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Released protease-nexin regulates cellular binding, internalization, and degradation of serine proteases

(thrombin/urinary plasminogen activator/endocytosis inhibitor/heparin/antithrombin III)

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ABSTRACT **Protease-nexin (PN), a component released by normal human fibroblasts into culture medium, forms covalent linkages with thrombin (Th) and the urinary plasminogen activator urokinase, apparently with their catalytic site serines. The present studies explored the function of PN by examining the interaction of protease-PN complexes with human fibroblasts and the consequences of this interaction. Th-PN and urokinase-PN complexes bind to cells *via* the PN portion of the complexes. The binding is selectively inhibited by heparin. Because PN has a heparin-binding site, this indicates that protease-PN complexes might bind to a cellular heparin-like site. After binding, the complexes are internalized. By inhibiting endocytosis with phenylarsine oxide, which does not affect cellular binding of Th-PN complexes, we showed that complexes must be internalized before they are degraded. Kinetic analysis of internalization and degradation of Th-PN showed that complexes are internalized more rapidly than they dissociate from the cell surface; by 120 min of incubation at 37°C most cell-bound Th-PN complexes are degraded to amino acids. The results are summarized in a model showing how PN mediates the cellular binding, internalization, and degradation of serine proteases through formation of protease-PN complexes. This series of events may be involved in the regulation of serine protease activity at the cell surface and in the extracellular environment.**

Recently we identified protease-nexin (PN), a component released from normal human foreskin (HF) cells that combines with serine proteases, including thrombin (Th) and urokinase (1). PN resembles antithrombin III (AT3), a serum inhibitor of Th. Both PN and AT3 form covalent linkages with serine proteases, and these linkages are identical in their sensitivity to various dissociating conditions. PN, like AT3, does not form a linkage with Th that has been derivatized at its catalytic-site serine with diisopropyl phosphate. Thus it appears that PN, like AT3, links to proteases at their catalytic sites and inactivates them. PN also resembles AT3 by having a high-affinity heparin-binding site, and its rate of linkage to Th is greatly enhanced by heparin. Nevertheless, PN (about 40 kilodaltons) and AT3 (about 65 kilodaltons) are clearly different in size and in immunological properties. In addition, PN is made by fibroblasts. These intriguing properties of PN, and our previous finding with HF cells that most of the specifically bound Th is found in Th-PN complexes (1, 2), prompted the present studies on the functions of PN.

MATERIALS AND METHODS

Materials. Highly purified human Th (about 3000 National Institutes of Health units/mg) was generously supplied by John W. Fenton II (3). Urokinase (low molecular weight form, 35 kilodaltons) was a gift from Collaborative Research (Waltham, MA). Heparin from porcine intestinal mucosa, soybean trypsin inhibitor (SBTI), and phenylarsine oxide (PhAsO) were products of Sigma. $Na^{125}I$ was obtained from Amersham, chloroglycouril from Pierce, and silica gel 60 aluminum sheets from Merck. We purchased Dulbecco–Vogt modified Eagle's medium (DV medium) from Flow Laboratories (Rockville, MD); trypsin solution, glutamine, and antibiotics from GIBCO; and calf serum from Irvine Scientific (Santa Ana, CA).

Cell Cultures, Gel Electrophoresis, and Radioiodination. HF fibroblast-like cells (passage 6–16) were cultured as described (4). Conditioned medium was obtained from confluent HF cultures that had been in serum-free DV medium for 48 hr. $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out as described (1) except that the sample buffer was 2 mM EDTA/7 mM dithiothreitol/1% $NaDodSO_4$/20 mM [bis(2-hydroxyethyl)-amino] tris(hydroxymethyl)methane, pH 7.5. This modification resulted in less breakdown of protease-PN complexes during electrophoresis. Mono[^{125}I]iodotyrosine (^{125}I-Tyr) and $^{125}I^-$ were separated as follows: Equal parts of culture medium and 2-fold concentrated electrophoresis sample buffer were mixed and heated at 100°C for 5 min. Mixtures (1 ml) were applied to $NaDodSO_4$/polyacrylamide gels consisting of a 2.5-cm 4% stacking gel and a 10-cm 10% separating gel. ^{125}I-Tyr migrated directly behind bromophenol blue and $^{125}I^-$ migrated with phenol red. ^{125}I-Tyr and $^{125}I^-$ were identified by applying extracts of gel slices (in acetone/acetic acid, 3:2 vol/vol) to silica gel 60 aluminum strips using a 1-butanol/acetic acid/water (10:1:1, vol/vol) developing solvent (5). Standards (prepared with $Na^{125}I$, chloramine-T, and tyrosine) were also applied. Radioiodination of Th and urokinase by using chloroglycouril (6) was performed as described (1).

