Purification of Protease Nexin II from Human Fibroblasts*

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Normal human fibroblasts secrete a protein named protease nexin II (PN II) which previously was shown to form thiation dodecyl sulfate (SDS)-stable complexes with epidermal growth factor-binding protein (EGF-BP). These complexes then bind to the same cells and are rapidly internalized and degraded (Knauer, D. J., and Cunningham, D. D. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2310–2314). Here we describe a procedure for purifying PN II to apparent homogeneity from serum-free culture medium conditioned by human fibroblasts. The first step employed dextran sulfate-phacryl resin to reveal homologies with other reported amino acid sequences. Further purification was achieved by ion-exchange chromatography on DEAE-Sepharose followed by gel filtration on Sephacryl S-400. Sequence analysis of purified PN II identified 33 amino-terminal amino acids; a computer search of several protein sequence data banks failed to reveal homologies with other reported amino acid sequences. Purified PN II had an apparent $M_\text{r}$ of 106,000 and an isoelectric point of approximately 7.2. It retained full activity after incubation in the presence of 0.05% SDS or at a pH of 1.5. PN II formed SDS-stable complexes with EGF-BP, the $\gamma$ subunit of 7 S nerve growth factor, and C-terminal fragments with estimated $M_\text{r}$ of 120,000, 120,000, and 110,000, respectively. PN II was metabolically labeled with $^{[35]}$Smethionine and purified; the metabolically labeled protein formed complexes with EGF-BP. Complexes between purified PN II and EGF-BP bound to human fibroblasts. These results show that the purified protein possesses the properties previously attributed to PN II in cell culture medium.

The PNs are protein protease inhibitors which are synthesized and secreted by human fibroblasts in culture and form stable 1:1 complexes with certain serine proteases. These PN-protease complexes then bind back to the cells from which the PNs were secreted, apparently via the PN moiety of the complex. Once bound to the cells, the PN-protease complexes are rapidly internalized and degraded to amino acids (1–7).

There is evidence that the linkage between a PN and its protease involves an ester bond with the catalytic site serine of the protease. First, PN-protease complexes are stable to boiling in SDS suggesting that they are covalent (1, 3, 4). Second, the complexes are disrupted by pH 12 or 1 M hydroxylamine (1, 9). Third, derivatization of the protease active site serine with diisopropyl fluorophosphate blocks the formation of complexes (1, 5, 8). The acyl-linked PN-protease complex is probably a stable intermediate of proteolysis of the PN since a fragment is released from native PN during complex formation (9).

Three PNs have been identified and have been shown to form 1:1 complexes with certain $^{[35]}$Smethionine-labeled serine proteases. PN I can rapidly complex several serine proteases including thrombin, trypsin, urokinase, and plasmin (1, 2, 9, 10). PN II previously was shown to complex EGF-BP (3, 5–7) and PN III was found to complex NGF-$\gamma$ (4–7).

Since the linkage of these proteases to their respective PN involves the catalytic site serine, this inactivates these proteases and provides a mechanism whereby cells can regulate the activity of certain proteases at or near the cell surface. Studies with purified PN I showed that it can modulate thrombin-stimulated cell division (11), levels of plasminogen activator at the cell surface (8), and prevent degradation of extracellular matrix by fibrosarcoma cells (12). The present purification of PN II was undertaken so that its biochemical properties and biological functions could also be examined.

PN I has been purified from serum-free culture medium conditioned by HF cells grown on microcarrier beads (9) or in tissue culture roller bottles (13). Here we describe the purification of PN II from serum-free culture medium conditioned by HF cells maintained in microcarrier cultures.

