Monoclonal Antibodies to Protease Nexin 1 That Differentially Block Its Inhibition of Target Proteases†

Steven L. Wagner,‡ William E. Van Nostrand,§ Alice L. Lau, and Dennis D. Cunningham*

Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92717

Received April 21, 1987; Revised Manuscript Received October 13, 1987

ABSTRACT: Protease nexin 1 (PN-1) is a protease inhibitor secreted by cultured fibroblasts that forms complexes with certain serine proteases; the complexes bind back to the cells and are internalized and degraded. In the present studies, a panel of PN-1 monoclonal antibodies (mAbs) was isolated; none showed detectable cross-reactivity with four related plasma protease inhibitors. Four purified mAbs (mAbp1, mAbp6, mAbp9, and mAbp18) were tested for their ability to block the formation of complexes between PN-1 and target proteases. mAbp1, as well as a rabbit polyclonal anti-PN-1 IgG preparation, did not block formation of ^{125}I-thrombin–PN-1 complexes. mAbp6, mAbp9, and mAbp18 blocked the formation of ^{125}I-thrombin–PN-1 and ^{125}I-urokinase–PN-1 complexes at stoichiometric concentrations of mAb and PN-1. Studies on their ability to block formation of ^{125}I-trypsin–PN-1 complexes showed that mAbp18 also blocked this reaction at stoichiometric concentrations with PN-1 whereas mAbp6 and mAbp9 blocked less effectively. Thus, mAbp18 appears to bind at or close to the reactive center of PN-1. The blocking mAbs should be useful in studies to probe physiological functions of PN-1.

Protease nexin 1 (PN-1)[1] is a 45-kDa protease inhibitor first detected in the medium from cultured human fibroblasts (Baker et al., 1980) and subsequently shown to be secreted by a variety of cultured cells (Eaton & Baker, 1983). It rapidly inhibits thrombin, urokinase, plasmin, and trypsin by forming stable 1:1 complexes at the protease catalytic serine residue (Baker et al., 1980; Scott et al., 1985). Several results indicate that PN-1 regulates proteases at and near the surface of cells in the extravascular compartment (Cunningham et al., 1986). First, it is detectable in only minute amounts in plasma (Baker & Gronke, 1986). Second, cells that secrete PN-1 rapidly bind, internalize, and degrade protease–PN-1 complexes (Low et al., 1981), providing a localized mechanism for their clearance. Third, the surface of fibroblasts accelerates the inactivation of thrombin by PN-1 (Farrell & Cunningham, 1986). This activity residues in the extracellular matrix and is mostly due to heparan sulfate (Farrell & Cunningham, 1987). Finally, active PN-1 is localized on the extracellular matrix of human fibroblasts (Farrell et al., 1988).

Studies on the functions of PN-1 have shown that it can modulate thrombin-stimulated cell division (Low et al., 1982), an activity that requires the proteolytic activity of thrombin (Glenn et al., 1980). Added PN-1 can also inhibit degradation of the extracellular matrix of smooth muscle cells brought about by urokinase that is released by fibrosarcoma cells (Bergman et al., 1986). PN-1 may also play an important regulatory role in the brain since a protease inhibitor that is identical with PN-1 is secreted by glioma cells and stimulates neurite outgrowth from neuroblastoma cells (Gloor et al., 1986).

This report describes mAbs that block PN-1 activity and will provide much needed tools to better define the above and other possible physiological functions of PN-1.

Materials and Methods

Materials. PN-1 was purified as previously described (Farrell et al., 1986). Purified antithrombin III and heparin cofactor II were provided by Dr. David Farrell. C1 inhibitor was purified from human plasma by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography, and heparin–Sepharose chromatography (W. E. Van Nostrand and D. D. Cunningham, unpublished results). Human α-thrombin (Fenton et al., 1977) was a gift of Dr. John W. Fenton, II (New York State Department of Health, Albany, NY). α_1-Protease inhibitor was a gift of Dr. James Travis, University of Georgia, Athens, GA. Urokinase (35-kDa form), bovine trypsin, and ovalbumin (grade V) were from Sigma, St. Louis, MO. The nonsecreting mouse myeloma P3X63-Ag8.653 (Kearney et al., 1979) was purchased from American Type Culture Collection, Rockville, MD. Microtiter 96-well tissue culture dishes were from Falcon Plastics, Oxnard, CA. All hybridoma reagents were purchased in the form of a kit (HyBRL Prep Kit) from Bethesda Research Laboratories, Bethesda, MD. Streptavidin–biotinylated horseradish peroxidase preformed complex and biotinylated sheep anti-mouse IgG were from Amersham, Arlington Heights, IL. Horseradish peroxidase conjugated goat anti-mouse IgG was from Cappel Laboratories, Malvern, PA.