Measurement of Cellular Binding and Internalization of ^{125}I-Labeled Th-PN (^{125}I-Th-PN) Complexes. Measurement of binding and linkage of the ^{125}I-labeled proteases ^{125}I-Th or ^{125}I-urokinase to HF cells was performed as described (1). The presence of intracellular Th-PN complexes was determined as follows. Cell cultures (2×10^5 cells per 35-mm dish) were rinsed four times with cold phosphate-buffered saline, pH 7.2 (P_i/NaCl) and incubated with trypsin (0.125% in DV medium) or DV medium without trypsin (1 ml each) for 5 min at 37°C. After addition of 0.2 ml of SBTI (5 mg/ml), the medium was aspirated and centrifuged at $10,000 \times g$ for 2 min; the resulting pellets were washed once in P_i/NaCl. The cells that remained attached to the culture dish were rinsed once with SBTI (1 mg/ml in P_i/NaCl) and once with P_i/NaCl and $NaDodSO_4$/polyacrylamide

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Abbreviations: PN, protease-nexin; HF cells, human foreskin cells; Th, thrombin; AT3, antithrombin III; SBTI, soybean trypsin inhibitor; PhAsO, phenylarsine oxide; DV medium; Dulbecco–Vogt modified Eagle's medium; P_i/NaCl, phosphate-buffered saline.

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gel electrophoresis sample buffer (200 μl) was then added. The cells were scraped with a rubber policeman and pooled with any cells sedimented from the medium; they were immediately heated for 5 min at 100°C. Radioactivity in trypsin-treated cultures was judged to be intracellular.

Cellular endocytosis was inhibited by pretreating cultures with 0.1 mM PhAsO (7) in serum-free DV medium for 20 min at 22°C. Because of the tendency of PhAsO-treated cells to detach around the edges of the dishes, the $NaDodSO_4$-soluble protein in each culture was determined. Measurements were then corrected for loss of protein.

RESULTS AND DISCUSSION

Binding of Protease-PN Complexes to Cells. We previously showed that cell-bound ^{125}I-Th-PN complexes were formed after addition of ^{125}I-Th to HF cell cultures (1, 2). We also showed that cells released PN into their culture medium, where it formed covalent complexes with ^{125}I-Th (1). This suggested the possibility that ^{125}I-Th-PN complexes formed in the culture medium might bind to the cells. To test this we added ^{125}I-Th to fresh serum-free medium or serum-free medium that had been "conditioned" by cells for 48 hr and that contained PN. These mixtures were then incubated with cells. About twice the amount of cell-bound ^{125}I-Th-PN was formed in incubations using conditioned medium compared to fresh medium (Fig. 1*A* and Table 1). If ^{125}I-Th-PN, present in conditioned medium but not in fresh medium, could bind to cells, this could account for the increase in cellular binding of complexes observed in incubations with conditioned medium. Alternatively, a factor in conditioned medium might increase the binding of free ^{125}I-Th to cellular PN. To discriminate between these possibilities we incubated ^{125}I-Th with conditioned medium for 30 min; after this incubation about 10% of the ^{125}I-Th was present in ^{125}I-Th-PN complexes. Then we added either excess nonlabeled Th or hirudin to specifically inhibit further linkage of ^{125}I-Th to PN, and we incubated these mixtures with cells. As shown in Table 1 (conditions 1–3), hirudin or nonlabeled Th prevented free ^{125}I-Th from forming ^{125}I-Th-PN complexes on the cells. However, cells incubated in conditioned medium that contained ^{125}I-Th-PN bound ^{125}I-Th-PN even in the presence of excess nonlabeled Th or hirudin (Table 1, conditions 4–6). The reduction in cell-bound ^{125}I-Th-PN seen in conditioned medium containing nonlabeled Th or hirudin can be accounted for by the inhibition of linkage of free ^{125}I-Th to PN (Table 1, conditions 1–3). We also found that ^{125}I-urokinase-PN complexes bound to cells in the presence of nonlabeled urokinase (Fig. 1*C*). These results show that ^{125}I-protease-PN complexes bound to the cells and that they bound via the PN moiety.

