Protease Nexin 1 Is a Potent Urinary Plasminogen Activator Inhibitor in the Presence of Collagen Type IV*

Robert J. Crisp, Mary F. Knauer, and Daniel J. Knauer‡

From the Department of Developmental and Cellular Biology, University of California, Irvine, California 92697

Protease nexin 1 (PN1) in solution forms inhibitory complexes with thrombin or urokinase, which have opposing effects on the blood coagulation cascade. An initial report provided data supporting the idea that PN1 target protease specificity is under the influence of collagen type IV (1). Although collagen type IV demonstrated no effect on the association rate between PN1 and thrombin, the study reported that the association rate between PN1 and urokinase was allosterically reduced 10-fold. This has led to the generally accepted idea that the primary role of PN1 in the brain is to act as a rapid thrombin inhibition and clearance mechanism during trauma and loss of vascular integrity. In studies to identify the structural determinants of PN1 that mediate the allosteric interaction with collagen type IV, we found that protease specificity was only affected after transient exposure of PN1 to acidic conditions that mimic the elution protocol from a monoclonal antibody column. Because PN1 used in previous studies was purified over a monoclonal antibody column, we propose that the allosteric regulation of PN1 target protease specificity by collagen type IV is a result of the purification protocol. We provide both biochemical and kinetic data to support this conclusion. This finding is significant because it implies that PN1 may play a much larger role in the modeling and remodeling of brain tissues during development and is not simply an extravasated thrombin clearance mechanism as previously suggested.

Protease nexin 1 (PN1)* is a 43-kDa member of the serine protease inhibitor (SERPIN) superfamily and has been shown to be a potent physiologic inhibitor of thrombin and urinary plasminogen activator (urokinase) (2, 3). PN1 is synthesized by astrocytes, smooth muscle, endothelial cells, and fibroblasts (3, 4) and is the only SERPIN found in physiologic quantities in the brain (5–9). Although most SERPINs are found in the plasma, PN1 is found primarily in tissues and has been shown to be associated with the extracellular matrix (10). Previous studies have shown that thrombin is able to induce apoptosis in astrocytes (11–13), and thus the localization of PN1 in the tissues surrounding the vasculature of the brain has led to a hypothesis that one of the primary physiological functions of PN1 is to act as a protective mechanism against thrombin that has escaped the vascular compartment during cerebrovascular trauma. This hypothesis has been supported by reports demonstrating that PN1 bound to collagen type IV is refractory to urokinase inhibition, thus leaving PN1 free to react with thrombin in the tissues. In the present report we show that this is due to the exposure of PN1 to acid during the purification protocol and that the protease inhibitory specificity of native PN1 is not altered by collagen type IV binding.

Like many other SERPINs, the rate of PN1 inhibition is under the influence of allosteric effectors. Cell surface proteoglycans are allosteric effectors for many SERPINs, including heparin cofactor II, antithrombin III, and PN1 (2, 14, 15). Interestingly, in the presence of heparin, PN1 increases its inhibitory activity toward thrombin by a factor of 1000, making PN1 a more efficient inhibitor of thrombin than even antithrombin III (2). Along with the proteoglycan-mediated acceleration there is also evidence of protein-protein interactions modifying the rate of SERPIN inhibitory action. A well characterized example of this is plasminogen activator inhibitor 1, a SERPIN with inhibitory activity toward plasminogen activators. Plasminogen activator inhibitor 1 becomes an effective inhibitor of thrombin only in the presence of the plasma borne cell adhesion protein vitronectin, increasing its rate of thrombin inhibition by a factor of 200 (16).

Collagen type IV helps form the core of basement membranes, the sheet-like extracellular structure that surrounds tissues and organs (17), and has been reported to be an allosteric effector of PN1 (1, 19). Whereas other forms of collagen are formed through interactions of their noncollagenous ends that result in long fibers with high tensile strength, collagen type IV forms a meshed, nonfibrillar network with interactions not only at the ends but also within the long collagenous triple helical region (17, 18). PN1 has been shown to colocalize with a component of the basement membrane, fibronectin (10). Further, PN1 secreted from cultured fibroblasts has been shown to copurify with a 120-kDa band that has been identified as collagen type IV (1). Studies on PN1 binding to fibroblast-secreted extracellular matrix led to the discovery that PN1 has altered inhibitory activity toward target proteases when bound to the extracellular matrix (19). It was later determined that collagen type IV was the component of the extracellular matrix that was causing this target protease specificity change in PN1. In the absence of collagen type IV, PN1 was able to form inhibitory complexes with thrombin, urokinase, and plasmin; however, when collagen type IV was added at a concentration of 1 μM, PN1 was no longer able to form complexes at the same rate with urokinase and plasmin (1, 19).