Preparation of Hybridomas. Eleven-week-old female BALB/c mice were immunized intraperitoneally with PN-1 (50 μg emulsified in 0.2 mL of Freund's complete adjuvant) every week for a period of 5 weeks. For three of the five immunizations, PN-1 was incubated at 100 °C for 2 h prior to emulsification with Freund's complete adjuvant. Four days after the last immunization, the mice were injected in the tail vein with 25 μg of PN-1 dissolved in 0.2 mL of phosphate-buffered saline (PBS). Three days later, splenocytes (1.1×10^8 cells) from an immunized mouse were fused with $2.8 \times$

† This work was supported by National Institutes of Health Research Grant GM 31609.

* Correspondence should be addressed to this author.

‡ Supported by National Institutes of Health Training Grant CA09054.

§ Recipient of a postdoctoral fellowship from the George E. Hewitt Foundation for Medical Research.

[1] Abbreviations: PN-1, protease nexin 1; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; kDa, kilodalton(s).

0006-2960/88/0427-2173$01.50/0

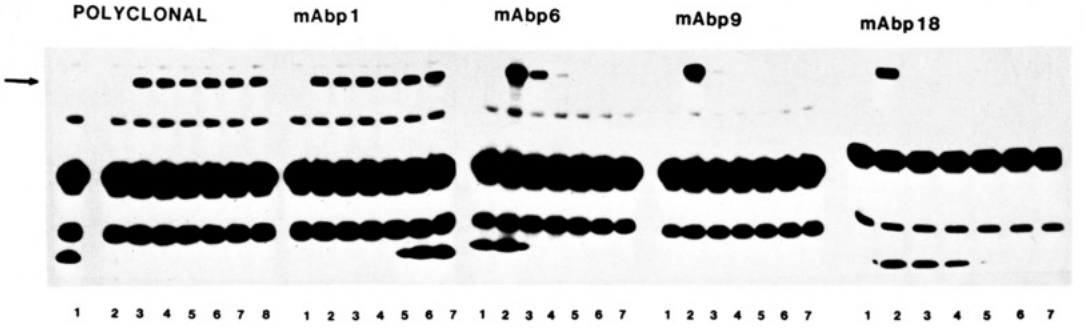

FIGURE 1: Effects of various anti-PN-1 antibodies on formation of ^{125}I-thrombin–PN-1 complexes. The experimental protocol is described under Materials and Methods. Rabbit polyclonal IgG: (lane 1) ^{125}I-thrombin; (lane 2) ^{125}I-thrombin + IgG (4.0 μM); (lane 3) ^{125}I-thrombin + PN-1; (lanes 4–8) ^{125}I-thrombin + PN-1 + 0.2, 0.4, 1.0, 2.0, or 4.0 μM IgG, respectively. mAbp1: (lane 1) ^{125}I-thrombin + mAbp1 (4.0 μM); (lane 2) ^{125}I-thrombin + PN-1; (lanes 3–7) ^{125}I-thrombin + PN-1 + 0.2, 0.4, 1.0, 2.0, or 4.0 μM mAbp1, respectively. mAbp6: (lane 1) ^{125}I-thrombin + mAbp6 (1.0 μM); (lane 2) ^{125}I-thrombin + PN-1; (lanes 3–7) ^{125}I-thrombin + PN-1 + 0.02, 0.1, 0.2, or 1.0 μM mAbp6, respectively. mAbp9: (lane 1) ^{125}I-thrombin + mAbp9 (2.0 μM); (lane 2) ^{125}I-thrombin + PN-1; (lanes 3–7) ^{125}I-thrombin + PN-1 + 0.1, 0.2, 0.4, 1.0, and 2.0 μM mAbp9, respectively. mAbp18: (lane 1) ^{125}I-thrombin + mAbp18 (4 μM); (lane 2) ^{125}I-thrombin + PN-1; (lanes 3–7) ^{125}I-thrombin + PN-1 + 0.2, 0.4, 1.0, 2.0, or 4.0 μM mAbp18, respectively. The arrow indicates the position of ^{125}I-thrombin–PN-1 complexes.