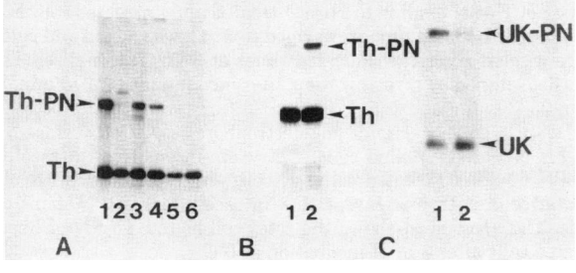

FIG. 1. Binding of protease-PN complexes to HF and mouse embryo cells. (*A*) ^{125}I-Th (500 ng/ml) was incubated for 30 min at 37°C with either fresh serum-free DV medium or serum-free DV medium that had been conditioned by HF cells. Samples of each then received heparin, Th, or no addition as indicated below. These samples were further incubated with HF cell cultures that had been rinsed with DV medium. After 30 min at 37°C, samples were prepared for $NaDodSO_4$/polyacrylamide gel electrophoresis. Incubation with conditioned medium, lanes 1–3; incubation with fresh DV medium, lanes 4–6. Lanes 1 and 4, no addition; lanes 2 and 5, heparin addition (30 ng/ml); lanes 3 and 6, Th addition (10 μg/ml). (*B*) ^{125}I-Th (500 ng/ml) was incubated for 30 min at 37°C with either serum-free DV medium or serum-free DV medium that was conditioned by mouse embryo cells. Each mixture was then further incubated with mouse embryo cell cultures that had been rinsed with serum-free DV. After 30 min at 37°C, samples were prepared for $NaDodSO_4$/polyacrylamide gel electrophoresis. Incubation with fresh DV medium, lane 1; incubation with conditioned medium, lane 2. (*C*) ^{125}I-urokinase (UK) (500 ng/ml) was incubated for 30 min at 37°C with conditioned medium. After addition of nonlabeled urokinase (10 μg/ml), one sample received heparin (30 μg/ml). Both mixtures were then further incubated with HF cell cultures that had been rinsed with serum-free DV medium. After 30 min at 37°C, samples were prepared for $NaDodSO_4$/polyacrylamide gel electrophoresis. Incubation without heparin, lane 1; incubation with heparin, lane 2.

Table 1. Effect of Th and hirudin on formation of cell-bound Th-PN

Condition	^{125}I-Th in Th-PN, cpm
1. Fresh medium	530
2. Fresh medium + Th	40
3. Fresh medium + hirudin	0
4. Conditioned medium	1190
5. Conditioned medium + Th	440
6. Conditioned medium + hirudin	620

Fresh medium or conditioned medium was incubated for 30 min at 37°C with ^{125}I-Th (500 ng/ml). These mixtures were incubated with HF cells for 30 min at 37°C in the absence or presence of nonlabeled Th (10 μg/ml) or hirudin (10 μg/ml).

Previously we showed that PN has a high-affinity heparin-binding site and that heparin greatly increases the rate of linking between Th and PN released from the cells (1). To determine if heparin also affected cellular binding of ^{125}I-Th-PN, we incubated ^{125}I-Th-PN complexes, preformed in conditioned medium, with cells in the presence or absence of heparin (30 μg/ml). Heparin greatly decreased the appearance of cell-bound ^{125}I-Th-PN at 37°C (Fig. 1*A*) or 0°C (arrowhead, Figs. 2 and 3). Heparin also markedly inhibited the binding of ^{125}I-urokinase-PN complexes to cells (Fig. 1*C*). It is unlikely that interaction of heparin with the protease caused this effect; although Th has a heparin-binding site, urokinase does not, judged by their ability to bind to heparin-derivatized Sepharose beads (unpublished results). This effect of heparin did not reflect a nonspecific inhibitory effect of polyanions on the binding of polypeptides to cells. Heparin at even 300 μg/ml did not detectably affect binding of ^{125}I-labeled epidermal growth factor to HF cells (unpublished results). Heparin might inhibit cellular binding of ^{125}I-protease-PN by changing the conformation of PN. Or, if protease-PN complexes bound to a cell surface component such as heparan sulfate, heparin would act as a competitive inhibitor of this process.

