). After removal of the 125I-proteinases, the ECM preparations were rinsed and solubilized in 250 μl of Laemmli sample buffer. The solubilized proteins were visualized by autoradiography following SDS-PAGE. Lane A, 125I-proteinase; lane B, 125I-proteinase plus purified protease nexin-1. The arrow denotes positions of the 125I-proteinase and the corresponding protease nexin-1-125I-proteinase complexes.
bovine serum albumin in the presence of a molar excess of heparin. To each solution was added 1 pmol of either the protease nexin-1 or the proteinase and the corresponding protease nexin-1-labeled protein. The reactions were stopped by adding an equal volume of Laemmli gel sample dilution buffer. The labeled proteins were visualized by autoradiography following SDS-PAGE. The arrows denote positions of the 125I-protease and the corresponding protease nexin-1-125I-proteinase complexes.

**Proteinase Specificity of Protease Nexin-1 Bound to the ECM**—Previous studies showed that about 60 to 80% of protease nexin-1 bound to cultured fibroblasts was localized to the ECM (14). Accordingly, experiments were conducted to determine if binding of protease nexin-1 to the ECM affected its target proteinase specificity. This was accomplished as described in the above experiments on binding of protease nexin-1 to intact cells. Fibroblast ECM was prepared using 0.25 M NH₄OH as described under “Experimental Procedures”; this inactivated ECM-bound protease nexin-1, obviating the need to treat with the blocking monoclonal antibody (Fig. 5, lane 0). Then, increasing amounts of purified protease nexin-1 were added to the ECM (Fig. 5, lanes 1 to 4), and these preparations were incubated with 125I-thrombin (panel A), 125I-urokinase (panel B), or 125I-plasmin (panel C). Fig. 5 shows that the added protease nexin-1 bound to the ECM and that it formed complexes with 125I-thrombin. In contrast, the ECM-bound protease nexin-1 did not form detectable complexes with either 125I-urokinase or 125I-plasmin.

Thus, the protease specificity of protease nexin-1 was similarly regulated by binding to intact cells or ECM prepared from them.

**Effect of Heparin on Reactivity of Protease Nexin-1 with Target Proteinases**—Previous studies showed that heparin accelerates the inactivation of thrombin by protease nexin-1 (1, 4). In addition, the ability of fibroblast ECM to accelerate the inactivation of thrombin by protease nexin-1 is mostly due to ECM heparan sulfate (15, 16). To determine if the blockage of protease nexin-1-125I-urokinase and protease nexin-1-125I-plasmin complex formation by fibroblasts and their ECM was due to heparin-like glycosaminoglycans, we examined the effect of heparin on formation of these complexes in solution. Fig. 6 shows that heparin enhanced the formation of protease nexin-1-125I-thrombin complexes, and that it slightly enhanced the formation of protease nexin-1-125I-urokinase complexes and protease nexin-1-125I-plasmin complexes. These results indicate that other ECM components are responsible for blocking the ability of protease nexin-1 to inhibit urokinase and plasmin.

**DISCUSSION**

The present results and other recent studies demonstrate that association of certain serine proteinase inhibitors with the cell surface and/or ECM markedly changes their functional properties. For protease nexin-1, this accelerates its inactivation of thrombin (15, 16), and, as shown here, blocks its ability to form complexes with urokinase and plasmin. Studies on plasminogen activator inhibitor-1 have shown that association with the ECM greatly increases its stability (25). It seems likely that the association of these inhibitors with the cell surface/ECM in vivo is even more important than in cell culture where the volume of the extracellular fluid phase is artificially very high.

Although protease nexin-1 is not present at significant levels in plasma, a protease nexin-1-like molecule has been identified at the surface of platelets (26). It is noteworthy that this molecule, designated platelet protease nexin, forms complexes with thrombin but not urokinase (26). If platelet protease nexin is identical with protease nexin-1, then association with the platelet surface also appears to block its ability to form complexes with urokinase.

The present experiments were designed to measure the reactivity of protease nexin-1 that was bound to the cell surface/ECM of fibroblasts. This was possible since dissociation of protease nexin-1 or protease nexin-1 protease complex from the cell surface or ECM could not be detected during these measurements. The experiments on reactivity of "endogenous" protease nexin-1 bound to the surface of cells as well as the experiments on purified protease nexin-1 bound to the fibroblast surface or ECM revealed that it reacted with thrombin, but not urokinase or plasmin. This is noteworthy in view of previous studies which showed that the association rate constants of protease nexin-1 with thrombin, urokinase, and plasmin in solution are approximately equal (4). It should be emphasized that our experiments on the regulation by the fibroblast surface/ECM employed a range of proteinase and protease nexin-1 concentrations. The data in Fig. 5 show that the concentrations of protease nexin-1 that led to detectable protease nexin-1-125I-thrombin complexes also led to detectable protease nexin-1-125I-urokinase and protease nexin-1-125I-plasmin complexes. Thus, these results are not due to concentrations of the proteinases or protease nexin-1 that would limit the ability to detect protease nexin-1-urokinase or protease nexin-1-plasmin complexes.

We recently showed that the ability of the cell surface/
ECM to accelerate the inactivation of thrombin by protease nexin-1 was mostly due to heparan sulfate (16). This is consistent with earlier studies which showed that protease nexin-1 possesses a heparin binding site (1) and that purified heparin (1, 4) and heparan sulfate (16) accelerate its inactivation of thrombin. In the present studies, it was of interest to determine if similar interactions blocked the ability of protease nexin-1 to form complexes with urokinase and plasmin. The finding that heparin slightly enhanced the formation of complexes between protease nexin-1 and both urokinase and plasmin leads to the conclusion that other ECM molecules are involved in this regulation. The nature of the interactions between the ECM component(s) and protease nexin-1 that block its ability to form complexes with urokinase and plasmin are not known, although it is not a blocking of the reactive center of protease nexin-1 since these interactions permit complex formation with thrombin. It should also be emphasized that the ECM molecules responsible for binding protease nexin-1 have not yet been identified.

The cell-conferred thrombin specificity of protease nexin-1 described here is consistent with two activities observed for protease nexin-1 in cell culture. First, added protease nexin-1 can modulate the mitogenic activity of thrombin; this depends on thrombin inhibition (7). Second, protease nexin-1 shares the same deduced amino acid sequence as a gliad-derived neurite promoting factor which stimulates neurite outgrowth from neuroblastoma cells and primary sympathetic neurons (10–13). Recent studies showed that thrombin can modulate and reverse the neurite outgrowth stimulated by protease nexin-1, indicating that this activity of protease nexin-1 also depends on thrombin inhibition (27).

REFERENCES