EXPERIMENTAL PROCEDURES

Materials—Gelatin microcarrier beads were obtained from KC Biological. Microcarrier flasks and stir plates were from Wheaton Scientific, Millville, NJ. Fetal bovine serum and all cell culture media were from Gibco. All tissue culture plasticware was from Corning. Sepharose CL-6B, DEAE-Sepharose, Sephacryl S-400, and dextran sulfate ($M_\text{r}$ = 500,000) were from Pharmacia P-L Biochemicals. 1,4-Butanediol diglycylic ether and $N,N'$-diallyltartardiamide were obtained from Aldrich. Aquacide II-A was from Behring Diagnostics. Ultrapure acrylamide and ampholytes were from BioRad. Isoelectric focusing marker proteins were from BDH Chemicals. Na$^{[35]}$I was from New England Nuclear and IODO-GEN was from Pierce Chemical Co. [$^{[35]}$S]Methionine was from Amersham Corp. Mono-Tris was synthesized and purified as previously described (14). All other chemicals were reagent-grade and obtained from Sigma.

Mouse EGF-BP was purified as previously described (15) and was iodinated by the chloroglycuril method using IODO-GEN and Na$^{[125]}$I (16). The specific activities ranged between 30,000 and 70,000 cpm/µg.

Synthesis of Dextran Sulfate-Sepharose—250 ml of Sepharose CL-6B was washed thoroughly with distilled water. The caked Sepharose
was then activated by adding 135 ml of 1 M NaOH containing 2 mg/ml NaBH₄ and 125 ml of the diisopropyl 1,4-butanediol diglycidyl ether; the mixture was subsequently mixed on a rocking platform for 8 h at room temperature. After activation, the 250 ml of epoxy-activated Sepharose was washed thoroughly with distilled water and added to 250 ml of 0.2 M sodium bicarbonate (pH 11) containing 10 g of dextran sulfate (M₀ = 500,000) and mixing was performed by mixing on a rocking platform at 37 °C for 16 h. Then, ethanolamine was added to a final concentration of 1 M and mixing was continued at 37 °C overnight to block any remaining reactive epoxide groups. The coupled Sepharose was then washed with 2 liters of 0.2 M sodium bicarbonate (pH 11), 2 liters of 0.1 M phosphate buffer (pH 7.4), and 2 liters of phosphate-buffered saline. The dextran sulfate-Sepharose was stored at 4 °C in phosphate-buffered saline containing 0.02% NaN₃.

Cell Culture—HF cells were isolated from explants of neonatal foreskins and were maintained in 100-mm culture dishes, as previously described (1). To set up 850-cm² roller bottle cultures, HF cells were treated with trypsin from two confluent 100-mm culture dishes and placed in 100 ml of DMEM buffered with 20 mM Hepes (pH 7.4) containing 100 units/ml of penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. The roller bottles were maintained at 37 °C in a roller bottle apparatus (0.7 rpm); the HF cells reached confluency for 10 h and then the culture was stirred at 40 rpm overnight. The culture was stirred for 60 s at 30 rpm and then allowed to settle for 2 h. This alternating procedure of stirring and settling was continued for 10 h and then the culture was stirred at 40 rpm overnight. The volume of medium was then increased to 3 liters and changed every 3-day periods, after each period HF cell "conditioned medium was removed by aspiration and replaced with 2 liters of DMEM buffered with 20 mM Hepes (pH 7.4) containing the antibiotics. The microcarrier beads were again allowed to settle; this rinse medium was removed by aspiration and replaced with 2 liters of DMEM buffered with 20 mM Hepes (pH 7.4) containing the antibiotics and 0.1% BSA. The culture was stirred for 60 s at 30 rpm and then allowed to settle for 2 h. This alternating procedure of stirring and settling was continued for 10 h and then the culture was stirred at 40 rpm overnight. The volume of medium was then increased to 3 liters and changed every 4 days. The cells reached confluence on the microcarrier beads in approximately 7–10 days.

Collection of Serum-free Conditioned Medium—To remove the serum from the confluent microcarrier cultures, the microcarrier beads were allowed to settle; the serum-containing medium was removed by aspiration and replaced with 2 liters of DMEM buffered with 20 mM Hepes (pH 7.4) containing the antibiotics and 10% fetal bovine serum. The culture was stirred for 60 s at 30 rpm and then allowed to settle for 2 h. This alternating procedure of stirring and settling was continued for 10 h and then the culture was stirred at 40 rpm overnight. The volume of medium was then increased to 3 liters and changed every 4 days. The cells reached confluence on the microcarrier beads in approximately 7–10 days.