Although the association of ^{125}I-protease-PN complexes with HF cells was heparin sensitive, after ^{125}I-Th-PN complexes became associated with the cell surface neither heparin nor a variety of other agents displaced them when incubations were carried out at 4°C. Table 2 shows that the complexes were not displaced by heparin at 30 μg/ml or extracted with 1 mM EDTA or 0.5 M urea, conditions that remove many peripheral membrane proteins (8). KCl at 3 M, an agent that extracts a portion of the membrane-bound HL-A histocompatibility antigen (9), removed 22% of the ^{125}I-Th-PN. A solution of 0.5% Nonidet P-40, which solubilizes many membrane proteins, extracted all of the ^{125}I-Th-PN complexes. The inability of the other treatments to extract ^{125}I-Th-PN was not caused by internalization

Table 2. High-affinity binding of Th-PN complexes to HF cells

Extraction agent	Th-PN extracted, %
P_i/NaCl	6
Trypsin (0.05% in P_i/NaCl)	95
Urea (0.5 M)	4
EDTA (1 mM)	8
Heparin (12 μg/ml)	6
KCl (3 M)	22
Nonidet P-40 (0.5% in 10 mM Tris/acetate, pH 8.2)	100

^{125}I-Th (200 ng/ml) was incubated with HF cells for 5 min at 37°C. Cells were harvested at 4°C with a rubber policeman and collected by centrifugation. Extraction agents were added to samples containing 2×10^5 cells. Cells were suspended and incubated at 4°C for 16 hr. Trypsin (0.05%) was added to one sample of cells in P_i/NaCl for 10 min at 37°C, followed by SBTI (1 mg/ml). After centrifugation (10,000 × *g*, 5 min), supernatant and pellet fractions were prepared for NaDodSO$_4$/polyacrylamide gel electrophoresis.

of the complexes by the cells during the 16-hr incubation at 4°C. Over 90% of these complexes were removed by a brief trypsin treatment after the 16-hr incubation, indicating that they were still present at the cell surface. These results demonstrated that ^{125}I-Th-PN complexes became bound to the cell surface with a high affinity, and, by the criteria used, behaved as integral components of the membrane.

We previously reported that a number of cell types, such as mouse embryo cells and Chinese hamster lung cells, linked ^{125}I-Th to cell surface components that were similar in size to HF cell PN (10). However, most cell types formed substantially fewer complexes than HF cells do. These studies were carried out by incubating the cells with ^{125}I-Th in fresh medium. In view of the ability of ^{125}I-Th-PN complexes to form in conditioned medium and bind to cells, we examined the amount of ^{125}I-Th-PN that became associated with mouse embryo and Chinese hamster lung cells when the ^{125}I-Th was added to cultures containing their respective conditioned serum-free medium. Under this condition, 3- to 4-fold more ^{125}I-Th-PN complexes became associated with mouse embryo cells (Fig. 1*B*) and Chinese hamster lung cells (data not shown). Thus, PN-mediated cellular binding of ^{125}I-Th was greatly increased in several cell types when binding was carried out in conditioned medium instead of fresh medium.

