When the plasminogen activator urokinase was radioiodinated and incubated at 40 ng/ml in medium conditioned by human foreskin (HF) cells, within 30 min over 80% of the added plasminogen activator was complexed to cell-released protease nexin (PN). The urokinase complexed to PN had little if any activity. Incubation of purified PN with urokinase confirmed that PN is an inhibitor of this plasminogen activator. However, a widely used plasminogen-dependent fibrinolytic assay for plasminogen activator indicated that abundant endogenous plasminogen activator activity co-existed with PN in HF cell-conditioned medium. The source of this activity was electromicrographically and immunologically indistinguishable from urokinase. Furthermore, gel exclusion chromatography showed that about 90% of the urokinase antigen detected in conditioned medium had a molecular weight similar to that of free active urokinase. These paradoxical findings are resolved by evidence that this "PN-resistant urokinase-like" plasminogen activator is actually urokinase proenzyme that is activated by plasmin or conditions in the fibrinolytic assay for plasminogen activator. It is shown that the activated form of HF cell plasminogen activator is sensitive to inhibition by PN. PN may thus be an important component in the cellular regulation of endogenous plasminogen activator activity.

A wide variety of cells and tissues secrete plasminogen activator(s) (1). Increased plasminogen activator secretion accompanies and may play a role in malignant transformation (2, 3) and hormone-directed changes in tissue functions (4, 5). The best characterized plasminogen activator is urokinase, which is secreted in relatively large quantities by the kidney. Urokinase exists in 54- and 60-kDa forms and also in a 35-kDa form generated by proteolysis of one or both of the higher molecular weight forms (6, 7). Although few plasminogen activators other than urokinase have been purified or extensively characterized, many cell types secrete 50–60-kDa plasminogen activator(s) that are immunologically similar and perhaps identical with urokinase (7, 8). The most widely used assay for plasminogen activators involves dilution of test material into wells coated with 125I-fibrin and containing plasminogen in Tris-Cl buffer. This assay has suggested that the urokinase-like plasminogen activators released by many cells are active. However, this evidence may be artifactual because the decreased concentration of NaCl combined with possible trace amounts of plasmin in the assay system can cause activation of otherwise inactive plasminogen activators (9, 10). Recently, a proenzyme form of urokinase that is identical in size with 54-kDa urokinase is activated in the 125I-fibrin well assay has been isolated from human tumor cell-conditioned medium (11).

Cellular regulation of plasminogen activator activity may involve protease inhibitors, as indicated by the existence of urokinase-inhibitory activity in the cytosol or media of a variety of cells (12–14). In studies involving human foreskin fibroblasts, we have identified a cellular component, protease nexin, that covalently binds urokinase and certain other serine proteases (15, 16). PN resembles the serum protease inhibitor antithrombin III in the nature of its linkage to bound proteases, its high affinity for heparin, and its increased rate of thrombin binding in the presence of heparin. However, PN is electromicrographically and immunologically distinct from antithrombin III. Protease-PN complexes bind to cell surface receptors which mediate endocytosis of the complexes and degradation of the bound proteases (17). Because human foreskin fibroblasts release urokinase-like plasminogen activator(s) (8), a physiological function of PN may be to regulate cellular expression of plasminogen activator activity. The purpose of the present work was to examine this possibility.

**Experimental Procedures**

_Materials—_Tissue culture materials were obtained from the following sources: Plasticware from Falcon; Cytodex 2 microcarrier beads from Pharmacia; media from Flow Laboratories; calf serum from M. A. Bioproducts; trypsin and stock solutions of penicillin and streptomycin from K. C. Biologicals; bovine serum albumin (Cohn Fraction V) from Sigma; epidermal growth factor was a gift from Patricia Comens (University of Kansas, Lawrence); human foreskin cells were isolated from explants of neonatal foreskins. Other materials were purchased as follows: 54-kDa urokinase (7.4 × 10⁵ CTA units/mg) from Winthrop Laboratories; 35-kDa urokinase (2.5 × 10⁵ CTA units/mg) from Collaborative Research; heparin and fibrinogen (B grade, plasminogen-free) from Calbiochem-Behring; BSA for the radioimmunoassay (fatty acid-free grade), disopropyl fluorophosphate, phenylmethylsulfonyl fluoride, Aprotinin, and Triton X-100 from Sigma; bovine pancreatic trypsin inhibitor from Worthington; 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (IodoGen) from Pierce. Plasminogen was purified by the procedure of Deutsch and Mertz (18). Goat antiserum against human 54-kDa urokinase was a gift from Dr. G. Murano (Bureau of Biologies, FDA), Bethesda, Maryland.