10^7 myeloma cells. The procedures employed were as described in the instruction manual provided with the HyBRL Prep Kit, except that poly(ethylene glycol) 1540 was used to fuse the cells. The fused cells were plated onto 96-well microtiter plates and grown in Dulbecco's modified Eagle's medium containing 15% fetal calf serum and hypoxanthine, aminopterin, and thymidine for 17 days as described in the HyBRL Prep Kit. Then, this medium was replaced with either growth medium or growth medium supplemented with hypoxanthine and thymidine. Approximately 21 days after fusion, hybridoma supernatants were tested for the production of antibody to PN-1 by enzyme-linked immunosorbent assay (ELISA). The positive hybridomas were cloned by limiting dilutions at an average cell density of 0.5–1 cell per well using 10^5 splenocytes per well as the feeder layer. Positive monoclonal cultures were expanded into 24-well plates and then into 25 cm^2 tissue culture flasks. Cells from confluent 25 cm^2 flasks were then injected intraperitoneally into mice which had been primed with 0.25 mL of pristane 3–14 days prior to inoculation to promote formation of antibody-rich ascites fluid.

Subtype Classification of mAbs. Antibody subclasses for each of the monoclonal hybridoma lines were identified with a Hybridoma Sub-Isotyping Kit (Behring Diagnostics, La Jolla, CA) according to the accompanying instruction manual.

Purification of mAbs. mAbs were purified by using an Affi-Gel Protein A MAPS II Kit (Bio-Rad, Richmond, CA) as described in the accompanying instruction manual.

Protein Determination. The concentration of polyclonal antibodies and purified mAbs was determined spectrophotometrically by using an extinction coefficient (1% w/v; 1 cm) of 14 (Ey et al., 1978). PN-1 concentration was estimated by using an extinction coefficient (1% w/v; 1 cm) of 16.2 (Scott et al., 1985).

Iodination of Proteins. Thrombin, trypsin, urokinase, and mAbs were iodinated as previously described (Glenn et al., 1980).

Inhibition Studies. PN-1 (0.2 μM) was incubated for 60 min at 37 °C with various concentrations (0.02–4.0 μM) of either mAb or polyclonal antibody in PBS containing 0.01% bovine serum albumin in a volume of 20 μL. In some experiments, heparin (0.2 mM final concentration) was incubated with PN-1 prior to addition of antibody. Following the antibody incubation, a 5-μL aliquot of ^{125}I-protease (diluted in 0.01% bovine serum albumin) was added to each tube and incubated with the PN-1/antibody solution for 15 min at 37 °C. The final concentration of ^{125}I-protease was 0.04 μM. The specific activities of the various proteases were 25 000 cpm/ng for thrombin and trypsin and 13 400 cpm/ng for urokinase. The reaction was quenched by adding 25 μL of Laemmli SDS–polyacrylamide gel sample dilution buffer. The individual samples were run on 7.5% SDS–polyacrylamide gels according to Laemmli (1970). Autoradiograms were then prepared from the gels. To quantitate protease–PN-1 complexes, the autoradiograms were aligned with the dried gels, the ^{125}I-labeled complexes were excised from the gels, and radioactivity was measured in a γ counter.

Results

Isolation and Properties of PN-1 mAbs. Eight microtiter wells out of 293 contained hybridomas which secreted anti-PN-1 antibodies. Subsequent cloning by limiting dilution resulted in 21 stable monoclonal hybridoma lines which secreted anti-PN-1. All 21 mAbs were shown to be specific for PN-1 by ELISA. Each clone was incubated with subclass-specific rabbit anti-mouse IgG in an ELISA to identify the type of antibody secreted. These studies showed that each clone secreted only one class of immunoglobin.

To determine the uniqueness of the PN-1 epitopes recognized by the mAbs, we tested the ability of all 21 mAbs to cross-react with 4 plasma inhibitors of serine proteases. The ELISA response for all 21 mAbs increased with increasing concentrations of PN-1 but showed no detectable binding to up to 1 μg of antithrombin III (Rosenberg & Damus, 1973), heparin cofactor II (Tollefsen et al., 1983), C1 inhibitor (Sim et al., 1980), or α_1-protease inhibitor (Beatty et al., 1980) (data not presented).