Transport of Th-PN Complexes into HF Cells. To determine if Th-PN was internalized by cells we took advantage of the finding, presented above, that ^{125}I-Th-PN complexes, preformed in conditioned medium, bound to cells in the presence of hirudin. Under these conditions, the hirudin prevented specific cellular binding and internalization of free ^{125}I-Th (data not shown) as well as its linkage to cellular PN (Table 1). Thus, preformed ^{125}I-Th-PN complexes were incubated with cells in the presence of hirudin at 0°C for 3 hr (Fig. 2). After this incubation all cell-bound ^{125}I-Th-PN was present at the cell surface as evidenced by its complete sensitivity to a brief trypsin treatment. These cells were then placed in medium without ^{125}I-Th-PN and were shifted to 37°C; the presence of intra-cellular and surface-bound ^{125}I-Th-PN was determined by treating the cells with trypsin. The results presented in Fig. 2 show that there was a rapid and progressive loss of cell surface ^{125}I-Th-PN. This loss of cell surface complexes could have been caused by extracellular destruction or dissociation of the complexes, by internalization of complexes, or by both processes. To measure these two processes some cell cultures were treated with PhAsO, a potent inhibitor of endocytosis in oocytes (7). PhAsO totally inhibited endocytosis of ^{125}I-Th-PN at 37°C (Fig. 2). However, it did not affect cellular binding of ^{125}I-Th-PN at 0°C (Fig. 2 *Inset*), indicating that dissociation of ^{125}I-Th-PN from the cell surface at 0°C was not detectably altered by PhAsO. After 5 min of incubation at 37°C, PhAsO-treated cultures lost about twice as much Th-PN from their cell surface as untreated cultures did (Fig. 2). Additionally, the amount of Th-PN internalized during the 5–10 min incubation was similar to the amount of Th-PN lost from the cell surface after 5 min in untreated cultures. These results indicated that internalization of complexes was more rapid than dissociation of complexes, because virtually all Th-PN was cell associated after untreated cultures were incubated for 5 min, whereas there was a significant loss of Th-PN from PhAsO-treated cultures after 5 min. Approximately one-sixth of total cellular ^{125}I-Th-PN became insensitive to trypsin during the 5–10 min incubation at 37°C (Fig. 2). However, a comparison of amounts of surface-bound and intracellular ^{125}I-Th-PN at 30-min and later time points showed that only a small fraction of the ^{125}I-Th-PN removed from the cell surface was recovered in the trypsin-insensitive (intracellular) fraction. These results

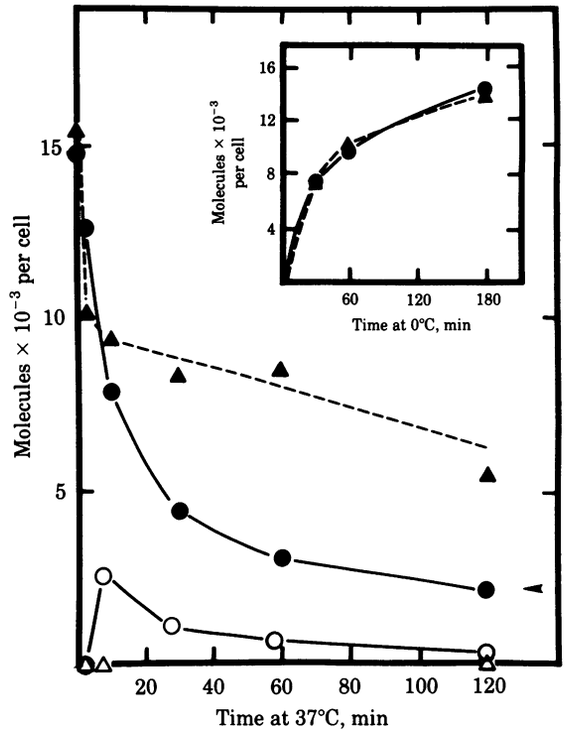

FIG. 2. Internalization at 37°C of ^{125}I-Th-PN bound to HF cells at 0°C. ^{125}I-Th (500 ng/ml) was incubated with serum-free conditioned DV medium for 30 min at 37°C; hirudin was then added (10 μg/ml). Some samples also received heparin (300 μg/ml). After cooling to 0°C, samples of the mixtures were incubated with confluent HF cultures that had been rinsed twice and incubated in serum-free DV medium for 2 days. The cultures were cooled to 0°C before mixture additions. Some cultures were pretreated with PhAsO. After 3 hr at 0°C, cultures were rinsed five times with 1 ml of P_i/NaCl. The *Inset* shows the time course of Th-PN binding to PhAsO-treated and untreated cells at 0°C. Serum-free DV medium, prewarmed to 37°C, was added and cultures were incubated for the indicated times at 37°C. Internalized Th-PN was determined by trypsinization after incubations for 0–5 min, 5–10 min, 25–30 min, and 115–120 min. Cultures were then processed for NaDodSO$_4$/polyacrylamide gel electrophoresis. ▲, Cell surface-bound Th-PN of PhAsO-treated cells; △, trypsin-insensitive (intracellular) Th-PN of PhAsO-treated cells; ●, cell surface-bound Th-PN of untreated cells; ○, trypsin-insensitive (intracellular) Th-PN of untreated cells. The arrowhead shows the total cell-bound Th-PN of heparin-treated cells after 3 hr of incubation at 0°C.