*The abbreviations used are: PN, protease nexin; HF, human foreskin cells; EGF, epidermal growth factor; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPPA, human plasmin plasminogen activator; DME, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.*
MD). The IgG fractions of serum were prepared as described by Paehy and McLaughlin (19). Protease nexin was obtained by purification. A detailed description of the purification and characterization of PN will be presented later. Briefly, medium from a serum-free 3-liter microcarrier culture supplemented with EGF and 0.1% BSA was poured into a siliconized beaker containing Triton X-100 (final concentration, 0.1%). The medium was then concentrated 30-fold by ultrafiltration and applied to a column containing heparin-Sepharose. Buffer containing 0.2 M NaCl and 20 mM sodium phosphate (pH 7.0) was run through the column for 12 h and PN was eluted with 1.0 M NaCl in 20 mM sodium phosphate (pH 7.0). The affinity-purified PN (30 kDa) was greater than 97% pure as judged from densitometric scans of stained SDS-polyacrylamide gels.

Cell Culture Methods—Stock cultures of HF cells in 10-cm diameter culture dishes were maintained in a humidified 37°C incubator containing 5% CO2, 95% air. The cultures were grown in Dulbecco’s modified Eagle’s medium containing penicillin (100 units/ml) and streptomycin at 100 μg/ml (DME medium) plus 5% calf serum. Confluent serum-free cultures were prepared by seeding 1–2×10^6 cells into 35-mm diameter dishes with DME medium plus 5% calf serum (2 ml). After 3–5 days, the cultures were examined microscopically and, if confluent, were rinsed and incubated with DME medium without serum. Cell number in these cultures remained stable for 4 days as described thereafter.

Microcarrier cultures of HF cells were prepared by seeding 3.0×10^6 cells onto 12.5-g microcarrier beads according to instructions of the manufacturer and incubated with 3 liters of DME medium containing 10% fetal calf serum. Cells grew to confluence on the beads in 9–12 days. Serum-free microcarrier cultures were prepared by allowing the beads to settle, decanting 2.5 liters of medium and rinsing the beads with five 1-liter volumes of phosphate-buffered saline. The cells were then cultured with DME medium containing BSA (0.1%) and EGF (10 ng/ml). Cell number remained stable on the beads for over 5 days under these conditions. Medium was collected after 3 days and the cultures were put back in 2.5% serum-containing DME medium for several days before beginning another incubation without serum.

Experimental Methods—BSA (0.02–0.1%) was added to culture dishes before collecting serum-free conditioned medium. Otherwise, recovery of PN from the culture was drastically reduced. To measure formation of complexes between urokinase and PN, PN was labeled with 125I by chloroglycylol-iodinated iodination as previously described (15) and was incubated with conditioned medium as described in the figure and table legends. 125I-urokinase-PN complexes were separated from unbound 125I-urokinase by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn (20) on 7.5% or 9% polyacrylamide slabs. 125I-urokinase-PN and 125I-urokinase in the gels were resolved by autoradiography and quantitated by measuring radioactivity in gel slices with a γ scintillation counter. Control experiments showed that when 125I-urokinase was present in excess over PN, addition of nonlabeled urokinase to the incubation mixture decreased the radioactivity in 125I-urokinase-PN by a factor approximately proportional to the decrease in 125I-urokinase specific activity (counts/min/μg of urokinase) (data not shown). This shows that the binding of 125I-urokinase to PN is not an artifact of iodination and is a valid indicator of interaction between native urokinase and PN.