Ability of Antibodies To Block Formation of Protease–PN-1 Complexes. The ability of the four purified mouse mAbs (mAbp1, mAbp6, mAbp9, and mAbp18) and the rabbit polyclonal IgG to block formation of ^{125}I-thrombin–PN-1 complexes was assessed in the experiments described in Figure 1. When various concentrations of purified anti-PN-1 polyclonal IgG were preincubated with PN-1 and then incubated with ^{125}I-thrombin, there was no apparent inhibition of ^{125}I-thrombin–PN-1 complex formation with a 20-fold molar excess of antibody over PN-1. (In subsequent experiments, even a 100-fold molar excess of antibody over PN-1 did not inhibit.) mAbp1 also did not detectably inhibit ^{125}I-thrombin–PN-1 complex formation over the concentration range shown. These results showed that the rabbit polyclonal IgG and mAbp1 bind to epitopes which are remote from the crucial thrombin interaction sites on PN-1. In contrast, mAbp6, mAbp9, and

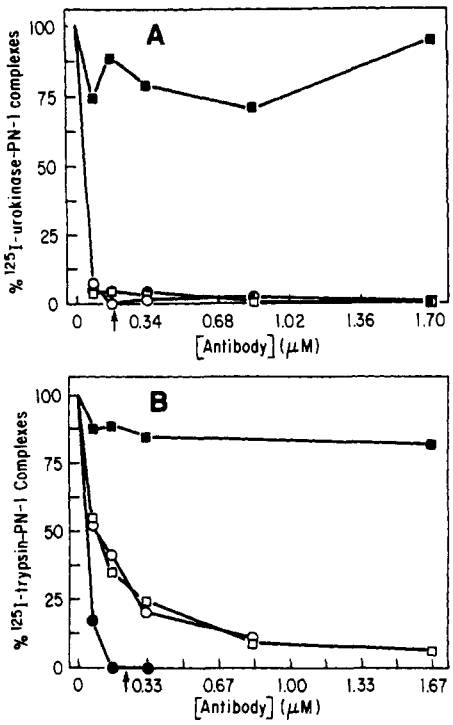

FIGURE 2: Effects of anti-PN-1 mAbs on formation of ^{125}I-urokinase–PN-1 complexes (panel A) and ^{125}I-trypsin–PN-1 complexes (panel B). The experimental protocol is described under Materials and Methods. (■) mAbp1; (□) mAbp6; (○) mAbp9; (●) mAbp18. The arrow on the abscissa indicates the concentration of PN-1 in the reaction mixture.

mAbp18 completely inhibited ^{125}I-thrombin–PN-1 complex formation at stoichiometric mAb and PN-1 concentrations (Figure 1).

The mAbs that prevented ^{125}I-thrombin–PN-1 complex formation could do this by binding to the reactive center. Alternatively, they could bind to other PN-1 regions required for binding or recognition of thrombin but perhaps not other proteases. We therefore tested the ability of mAbp6, mAbp9, and mAbp18 to block formation of other protease–PN-1 complexes. Figure 2 (panel A) shows that similar results were obtained with urokinase: mAbp6, mAbp9, and mAbp18 blocked formation of ^{125}I-urokinase–PN-1 complexes at stoichiometric concentrations of mAb and PN-1, while mAbp1 did not block this reaction. Since thrombin and urokinase are similar in size (36 and 35 kDa, respectively), the smaller protease trypsin (22 kDa) was used to further probe the binding of these mAbs. As shown in Figure 2 (panel B), mAbp6 and mAbp9 equally inhibited ^{125}I-trypsin–PN-1 complex formation, although a molar ratio of mAb to PN-1 of 5.0 was required for complete inhibition. In contrast, mAbp18 blocked formation of PN-1 complexes with ^{125}I-trypsin at stoichiometric mAb and PN-1 concentrations as described above with thrombin and urokinase. Therefore, mAbp6 and mAbp9 interact similarly with PN-1. mAbp18 may bind to an epitope on PN-1 which is closer to the reactive center than the sites for mAbp6 and mAbp9.

Discussion

The present studies on anti-PN-1 mAbs focused on their ability to block the inhibition of target proteases by PN-1. This is because the reactivity of PN-1 with a spectrum of serine proteases has been studied (Scott et al., 1985), and because antibodies that block these reactions will be valuable to probe the physiological roles of PN-1. To increase our chance of obtaining blocking mAbs, we immunized mice with both native and denatured PN-1 with the expectation that this might increase the number of PN-1 epitopes available to their immune system. The polyclonal rabbit IgG directed against highly purified native PN-1 failed to block its ability to form complexes with thrombin even at a 100-fold molar excess of IgG over PN-1. These antibodies, however, are useful for immunoprecipitating both PN-1 and protease–PN-1 complexes as well as reacting with PN-1 in immunoblots, immunofluorescence studies, and ELISA (Farrell et al., 1987). We also isolated a mAb (mAbp1) that did not block the ability of PN-1 to complex thrombin. It effectively immunoprecipitated thrombin–PN-1 complexes (S. L. Wagner, unpublished results), consistent with the interpretation that the PN-1 epitope it recognized was remote from the PN-1 reactive center as well as recognition/binding domains that directly interact with thrombin.