indicated that ^{125}I-Th-PN not only was rapidly internalized but also was rapidly degraded.

Degradation of ^{125}I-Th-PN. To determine the kinetics of cellular degradation of ^{125}I-Th-PN, preformed ^{125}I-Th-PN complexes were bound to the cell surface at 0°C and cells were then shifted to 37°C as described above for Fig. 2. At various times, medium was assayed for the presence of ^{125}I-Tyr and $^{125}I^-$. The results presented in Fig. 3 showed that $^{125}I^-$ was released into the medium. This $^{125}I^-$ was probably not derived from cell-bound ^{125}I-Th-PN or ^{125}I-Th, because $^{125}I^-$ levels did not change with increasing incubation times. Additionally, similar amounts of $^{125}I^-$ were released from PhAsO-treated and untreated cells, indicating that internalization was not required for $^{125}I^-$ release. Because about 10% of the total ^{125}I radioactivity in the binding medium was in the form of $^{125}I^-$, it seems likely that some $^{125}I^-$ in this mixture became associated with cells or dishes at 0°C, but rapidly dissociated at 37°C.

Measurement of ^{125}I-Tyr released into the medium indicated that at least the Th portion of ^{125}I-Th-PN was rapidly degraded to free amino acids by cells. After 10 min of incubation at 37°C there was no detectable ^{125}I-Tyr release (Fig. 3*A*). However, after 30 min of incubation, ^{125}I-Tyr release from cells was detectable, and further release occurred by 120 min of incubation (Fig. 3 *B* and *C*). Importantly, PhAsO-treated cells did not release ^{125}I-Tyr into the medium even after 120 min of incubation (Fig. 3*C*). Thus, ^{125}I-Th-PN complexes must be internalized before they are degraded to amino acids.

The results presented in Fig. 3*E* show that, by 120 min of incubation, most of the cell-bound ^{125}I-Th-PN was degraded to ^{125}I-Tyr. However, a small portion of the ^{125}I-Tyr in the medium could have been generated from unlinked ^{125}I-Th that bound to cells at 0°C in the presence of hirudin, and was thus nonspecifically bound (Fig. 3*E*). To determine what proportion of ^{125}I-Tyr was generated from free ^{125}I-Th, ^{125}I-Th-PN complexes were incubated with cells at 0°C in the presence of heparin. Under these conditions, only a small fraction of ^{125}I-Th-PN (8% of the control culture that did not receive heparin) was cell bound after 3 hr (arrowhead, Fig. 3*E*), whereas about 42% of the ^{125}I-Th remained cell bound (data not shown). Fig. 3*D* shows that under these conditions ^{125}I-Tyr was not generated after 30 min of incubation. However, after 120 min of incubation, cells released a small amount of ^{125}I-Tyr. Degradation of ^{125}I-Th at 120 min but not at earlier times was supported by cellular binding results (Fig. 3*E*). Thus, in Fig. 3*E* about 8000 cpm of the ^{125}I-Tyr generated after 120-min incubation was probably due to degradation of nonspecifically bound ^{125}I-Th.

Together these results showed not only that ^{125}I-Th-PN must be internalized before degradation to amino acids but also that degradation was rapid. Over 40% of the ^{125}I-Tyr resulting from degradation of ^{125}I-Th-PN was generated after only 30 min of incubation. Importantly, Fig. 3*E* shows that after 120 min of incubation most cell-bound ^{125}I-Th-PN had been degraded, as evidenced by the production of ^{125}I-Tyr. This result supports our interpretation, presented above, that internalization of Th-PN is more rapid than dissociation of Th-PN, because we have also shown that degradation of complexes cannot occur unless they are internalized.