Plasminogen activator activity assays based on fibrin cleavage were carried out as described by Loskutoff and Edgington (13) in plastic wells coated with 125I-fibrin and containing 5 μg/ml of plasminogen plus 0.1% gelatin in 0.1 M Tris-C1 buffer, pH 8.1 (125I-fibrin well assay). To measure plasminogen activator activity following SDS-PAGE, the gel was soaked with 1 h in 2.5% Triton X-100 at room temperature and the 125I-fibrin well assay was carried out on gel slices. To directly assay plasminogen activator activity by cleavage of plasminogen, the method of Dan0 and Reich (21) was modified as described in the figure legends.

Radioimmunoassay for urokinase was carried out by incubating antiserum to urokinase (1:16,000 dilution of IgG fraction from antourokinase antiserum) in plastic assay wells (Corning assay wells strips) for 4 h at 5°C. The antibody-coated wells were then incubated for 1 h with PBS containing 3% BSA. Material to be assayed was diluted in PBS containing 2% BSA (0.25 ml total volume) and incubated in the wells for 2 h at 5°C. 125I-urokinase (1.5–3.0 ng in 50 μl) was added to the wells, which were then gently shaken on a rocker platform for 20 min and incubated overnight at 5°C. The wells were rinsed several times with PBS and counted in a γ counter to determine the total radioactivity bound to the wells. 125I-urokinase bound to antibody was defined as the difference between the radioactivity bound to the wells in the presence and absence of antibody. Wells coated with preimmune or non-urokinase hyperimmune rabbit IgG bound about the same amount of 125I-urokinase as uncoated wells.

RESULTS

Interaction between Urokinase and PN—Binding of 125I-labeled proteases to PN in cell culture medium can be monitored by using SDS-PAGE to resolve the 125I-protease-PN complexes, which migrate on SDS-polyacrylamide gels with molecular weights approximately 40,000 larger than the molecular weights of the free 125I-protease (15–17). The complex formed between 54-kDa 125I-urokinase and PN present in HF cell-conditioned culture medium is shown in Fig. 1, inset. Formation of 125I-urokinase-PN complexes did not occur when the binding incubation was carried out in the presence of phenylmethanesulfonyl fluoride (Fig. 1, inset), suggesting that the urokinase active site is involved in the binding of the protease to PN.

Fig. 1 shows the time course of 125I-urokinase-PN formation when 54-kDa 125I-urokinase (40 ng/ml) was incubated in conditioned medium at 37°C. PN complexed 50% of the 125I-urokinase within 2 min and 80% within 30 min. As shown, heparin did not significantly affect the rate of 125I-urokinase-PN formation. This contrasts with the heparin-dependent formation of complexes between 125I-t-thrombin and PN (15). PN also rapidly formed complexes with the 35-kDa form of urokinase and this reaction also was not influenced by heparin (data not shown).

As occurs with other serine proteases, urokinase activity can be recovered from gels after SDS-PAGE (22). This allowed us to examine whether urokinase bound to PN in conditioned medium was active. Conditioned medium was incubated with 1 h with either 35-kDa 125I-urokinase or nonlabeled 35-kDa urokinase at 0.1 μg/ml. (The 54-kDa form of urokinase was not used in this experiment to avoid co-migration with an endogenous plasminogen activator that is described below.) Samples of each mixture were electrophoresed and gel slices were assayed for radioactivity or plasminogen activator activity. As shown in Fig. 2 (lower panel), at this...
Aliquots of this 35-kDa urokinase (0.1 pg/ml) for 1 h at 37°C solubilization buffer. SDS-PAGE was carried out in a slab gel and from 24-h serum-free 35-mm cultures of HF cells well assay (3.5-h fibrinolysis incubation). The pooled conditioned medium was incubated with fresh 0.1 pg/ml plasminogen activator activity in gel slices was assayed by 125I-fibrin well assay (3.5-h fibrinolysis incubation). Lower panel, an aliquot of the pooled conditioned medium was incubated with fresh 0.1 μg/ml of 35-kDa 125I-urokinase for 1 h at 37°C. SDS-PAGE was carried out on the same slab gel described in the upper panel and radioactivity in the gel slices was quantitated. Gel slices are numbered from the top of the gel. The arrow marks the position of free 125I-urokinase.