Three of the mAbs studied (mAbp6, mAbp9, and mAbp18) blocked the ability of PN-1 to form complexes with thrombin and urokinase at stoichiometric concentrations of the mAbs and PN-1. This suggested that they might recognize epitopes at or close to the reactive center of PN-1. To explore the possibility that they might interact differently with PN-1, we examined their ability to block the formation of protease–PN-1 complexes involving a smaller protease. These studies showed that mAbp18 also blocked the formation or ^{125}I-trypsin–PN-1 complexes at stoichiometric concentrations of mAbp18 and PN-1. In contrast, mAbp6 and mAbp9 blocked this reaction only when the molar ratio of mAb to PN-1 reached 5.0. This difference was also observed in experiments which showed that mAbp18 inhibited the rate of formation of complexes between ^{125}I-trypsin and PN-1 more effectively than mAbp9 (S. L. Wagner, unpublished results).

The PN-1 mAbs will be valuable for several studies. One of them has already been used to immunopurify PN-1 and a form of PN-1 that binds heparin with a low affinity (Van Nostrand et al., 1988). This will facilitate isolation and study of these molecules from specific fluids and tissues. The mAbs which block the antiprotease activity of PN-1 will be particularly useful in defining its physiological functions. For example, PN-1 binds to the extracellular matrix of fibroblasts (Farrell et al., 1987); the blocking mAbs should facilitate studies to determine if it regulates degradation of the matrix.

Acknowledgments

We thank Dr. John W. Fenton, II, for thrombin, Dr. James Travis for α_1-protease inhibitor, and Dr. David H. Farrell for antithrombin III and heparin cofactor II. We are grateful to Bradley Hoo for technical assistance.

References

Baker, J. B., & Gronke R. S. (1986) *Semin. Thromb. Hemostasis 12*, 216–220.

Baker, J. B., Low, D. A., Simmer, R. L., & Cunningham, D. D. (1980) *Cell (Cambridge, Mass.) 21*, 37–45.

Beatty, K., Bieth, J., & Travis, J. (1980) *J. Biol. Chem. 255*, 3931–3934.

Bergman, B. L., Scott, R. W., Bajpai, A., Watts, S., & Baker, J. B. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 996–1000.

Cunningham, D. D., Van Nostrand, W. E., Farrell, D. H., & Campbell, C. H. (1986) *J. Cell. Biochem. 32*, 281–291.

Eaton, D. L., & Baker, J. B. (1983) *J. Cell. Physiol. 117*, 175–182.

Ey, P. L., Prowse, S. J., & Jenkin, C. R. (1978) *Immunochemistry 15*, 429–436.

Farrell, D. H., & Cunningham, D. D. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6858–6862.

Farrell, D. H., & Cunningham, D. D. (1987) *Biochem. J. 245*, 543–550.
Farrell, D. H., Van Nostrand, W. E., & Cunningham, D. D. (1986) *Biochem. J. 237*, 907–912.
Farrell, D. H., Wagner, Y. L., Yuan, R. H., & Cunningham, D. D. (1988) *J. Cell. Physiol. 134*, 179–188.
Fenton, J. W., II, Fasco, M. J., Stackrow, A. B., Aronson, D. L., Young, A. M., & Finlayson, J. S. (1977) *J. Biol. Chem. 252*, 3587–3598.
Glenn, K. C., Carney, D. H., Fenton, J. W., II, & Cunningham, D. D. (1980) *J. Biol. Chem. 255*, 6609–6616.
Gloor, S., Odink, K., Guenther, J., Hanspeter, N., & Monard, D. (1986) *Cell (Cambridge, Mass.) 47*, 687–693.
Kearney, J. F., Radbruch, A., Liesegang, B., & Rajewsky, K. (1979) *J. Immunol. 123*, 1548–1550.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Low, D. A., Baker, J. B., Koonce, W. C., & Cunningham, D. D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2340–2344.
Low, D. A. Scott, R. W., Baker, J. B., & Cunningham, D. D. (1982) *Nature (London) 298*, 476–478.
Rosenberg, R. D. & Damus, P. S. (1973) *J. Biol. Chem. 248*, 6490–6505.
Scott, R. W., Bergman, B. L., Bajpai, A., Hersh, R. T., Rodriguez, H., Jones, B. N., Barreda, C., Watts, S., & Baker, J. B. (1985) *J. Biol. Chem. 260*, 7029–7034.
Sim, R. B., Arlaud, G. J., & Colomb, M. G. (1980) *Biochim. Biophys. Acta 612*, 433–449.
Tollefsen, D. M., Majerus, D. W., & Blank, M. K. (1982) *J. Biol. Chem. 257*, 2162–2169.
Van Nostrand, W. E., Wagner, S. L., & Cunningham, D. D. (1988) *Biochemistry* (following paper in this issue).