Possible Functions of PN. In this paper we describe a series of events that occur upon release of PN from HF cells. Our results, summarized in Fig. 4, show that released PN (step 1) forms a linkage (step 2) with serine proteases (Th and urokinase)

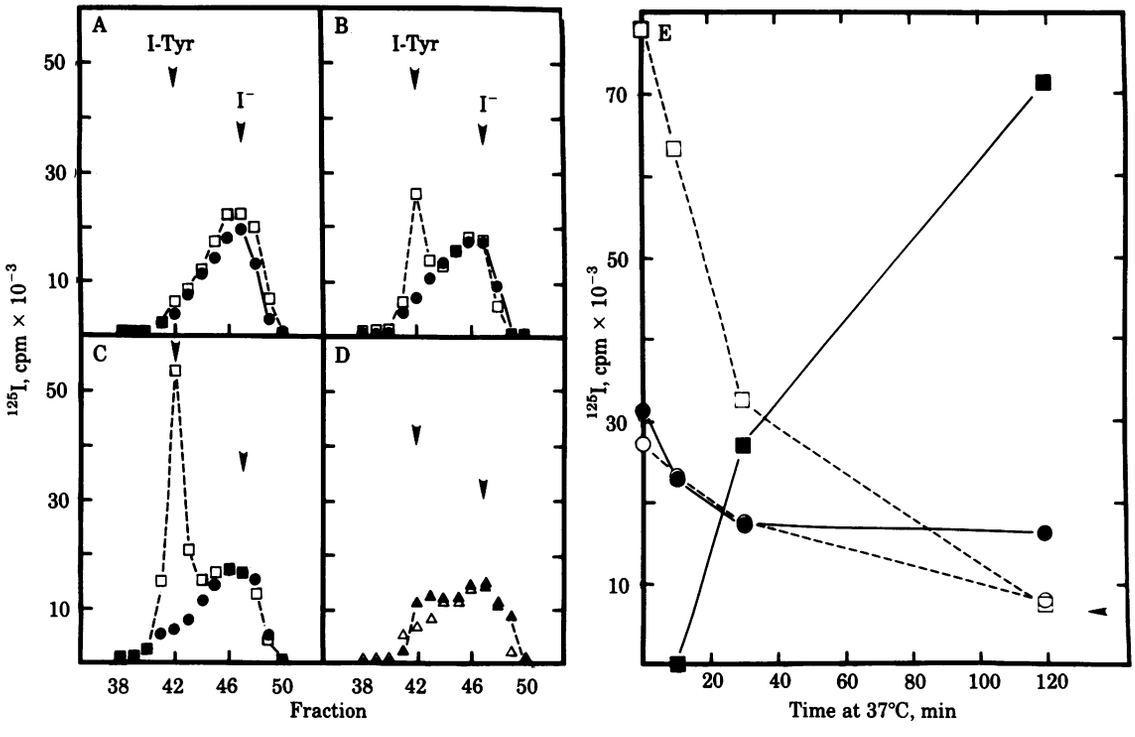

FIG. 3. Degradation of ^{125}I-Th-PN complexes by HF cells. ^{125}I-Th-PN complexes were incubated with HF cells at 0°C in the presence of hirudin (10 μg/ml) for 3 hr; the cultures were then shifted to 37°C as described for Fig. 2. Additionally, some cultures received heparin (300 μg/ml). After various times, medium was processed for NaDodSO$_4$/polyacrylamide gel electrophoresis and iodinated compounds were separated and identified by thin-layer chromatography. Positions of standards are indicated by vertical arrowheads. Each fraction is 2 mm. (*A*) Medium after 10 min of incubation, (*B*) medium after 30 min of incubation, (*C*) medium after 120 min of incubation; □, untreated cells; ●, PhAsO-treated cells. (*D*) Heparin addition to cells (300 μg/ml); △, medium after 30 min of incubation; ▲, medium after 120 min of incubation. (*E*) Summary of results: ■, ^{125}I-Tyr released from untreated cells into the medium; □, cell-bound ^{125}I-Th-PN of untreated cells; ● cell-bound ^{125}I-Th of PhAsO-treated cells; ○, cell-bound ^{125}I-Th of untreated cells. The arrowhead shows the total cell-bound ^{125}I-Th-PN of heparin-treated cells after 3 hr of incubation at 0°C.