relatively high 125I-urokinase concentration about 60% of the protease became linked to PN (72-kDa complexes) and the remainder migrated as free 35-kDa 125I-urokinase (arrow). SDS-PAGE revealed four sources of plasminogen activator activity in conditioned medium incubated with 35-kDa urokinase at 0.1 μg/ml. These had approximate molecular weights of 35,000 51,000, 72,000, and 90,000. The 51- and 90-kDa activities represented plasminogen activators released into the medium by HF cells (see below). The 35- and 72-kDa activities represented activity of the added urokinase: they were present only in conditioned medium containing added urokinase and co-migrated with 125I-urokinase and 125I-urokinase-PN, respectively. Significantly, the 72-kDa activity peak contained only a small fraction of the total urokinase activity in the medium even though most urokinase added to the medium was present in 72-kDa complexes with PN (Fig. 2). This indicates that urokinase linked to PN had lost activity. In fact, even the limited urokinase activity apparently associated with urokinase-PN could be artifactual because a significant amount of 35-kDa 125I-urokinase was released from 125I-urokinase-PN complexes extracted from SDS-polyacrylamide gels and re-submitted to SDS-PAGE (data not shown). Urokinase incubated in conditioned medium had less activity than urokinase incubated in fresh medium (Fig. 2), which demonstrates the presence of a urokinase inhibitor in conditioned medium.

Purified PN inhibited the ability of urokinase to activate plasminogen. Fig. 3 shows the urokinase-mediated conversion of plasminogen (90 kDa) to plasmin (70- and 25-kDa chains). Bovine pancreatic trypsin inhibitor (lane 2) or soybean trypsin inhibitor (lane 3) were added to inhibit plasmin. When urokinase and plasminogen were co-incubated in the absence of plasmin inhibitors, the generated 70- and 25-kDa chains of plasmin were degraded by plasmin autolysis (lane 4). Significantly PN, added in 2-fold molar excess over urokinase, completely blocked urokinase-mediated plasminogen activation (lane 5).

Inactive Urokinase-like Plasminogen Activator Released by Human Fibroblasts—In a standard 125I-fibrin well plasminogen activator assay, conditioned medium contained substantial plasminogen activator activity. SDS-PAGE showed that about 90% of this activity was associated with a single component (Fig. 4). The activity of this human fibroblast plasminogen activator in the 125I-fibrin assay was roughly equivalent to the activity of the 35-kDa urokinase at 10–20 ng/ml (see Fig. 2 above). Based on the mobility of HFPA relative to 35-kDa urokinase and 72-kDa urokinase-PN (see Fig. 2 above), we estimated its molecular mass as approximately 51 kDa. In view of the similar sizes of HFPA and urokinase, we examined
whether anti-urokinase antibody inhibited HFPA. Goat IgG containing anti-urokinase antibody inhibited conditioned medium plasminogen activator activity by 60% at 0.07 \( \mu \)g/ml of IgG and by 98% at 6.7 \( \mu \)g/ml of IgG. Normal goat IgG at 67 \( \mu \)g/ml did not significantly affect HFPA activity. The immunological relationship between HFPA and urokinase was further explored by radioimmunoassay for 54-kDa urokinase. Conditioned medium inhibited binding of \(^{125}\text{I}\)-urokinase to the anti-urokinase antibody in the radioimmunoassay (Fig. 5). The inhibition curve generated by increasing volumes of conditioned medium paralleled the inhibition curve generated by increasing amounts of urokinase. This suggested that HFPA was immunologically very similar to urokinase. Because PN binds urokinase, an alternative possibility was that PN blocked binding of \(^{125}\text{I}\)-urokinase to anti-urokinase antibody, thereby artificially suggesting the presence of urokinase-like antigen in conditioned medium. The latter possibility is ruled out because (1) \(^{125}\text{I}\)-urokinase-PN bound to the antibody as effectively as free \(^{125}\text{I}\)-urokinase and (2) addition of either thrombin (1 \( \mu \)g/ml) or the urokinase inhibitor \( \beta \)-nitrophenyl guanidinobenzoate (10 \(^{-7}\) M) to conditioned medium did not significantly alter the radioimmunoassay results even though both completely blocked formation of \(^{125}\text{I}\)-urokinase-PN complexes (data not presented). Numerous radioimmunoassays, including the one shown in Fig. 5, indicated that urokinase-like antigen in conditioned medium was present at concentrations between 25 and 100 ng/ml.