Purification of a Form of Protease Nexin 1 That Binds Heparin with a Low Affinity†

William E. Van Nostrand,‡ Steven L. Wagner,§ and Dennis D. Cunningham*
Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92717
Received April 21, 1987; Revised Manuscript Received August 20, 1987

ABSTRACT: A form of protease nexin 1 (PN-1) that binds heparin with a low affinity (L-PN-1) was purified and studied since altered interactions with glycosaminoglycans could affect its inhibition of certain serine proteases. Purification of L-PN-1 and PN-1 was achieved by fractionating serum-free conditioned culture medium from human fibroblasts over dextran sulfate–Sepharose followed by immunoaffinity fractionation over a PN-1 monoclonal antibody–Sepharose column. The first step separated L-PN-1 from PN-1, and the second step resulted in apparently homogeneous L-PN-1 and PN-1. Comparisons of the two proteins showed that they could not be distinguished by the following properties: (a) molecular weight; (b) proteases complexed; (c) molecular weights of protease–L-PN-1 and protease–PN-1 complexes; (d) CNBr peptide maps; and (e) immunological cross-reactivity. Studies on activities that depend on the heparin binding domain revealed that heparin equally accelerated the rate of formation of ^{125}I-thrombin–L-PN-1 and ^{125}I-thrombin–PN-1 complexes even when the ratio of heparin to L-PN-1 or PN-1 was varied from 0.01 to 100. A functional difference, however, between L-PN-1 and PN-1 was observed in studies on the ability of the fibroblast surface to accelerate their reactions. Fixed fibroblasts accelerated the formation of ^{125}I-thrombin–L-PN-1 complexes 2-fold, whereas they accelerated the formation of ^{125}I-thrombin–PN-1 complexes 5-fold. The availability of purified L-PN-1 will permit studies on its functional relationship to PN-1.

Protease nexin 1 (PN-1)[1] is a 45-kDa serine protease inhibitor that is synthesized and secreted by cultured human fibroblasts (Baker et al., 1980, 1986; Scott et al., 1985) and several other cultured nonvascular cells (Eaton & Baker, 1983). PN-1 rapidly complexes thrombin, urokinase, plasmin, and trypsin (Scott et al., 1985). Previous studies showed that PN-1 binds to heparin with a high affinity (Baker et al., 1980; Scott & Baker, 1983; Scott et al., 1985) and that in the presence of heparin the rate of thrombin inactivation by PN-1 is markedly accelerated (Baker et al., 1980; Scott et al., 1985). This affinity for heparin has been employed in the procedures to purify PN-1 (Scott & Baker, 1983; Scott et al., 1985; Farrell et al., 1986). During these purifications, a form of PN-1 was noted which has a relatively low affinity for heparin. We have designated this form L-PN-1. Little is known about L-PN-1 because it has not been purified.

There are several important activities of PN-1 that likely depend on its heparin binding site and which could be modified in L-PN-1. For example, the surface of fibroblasts accelerates the formation of complexes between PN-1 and thrombin; this is due to glycosaminoglycans in the extracellular matrix (Farrell & Cunningham, 1986, 1987). Also, PN-1 is localized on the fibroblast extracellular matrix (D. H. Farrell et al., unpublished results). The molecules responsible for this association are not known but could involve heparin-like gly-

† This work was supported by National Institutes of Health Research Grant GM31609.
* Correspondence should be addressed to this author.
‡ Recipient of a postdoctoral fellowship from the George E. Hewitt Foundation for Medical Research.
§ Supported by National Institutes of Health Training Grant CA09054.

[1] Abbreviations: PN-1, protease nexin 1; L-PN-1, protease nexin 1 that binds heparin with low affinity; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; kDa, kilodalton(s); Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

0006-2960/88/0427-2176$01.50/0