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Anion Exchange Chromatography—PH II-containing fractions from dextran sulfate-Sepharose were diluted with 20 mM potassium phosphate (pH 7.4) to reduce the conductivity to that of the starting buffer of the next column, 20 mM potassium phosphate, 0.2 M NaCl (pH 7.4). The resulting solution was applied to a column (0.5 x 12 cm) of DEAE-Sepharose equilibrated in the above starting buffer at a flow rate of 20 ml/h. The column was then washed with 4 column volumes of starting buffer and PH II was eluted with 20 mM potassium phosphate, 0.75 M NaCl (pH 7.4). Fractions of 2 ml were collected at a flow rate of 5 ml/h. Aliquots of each fraction were analyzed by SDS-PAGE; fractions containing apparently homogeneous PH II were pooled, aliquoted, and stored at −70 °C.

Electrophoretic Techniques—SDS-PAGE was performed according to the method of Laemmli (19). Either 7.5% or 10% total acrylamide was used as the gel slab. Gels containing 15% acrylamide were cast as 15 x 8 x 0.7 mm slabs. Phlase concentrations contained 5% mercaptoethanol. Gels were stained in methanol/acetic acid/water (5:1.5) containing 0.05% Coomassie Brilliant Blue R-250, followed by destaining in 5% ethanol, 7% acetic acid. Silver staining of gels was carried out by the method of Rubin et al. (20). The gel slices were dehydrated in the classical manner, the gel slices were overlaid with distilled water, and waterpolymersized under fluorescent light. The upper reservoir buffer consisted of 20 mM NaOH and the lower reservoir buffer consisted of 10 mM H₂PO₄. Samples were focused at 250 V for 18 h at 4 °C. When focusing was complete, the gel slices were washed twice with 10% acetic acid and extracted into 0.2 ml of distilled water for 24 h at 4 °C. The pH of each gel slice extract was measured. 0.1 ml of phosphate-buffered saline was then added to each extract and aliquots of each extract were tested for PH II activity as described above.

PN II Assay—Known quantities of ¹²⁵I-EGF-BP were incubated with aliquots of samples containing PH II for 20 min at 37 °C. An equal volume of SDS sample buffer was then added; the mixtures were subjected to SDS-PAGE as described above. After autoradiography, PH II activity was monitored by the formation of a 120-kDa complex with the ¹²⁵I-EGF-BP. To quantitate PH II activity, the autoradiograms were aligned with the dried gels, the ¹²⁵I-labeled complexes were located, excised, and measured in a γ-counter.

Protein Sequencing—Amino-terminal sequencing was performed as described above. After autoradiography, PH II activity was monitored by the formation of a 120-kDa complex with the ¹²⁵I-EGF-BP. To quantitate PH II activity, the autoradiograms were aligned with the dried gels, the ¹²⁵I-labeled complexes were located, excised, and measured in a γ-counter.

Stability of PH II—To investigate the stability of PH II under denaturing conditions, the purified protein (30 μg/ml) was incubated at 55 °C and the ¹²⁵I-EGF-BP complexes with the PH II were monitored by SDS-PAGE as described above. After autoradiography, PH II activity was monitored by the formation of a 120-kDa complex with the ¹²⁵I-EGF-BP. To quantitate PH II activity, the autoradiograms were aligned with the dried gels, the ¹²⁵I-labeled complexes were located, excised, and measured in a γ-counter.
incubated in 30 μl of 0.2 M glycine-HCl (pH 1.5) for 60 min at 37 °C. After that, 10 ml of 3 M Tris-HCl (pH 8.3) and 20 ng of [125I]-EGF-BP were added and incubated for an additional 20 min at 37 °C. The samples were subjected to SDS-PAGE and the [125I]-EGF-BP.PN II complexes were quantitated as described above. In each study, samples incubated with phosphate-buffered saline instead of SDS or glycine-HCl served as controls.