Conditioned medium plasminogen activator activity in \(^{125}\text{I}\)-fibrin well assays was unaffected by incubation of conditioned medium at 37 \(^\circ\)C for several hours before assay (Fig. 6A) even though active PN was present in conditioned medium over this time course (Fig. 6B). As shown in Fig. 6B, PN is permanently inactivated by a 2-h incubation at pH 3 on ice. Whether HFPA was preincubated in untreated or acid-treated conditioned medium, the conditioned medium plasminogen activator activity measured in \(^{125}\text{I}\)-fibrin well assays was similar.\(^2\) These results suggest that unlike urokinase, secreted HFPA is not complexed by PN. As an independent test of this conclusion, we fractionated conditioned medium on Sephadex G-200 and used radioimmunoassay for urokinase to detect HFPA. Significantly, approximately 90% of the urokinase-like antigen eluted from the column in the same volume as free, active 50-kDa urokinase (Fig. 7). This antigen peak coincided with HFPA activity. The remaining 10–15% of the antigen co-eluted with the 90-kDa marker protein and might represent either HFPA complexed to PN or an inactive high molecular weight form of urokinase. Thus, most and possibly all of the urokinase-like antigen in conditioned medium corresponded to plasminogen activator that was not complexed to PN or other macromolecular inhibitors.

When PMSF was incubated with urokinase and unreacted PMSF removed by dialysis, the PMSF-treated urokinase was inactive (Table I). In contrast, similar treatment of conditioned medium did not reduce its plasminogen activator activity measured in the \(^{125}\text{I}\)-fibrin well assay system. Paradoxically, PMSF pretreatment increased conditioned medium

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\(^2\) When the concentration of PN exceeds \( \approx 10\) ng/ml, PN significantly inhibits the plasmin generated in the \(^{125}\text{I}\)-fibrin well assay (D. L. Eaton and J. B. Baker, unpublished results). In such cases, pH 3 treatment of conditioned medium increases its fibrinolytic activity. In the experiment shown in Fig. 6, conditioned medium was diluted by 20-fold or 100-fold in the \(^{125}\text{I}\)-fibrin wells to prevent significant PN-mediated inhibition of plasmin. The apparent transient increase in plasminogen activator activity that Fig. 6 shows in control-conditioned medium was not reproducible.
plasminogen activator activity in this assay system (see "Discussion"). The evidence that neither PN nor PMSF reacts with HFPA in conditioned medium indicates that HFPA is actually inactive but is activated by conditions in the 125I-

**TABLE I**

| Sample Description                  | Assay Buffer (1 ml) | % 125I-Fibrin Hydrolyzed |
|-------------------------------------|---------------------|--------------------------|
| Conditioned medium (50 μl)          | 0.1 M Tris-Cl, pH 8.1 | 105                      |
| Conditioned medium (50 μl)          | DME medium          | 2                        |
| Urokinase (0.4 ng)                  | DME medium          | 84                       |
| Urokinase (0.1 ng)                  | DME medium          | 72                       |
| Urokinase (0.1 ng)                  | DME medium plus 50 μl conditioned medium | 19 |