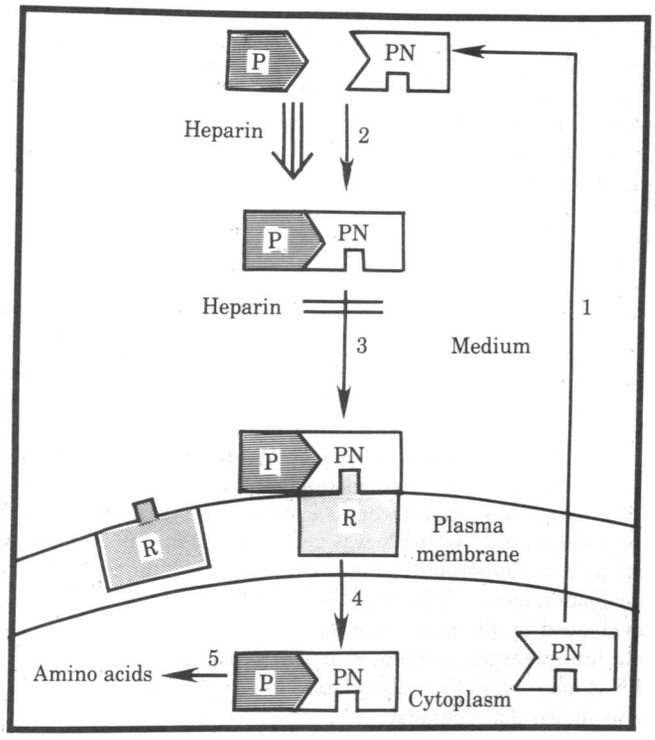

FIG. 4. Summary of interactions of PN with serine proteases and cells. Step 1, release of PN into the culture medium; step 2, linking of PN with serine protease (P); step 3, cellular binding of serine protease-PN complexes to cell surface receptors (R); step 4, internalization of complexes; step 5, degradation of complexes.

present in the culture medium. As previously shown, the rate of linkage formation between Th and PN is greatly increased by addition of heparin (1). Protease-PN complexes formed in cell-conditioned medium bind tightly to HF cells (step 3). Binding is markedly reduced in the presence of heparin at 30 μg/ml. Our results indicate that binding is mediated by the PN portion of protease-PN complexes. Thus, PN appears to be a mediator of protease binding and not a cell surface binding site as we originally suggested (2). After cellular binding, Th-PN complexes are rapidly internalized (step 4) and degraded (step 5).

Because Th linked to PN is rapidly degraded by the cells (Fig. 3), it seems unlikely that PN transports serine proteases into cells. Also, PN probably does not play a direct role in the generation of the mitogenic signal by Th. Cellular binding of Th-PN complexes can be almost totally inhibited by heparin addition with no apparent effect on the ability of Th to stimulate HF cells and mouse embryo cells to divide (unpublished data). However, it seems likely that PN is involved in the regulation of serine protease levels in the extracellular and cell-surface environments. This regulation would occur via the cellular degradation of proteases outlined in Fig. 4 and also by direct inhibition of proteases by PN. Our studies with urokinase indicate that this plasminogen activator loses its fibrinolytic activity when linked to PN (11). The presence of binding sites for PN at the cell surface could play the important role of increasing the concentration of PN at this critical location. For example, at this site PN could directly modulate thrombin-stimulated cell division, because proteolysis by thrombin at the cell surface is sufficient to produce a proliferative response (12, 13). PN may be identical to a recently described mouse cytosol protein called trypsin combining protein (14). Finally, it is noteworthy that α_2-macroglobulin, a serum protease inhibitor, forms complexes with proteases and mediates their binding, internalization, and degradation. However, α_2-macroglobulin does not form a covalent linkage to the catalytic site of the protease (15) and it is supplied by plasma or serum rather than the cells that bind and internalize the complexes (16–18). Because PN is made by the same cells that bind, internalize, and degrade protease-PN complexes, it could provide a mechanism for autoregulation by these cells of serine protease levels at their cell surface.

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