Metabolic Labeling of PN II by HF Cells—PN II was also purified from conditioned medium collected from HF cells that were cultured in the presence of [35S]methionine. Three confluent 100-mm cultures of HF cells were each incubated for 24 h in 10 ml of a minimal essential medium buffered with 20 mM Hepes (pH 7.4) containing 0.1% BSA and only 15% of the normal methionine concentration. This medium was then replaced with 10 ml/dish of the same medium containing 10 μCi/ml of [35S]methionine (1450 Ci/mmol). After a 4-day incubation, the medium was collected as described above, combined with 90 ml of unlabelled conditioned medium, and loaded onto a 5-ml dextran sulfate-Sepharose column equilibrated with phosphate-buffered saline. The column was washed with 30 ml of phosphate-buffered saline and the protein was eluted with a 40-ml linear gradient of 0.15–1.2 M NaCl in phosphate-buffered saline. Fractions were assayed for PN II activity by incubation with [125I]-EGF-BP as described above; the appropriate fractions were then subjected to preparative SDS-polyacrylamide tube gel electrophoresis employing the mono-Tris/bis-Tris/Bicine/SDS gel system as described above. Since PN II retained full activity in the presence of 0.65% SDS the preparative gel electrophoresis was performed at this SDS concentration. After electrophoresis, the gels were frozen, sliced into 2-mm sections, and extracted with 0.3 ml of phosphate-buffered saline for 24 h at 4 °C. Each gel slice extract was then tested for PN II activity with [125I]-EGF-BP as described above. The resulting gel slice extracts that contained PN II were then stored at −20 °C.

Cellular Binding of [125I]-EGF-BP.PN II Complexes—[125I]-EGF-BP (150 ng) was incubated alone or with an equimolar or 4-fold molar excess of purified PN II for 30 min at 37 °C in 200 μl of 20 mM potassium phosphate, 0.2 M NaCl, (pH 7.4). Each of these incubation mixtures was then added to 2.8 ml of DMEM buffered with 20 mM Hepes (pH 7.4) containing 0.1% BSA (binding medium). The serum-containing growth medium on 35-mm culture dishes of confluent HF cells was replaced with binding medium for 24 h at 37 °C. Each gel slice extract was then tested for PN II activity with [125I]-EGF-BP as described above. The resulting gel slice extracts that contained PN II were then stored at −20 °C.

RESULTS

Purification of PN II—We purified PN II from serum-free culture medium conditioned by HF cells maintained in microcarrier cultures utilizing the procedures outlined under "Experimental Procedures." Table I summarizes the purification. An early observation that PN II adsorbs to highly sulfated proteins such as heparin and dextran sulfate provided a useful initial step in the purification. Earlier we synthesized sulfated-dextran beads according to the procedure of Mileitch et al. (23) which utilizes Sephadex G-50. The major drawback of this affinity gel is that the column bed volume decreases drastically upon exposure to high ionic strength, thus preventing the use of a salt gradient to elute the adsorbed proteins. To circumvent this problem, we coupled dextran sulfate to epoxy-activated Sepharose CL-6B to produce a gel which possesses a high affinity for PN II and is unaffected by changes in ionic strength. This affinity matrix not only permitted enrichment for PN II, but also provided a means for concentrating the large volume of starting material. Four liters of HF cell serum-free conditioned medium were applied directly to this dextran sulfate-Sepharose column followed by elution of the adsorbed protein with a linear salt gradient. PN II eluted from the column between 0.5 and 0.6 M NaCl (Fig. 1) with a yield of 95% (Table I). Aliquots of each fraction were incubated with [125I]-EGF-BP and analyzed by SDS-PAGE with subsequent autoradiography as described under "Experimental Procedures." PN II-containing fractions were identified by the appearance of a 120-kDa [125I]-EGF-BP.PN II complex.

The second step of the purification took advantage of the tenacious binding that PN II exhibits towards DEAE-Sepharose. The pooled PN II-containing fractions from dextran sulfate-Sepharose were diluted to the conductivity of the starting buffer (0.2 M NaCl) and the resulting solution was applied to the column. After washing, PN II was eluted isocratically with 0.75 M NaCl resulting in an approximately 4-fold purification (Table I). Use of the high salt starting buffer helped prevent nonspecific losses of PN II and resulted in a very high yield for this step in the purification. SDS-PAGE analysis of PN II eluted from DEAE-Sepharose revealed the presence of only high M₆, contaminating proteins.