**FIG. 7. Fractionation of urokinase-like antigen and HFPA activity in conditioned medium.** Medium conditioned for 3 days in HF cell micrcarrier culture was concentrated 5-fold by ultrafiltration using an Amicon PM10 membrane and 1 ml was fractionated at 5 °C on a Sephadex G-200 column (1.5 × 90 cm) equilibrated with a buffer containing 0.3 M NaCl, 0.01 M Tris-Cl, and 0.1% Triton X-100. Column fractions (approximately 2.5 ml each) were assayed for urokinase-cross-reactive antigen by radioimmunoassay and for plasminogen activator activity by 125I-fibrin well assay. To detect plasminogen activator activity, samples were pretreated at pH 3 as in Fig. 6 in order to inactivate PN, which eluted in fractions 34–40. PN at the concentrations present in fractions 34–40 inhibited plasmin and prevented 125I-fibrin digestion in the plasminogen activator assay (our unpublished data). The column was calibrated with dextran blue (excluded volume), plasminogen (90 kDa), 125I-urokinase (54 kDa), and cytochrome c (14 kDa).

**Effect of phenylmethanesulfonyl fluoride on the fibrinolytic activity of urokinase and HF cell-conditioned medium**

Conditioned medium from microcarrier cultures or 54-kDa urokinase (0.1 μg/ml in fresh DME medium) were incubated at 37 °C with or without 1 mM PMSF for the times shown. These mixtures were then dialyzed against fresh DME medium for 24 h at 5 °C and plasminogen activator activity of 0.1-ml aliquots was measured by the 125I-fibrin well assay. In experiment I, before PMSF treatment, the conditioned medium was pretreated at pH 3 as described in Fig. 6.

**FIG. 8. Activation of conditioned medium plasminogen activator by plasmin and inhibition by PN.** Medium incubated with 35-mm HF cell cultures for 3 days was concentrated 10-fold by ultrafiltration through an Amicon PM10 membrane, diluted 10-fold with 0.1 M Tris-Cl buffer (pH 8.1), and reconstituted. Aliquots of this or Tris-Cl buffer were incubated for 20 min at 37 °C either alone or with plasmin at 1.0 μg/ml. Bovine pancreatic trypsin inhibitor (0.125 mg/ml) and 125I-plasminogen (0.5 mg/ml) with or without PN (1.0 μg/ml) were added. After 14 h at 37 °C, the mixtures were solubilized and heated in SDS sample buffer. Radioactive proteins were resolved by SDS-PAGE and autoradiography. The autoradiogram shows 125I-plasminogen incubated with: Tris-Cl buffer containing urokinase (10 ng (0.75 CTA unit)/ml) (lane 1), Tris-Cl buffer (lane 2), Tris-Cl buffer preincubated with plasmin (lane 3), conditioned medium (lane 4), conditioned medium preincubated with plasmin (lane 5), or conditioned medium preincubated with plasmin and incubated with PN (lane 6).
HF cell-conditioned medium did not convert $^{125}$I-plasminogen to $^{125}$I-plasmin (lane 4). However, when HF cell-conditioned medium was preincubated with plasmin, it then contained active plasminogen activator (lane 5). This was not plasmin itself because plasmin inhibitor added after preincubation of conditioned medium with plasmin prevented further plasmin action (lane 3).

The conditioned medium used in this experiment had been depleted of PN by preincubation at pH 3 in order to eliminate possible inhibitory effects of PN. When purified PN (1.0 $\mu$g/ml) was added to PN-depleted conditioned medium containing plasmin-activated plasminogen activator and this mixture was incubated with $^{125}$I-plasminogen, no cleavage of $^{125}$I-plasminogen occurred (Fig. 8, lane 6).