Gel filtration chromatography was employed for the final step in the purification. PN II-containing fractions from DEAE-Sepharose were pooled and concentrated against Aquacide II-A as described under "Experimental Procedures." The resulting concentrated solution was applied to a column of Sephacryl S-400. Again, the use of a high salt concentration in these final steps of the purification resulted in very little loss of activity (Table I). It should be stressed that earlier attempts at using low salt buffers in these steps resulted in severe losses of PN II (data not shown). This gel filtration step effectively removed the high M₆ contaminants (Fig. 2) and resulted in apparently homogeneous protein as assessed by SDS-PAGE and silver stain analysis (Fig. 3, lane 1). In addition, amino-terminal sequence analysis of purified PN II yielded a single sequence of 33 amino acids, further demonstrating the homogeneity of this protein (Table II).

Preliminary Characterization of PN II—PN II is a single-chain polypeptide with an estimated M₆ of 106,000 (Fig. 3, lanes 1 and 2). When PN II was electrophoresed under reducing conditions it migrated slower on the SDS gel with an estimated M₆ of 112,000 (Fig. 3, lane 3), suggesting that PN

| Step | Volume | Protein | Units* | Specific activity | Yield | Purification |
|------|--------|---------|--------|------------------|-------|-------------|
|      | ml     | ng      | units/mg | units/mg | %     | fold       |
| Conditioned medium | 4,000 | ~4,400b | 42,880 | 9.75 | 100 | 1 |
| Dextran sulfate-Sepharose | 174 | 14.1 | 40,900 | 2,894 | 95 | 297 |
| DEAE-Sepharose | 12 | 3.9 | 40,640 | 10,420 | 94 | 1,070 |
| Sephacryl S-400 | 16 | 0.621 | 35,680 | 58,300 | 83 | 5,960 |

* One unit is defined as the amount of PN II that will complex 1 ng of [125I]-EGF-BP as quantitated by SDS-PAGE as described under "Experimental Procedures."
denaturing conditions was examined. Incubation of purified PN I1 was reported amino acid sequences. When purified PN I1 was type of behavior on SDS gels after reduction (10). The isoelectric point of PN I1 was determined as described under "Experimental Procedures." Fractions 26-34 containing peak PN I1 activity were pooled for anion-exchange chromatography. , absorbance at 280 nm; O, PN II activity; - - -, m NaCl.

FIG. 2. Gel filtration of PN II on Sephacryl S-400. The protein was chromatographed on the column (1.1 × 110 cm) equilibrated with 20 mM potassium phosphate, 0.5 M NaCl (pH 7.4). Fractions of 2 ml were collected at a flow rate of 5 ml/h. , absorbance at 280 nm; O, PN II activity measured with 125I-EGF-BP and analysis on SDS-PAGE with subsequent autoradiography as described under "Experimental Procedures." Fractions 26-34 containing peak PN II activity were pooled for anion-exchange chromatography. , absorbance at 280 nm; O, PN II activity; - - -, m NaCl.

FIG. 3. SDS-PAGE of purified PN II. Aliquots of purified PN II were electrophoresed on 7.5% SDS gels in the absence (lanes 1 and 2) or presence (lane 3) of /β-mercaptoethanol as described under "Experimental Procedures." Lane 1 was silver stained according to the procedure of Rubin et al. (20). Lanes 2 and 3 were stained with Coomassie Brilliant Blue.

The stability of the complexing activity of PN II under denaturing conditions was examined. Incubation of purified PN II in 0.2 M glycine-HCl (pH 1.5) for 60 min at 37 °C had no effect on its ability to complex 125I-EGF-BP (102 ± 3.0% of control value; data not shown). The ability of PN II to form complexes with 125I-EGF-BP was not significantly af-

affected upon incubation with 0.05% SDS for 60 min at 37 °C (data not shown). Incubation with higher concentrations of SDS did begin to affect the inhibitor activity of PN II, but even at 0.1% SDS, greater than 80% of the control activity remained. Together, these data suggest that PN II is a stable protein. In contrast, similar treatments readily destroy the inhibitor activity of PN I (24).

Purified PN II formed 1:1 SDS-stable complexes when incubated with EGF-BP, the closely related NGF-γ (25), and bovine trypsin (Fig. 5). In contrast, no complex formation was observed when PN II was incubated with numerous other serine proteases including chymotrypsin, elastase, thrombin, plasmin, urokinase, plasma kallikrein, or factor XIIa (data not shown).

Purification of PN II from HF Cells Cultured in the Presence of [35S]Methionine—Since cultured cells have been shown to internalize serum protease inhibitors and subsequently release them back into the culture medium (26-29), we determined whether HF cells actually synthesize PN II. To do this we incubated 100-mm confluent culture dishes with [35S]methionine. Since PN II was stable in 0.05% SDS, we purified 35S-PN II employing preparative SDS-PAGE as described under "Experimental Procedures." In Fig. 6, lane 1 is an autoradiogram of purified PN II demonstrating that it was metabolically labeled with [35S]methionine. Lane 2 of the same figure is an autoradiogram of the purified 35S-PN II incubated with unlabeled EGF-BP, clearly showing the formation of a 120-kDa 35S-PN II-EGF-BP complex. The metabolic labeling of the protein clearly shows that it is indeed a biosynthetic product of the HF cells. The formation of an SDS-stable 120-kDa complex with EGF-BP correctly identified this metabolically labeled protein as PN II.

Cellular Binding of 125I-EGF-BP·PN II Complexes—We investigated whether complexes formed between EGF-BP and purified PN II bound to HF cells. 125I-EGF-BP was incubated with an equimolar or 4-fold molar excess of PN II and the resulting 125I-EGF-BP·PN II complexes were incubated with duplicate confluent cultures of HF cells for 20 min at 37 °C. After rinsing the cells, the cell-associated radioactivity was measured as described under "Experimental Procedures." As shown in Fig. 7, when free 125I-EGF-BP was incubated with the cultures some of the protease was bound to cells via PN II as evidenced by the small amount of 120-kDa complex (lanes 1 and 4). This binding probably occurred from PN II
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TABLE II
Amino-terminal amino acid sequence of purified PN II

| Residue number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|----------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|
| PN II          | Leu| Glu| Val| Pro| Thr| Asp| Gly| Asn| Ala| Gly| Leu| Leu| Ala| Glu| Pro| Gln| Ile|
| 18             | 19| 20| 21| 22| 23| 24| 25| 26| 27| 28 | 29 | 30 | 31 | 32 | 33 |
| Ala            | Met| Phe| Cys| Gly| Arg| Leu| Asn| Met| [Phe]|Met| Asn| Val| Gln| Asn| Gly|    |    |

* Brackets indicate determination with less than full confidence.

FIG. 4. Formation of EGF-BP-PN II complexes. EGF-BP (50 µg/ml) was incubated with or without purified PN II (100 µg/ml) in 20 mM potassium phosphate, 0.5 M NaCl (pH 7.4) for 30 min at 37 °C. The samples were electrophoresed under nonreducing conditions on a 10% polyacrylamide gel as described by Laemmli (19). Lane 1, EGF-BP; lane 2, PN II; lane 3, EGF-BP + PN II.

FIG. 5. Formation of [125I] labeled protease-PN II complexes. [125I]-EGF-BP, [125I]-NGF-γ, and [125I]-trypsin were each incubated with aliquots of purified PN II for 20 min at 37 °C. The samples were subjected to SDS-PAGE under nonreducing conditions and analyzed by autoradiography. Lane 1, EGF-BP; lane 2, EGF-BP + PN II; lane 3, NGF-γ; lane 4, NGF-γ + PN II; lane 5, trypsin; lane 6, trypsin + PN II.