**DISCUSSION**

When a number of serine proteases, including the plasminogen activator urokinase, are added to cultures of human foreskin fibroblasts, they become bound to the secreted protease-binding protein PN (15–17). In our efforts to identify physiological function(s) of PN, we have focused on the role of PN in the regulation of plasminogen activator activity because foreskin fibroblasts as well as other cell types produce urokinase-like protease. Here we have shown that urokinase is inhibited by PN, either in conditioned medium or in a purified system. This interaction involves the urokinase active site because urokinase preincubated with diisopropyl fluorophosphate is not complexed by PN. Previous studies carried out with thrombin indicate that protease-PN complexes include an intermolecular carboxyester linkage (15). We show that formation of urokinase-PN complexes is essentially irreversible.

Although these results imply that HF cells are a source of anti-activator, medium conditioned by these cells exhibits substantial plasminogen activator activity in the standard two-step assay system in which plasmin-mediated hydrolysis of $^{125}$I-fibrin is measured ($^{125}$I-fibrin well assay). The source of this activity is a plasminogen activator (HFPA) that is immunologically and electrophoretically similar to urokinase. When conditioned medium is fractionated by gel exclusion chromatography, all the HFPA activity and about 90% of the HFPA preincubated with phenylmethanesulfonyl fluoride is active in $^{125}$I-fibrin well assays. These apparently paradoxical findings are resolved by evidence that HFPA is actually inactive in conditioned medium and therefore does not interact with inhibitors. Artificial activation of HFPA, as with plasminogen activators released by certain other cell types, probably results from a combination of decreased NaCl concentration and trace amounts of plasmin in the $^{125}$I-fibrin well assay system (10, 11). Once active endogenous plasminogen activator is generated in conditioned medium, it is sensitive to inhibition by PN. Thus, HF cells control the activity of secreted plasminogen activator both by secreting the protease in an inactive form (proenzyme) and by secreting an inhibitor of the active protease. These findings suggest that the increased expression of plasminogen activator activity by malignant cells (2, 3) could be caused by activation of inactive plasminogen activator or by decreased levels of PN or other protease inhibitors.

HFPA may be identical with a recently purified and characterized 53-kDa urokinase proenzyme secreted from human epidermoid carcinoma cells (11). Both are inactive in conditioned medium but are activated by plasmin or conditions in the $^{125}$I-fibrin well assay. The tumor cell urokinase proenzyme is composed of a single polypeptide chain that is cleaved by plasmin to form the 35- and 18-kDa chains of active urokinase.

We are currently purifying HFPA for further characterization. Heparin-Sepharose chromatography results in approximately 100-fold purification of HFPA. This material is inactive and can be activated by plasmin. Attempts to purify HFPA by absorption to p-aminobenzamidine-Sepharose, which binds urokinase at its active site (23), have failed, presumably because the active site of HFPA is inaccessible. The present results show that the amount of HFPA detected in conditioned medium increases when conditioned medium is incubated with PMSF. This suggests that conditioned medium may contain an HFPA-degrading protease.

Because PN is the major urokinase-binding protein released by HF cells, the evidence that HF cells release urokinase proenzyme suggests that a physiological function of PN is to regulate urokinase. PN has a remarkable affinity for urokinase. Unpublished studies show that although serum contains a wealth of protease inhibitors, some of which bind urokinase (24, 25), serum at concentrations up to 20% does not competitively inhibit formation of urokinase-PN complexes in conditioned medium. In contrast, serum at just 0.5% prevents formation of thrombin-PN complexes in conditioned medium because thrombin is complexed by serum inhibitors. We have found that thrombin, EGF, and phorbol myristate acetate stimulate HFPA secretion with an order of effectiveness: thrombin < EGF < phorbol myristate acetate. Consistent with a role for PN in inhibition of endogenous urokinase, these agents also stimulate PN secretion with the same order of effectiveness. The present experiments detected little material in conditioned medium that could have represented complexes between PN and endogenous urokinase (activated HFPA). However, because protease-PN complexes are rapidly endocytosed, such complexes may form but be effectively cleared from the medium. The extent to which PN regulates urokinase secreted by HF cells depends on the frequency that the inactive form of the secreted protease becomes activated. The physiological circumstances under which activation occurs are presently unclear.

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