DISCUSSION

This report shows that PN II has been purified to apparent homogeneity from serum-free medium conditioned by HF cells maintained in microcarrier cultures. Initial attempts to purify PN II from HF cell monolayer cultures maintained in roller bottles resulted in low yields due to the low concentrations of PN II in the starting conditioned medium. To increase the concentration of PN II in conditioned medium, Scott and Baker (9) maintained HF cells in microcarrier cultures using Cytodex-2 microcarrier beads. Therefore, we attempted to purify PN II from serum-free culture medium conditioned by HF cells cultured on the same microcarrier beads. However, we found very low levels of PN II activity in the conditioned medium collected from these cultures. The Cytodex-2 microcarrier beads are composed of a cross-linked dextran matrix which is coated with positively charged N,N,N-trimethyl-2-hydroxyaminopropyl groups (30). These positively charged groups apparently enhance the adherence of cells to the beads. In the present studies we showed that PN II binds very tightly to the positively charged groups of DEAE-Sepharose. Therefore, a likely explanation for the very low levels of PN II activity in the conditioned medium is that PN II released by the HF cells was adsorbed to the microcarrier beads. To circumvent this problem we maintained HF cells on gelatin microcarrier beads and indeed found much more PN II in the conditioned medium. Employing a three-step procedure, an approximate 6000-fold purification was achieved with a recovery of 83%. Using dextran sulfate-Sepharose affinity chromatography for the initial step not only concentrated and enriched for PN II, we found in Cytodex-2 microcarrier cultures that PN II released by the HF cells was adsorbed to the microcarrier beads. To circumvent this problem we maintained HF cells on gelatin microcarrier beads and indeed found much more PN II in the conditioned medium. Employing a three-step procedure, an approximate 6000-fold purification was achieved with a recovery of 83%. Using dextran sulfate-Sepharose affinity chromatography for the initial step not only concentrated and enriched for PN II, but also produced a very high yield of 95%. Further purification was achieved by taking advantage of the very tight binding that PN II exhibits toward DEAE-Sepharose. Apparent homogeneity was achieved by gel filtration.
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was added to triplicate culture dishes of confluent HF cells. The resuspended to a final volume of protein that formed complexes with EGF-BP that were specifically demonstrate that PN

possesses an extremely anionic region which is responsible for its binding to lectin-Sepharose columns (data not shown). Furthermore, EGF-BP under these conditions reveals that it is a highly negatively charged protein. In contrast, several plasma serine protease inhibitors, is a extremely stable protein.

FIG. 7. Binding of 125I-EGF-BP-PN II complexes to HF cells. 125I-EGF-BP (0.75 μg/ml) was incubated alone or with purified PN II (3.0 μg/ml or 12.0 μg/ml) in 20 mM potassium phosphate, 0.2 M NaCl (pH 7.4) for 30 min at 37°C. Each of these mixtures was resuspended to a final volume of 5 ml with binding medium and 1 ml was added to triplicate culture dishes of confluent HF cells. The cultures were incubated for 20 min at 37°C and rinsed five times with phosphate-buffered saline containing 0.1% BSA. For each sample, the cells from two of the dishes were solubilized in SDS sample buffer and subjected to nonreducing SDS-PAGE followed by autoradiography as described under “Experimental Procedures.” Lane 1, 125I-EGF-BP alone; lane 2, 125I-EGF-BP + 3.0 μg/ml PN II; lane 3, 125I-EGF-BP + 12.0 μg/ml PN II. Lanes 4, 5, and 6 of the accompanying autoradiogram are aliquots of samples in lanes 1, 2, and 3, respectively.

Although PN II was originally identified using 125I-EGF-BP as a probe (3), the present studies showed that purified PN II also formed complexes with NGF-γ and bovine trypsin (Fig. 7). We also investigated whether other serine proteases which possess trypsin-like activities are complexed by PN II. Screening of numerous proteases from the coagulation, fibrinolytic, and complement pathways, as well as several chymotrypsin-like and elastase-like proteases failed to reveal any which formed SDS-stable complexes with PN II. Although no complex formation was observed, noncovalent inhibition of these proteases may occur. In contrast, PN I appears to have a broader specificity towards serine proteases (9). The reasons for the more limited specificity of PN II are as yet unclear. However, now that PN II can be purified in relatively large quantities, it will be possible to conduct further biochemical and structural studies on it, including its protease specificity, so that physiological roles for this protein may be determined.

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