The original purpose of this study was to identify the specific structural determinants in PN1 that mediate the alteration of its target protease specificity when in complex with collagen.
type IV. However, we had no success reproducing the alteration of PN1 target protease specificity through interaction with collagen type IV. What we found was that the collagen type IV allosteric regulation of PN1 target protease specificity was the result of exposing the SERPIN to acidic conditions that were required for the purification of PN1 from a monoclonal antibody column. Because all of the studies to date on the regulation of PN1 target protease specificity with collagen type IV have used PN1 that was purified on a monoclonal antibody column, we propose that the observed allosteric effect is the result of acid exposure of PN1 during the monoclonal antibody column purification protocol and thus may not be physiologically relevant. In all of our previous studies we have used a heparin-Sepharose affinity chromatography protocol to purify PN1. We show here that there is no regulation of PN1 target protease specificity by collagen type IV and provide strong evidence that the allosteric effect of collagen type IV is the result of exposure to nonphysiological conditions required for the elution of PN1 from a monoclonal antibody column. Thus, PN1 is still a potent urokinase inhibitor in the basement membrane when bound to collagen type IV. These data are very important because they re-open the possibility that PN1 may play a key regulatory role in the urokinase receptor-mediated activation of signaling pathways that control cell migration during tissue repair/remodeling.

EXPERIMENTAL PROCEDURES

Materials—The cell culture media and reagents were purchased from Irvine Scientific and JRH Scientific. The cell culture plastics were from Corning. Thrombin (3,000 NIH units/mg) high molecular mass urokinase (80,000 IU/mg), and plasmin (27 units/mg protein) were purchased from Calbiochem. Collagen type IV was purchased from Calbiochem and from Sigma (the Calbiochem samples were used in all of the experiments shown here). High trap heparin-Sepharose affinity column chromatography was performed from Amersham Biosciences. Enzymes used for molecular biology were from Fisher/Promega or Invitrogen. All of the other common laboratory supplies were purchased from either Irvine Scientific or Sigma.

Cell Culture and Extracellular Matrix Preparation—Human foreskin fibroblasts were grown and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum as described previously (20–22). The experimental cultures were seeded at 1.0 × 10^5 cells/well into 24-well plates and grown to confluency in the presence of 50 μg/ml tetracycline. Following a 50-ml wash with PBS, the cells were incubated for 30 min at 37°C to allow for color development as a measure of residual thrombin activity. The absorbance measurements were taken at 405 nm to quantify activity colorimetrically.

The kinetic measurements were done using a protocol previously described (2). Briefly, approximately a 200-fold molar excess PN1 (8 nM) was preincubated with urokinase (0.3 nM) in a final volume of 100 μl of Tyrode's buffer. At the indicated times, the reaction was diluted with Tyrode's buffer to a final volume of 2 ml containing 100 μM Z-Gly-Gly-Arg-AMC, a quenched fluorogenic substrate of urokinase. Urokinase that was not inactivated by PN1 during the preincubation is free to cleave the fluorogenic substrate, providing a quantitative measurement of the amount of active urokinase remaining. The change in relative fluorescence was monitored over a 480-s period with a sampling frequency of 20 s. The samples were excited at 350 nm, and emission was monitored at 450 nm. The k_{max} rates were calculated using the following equation (3).

$$k_{max} = \frac{\ln[1 - [P]/[P]_o]}{[H]^n}$$

Eq. (1)

Transient Exposure of PN1 to Acidic Conditions—PN1 elution from a monoclonal antibody column transiently exposes it to a strong acid at pH 3.0 (1). To mimic these conditions, we exposed samples of PN1 purified on a heparin-Sepharose column to 100 mM glycine HCl, pH 3.0, for 1 min, followed by neutralization by the addition of 200 mM NaPO₃, pH 7.4. Longer exposure of PN1 to these conditions (15–30 min) resulted in an 80% loss of PN1 inhibitory activity (data not shown). PN1 exposed to acid for 1 min was dialyzed into PBS and then evaluated using the linkage assay described above.

Collagen Type IV Inhibition Assay—The protocol for this assay is described elsewhere (1) and is summarized here. Dialyzed protease at 20 nM was incubated with 5 nM PN1 with no collagen type IV or in the presence of increasing concentrations of collagen type IV for 15 min. The reactions were terminated by the addition of 40 mM EDTA to 100 mM NaPO₃, pH 7.4. The product was digested using EcorI and Smal, ligated into the pVL1393 baculovirus shuttle vector, and cotransfected into S9 insect cells along with BaculoGold baculovirus. Recombinant viruses were purified by a single round of plaque purification. For protein expression, S9 cells grown in T175 flasks were infected at a multiplicity of infection of A91. Four to seven days later the medium was harvested, centrifuged to remove dead cells, and concentrated using an Amicon concentrator with a molecular mass cut-off of ~10 kDa. The PN1 sample was then dialyzed against PBS using dialysis tubing with a cut-off of 12 kDa and further concentrated within the dialysis tubing using Calbiochem Aquacide. The PN1 was finally brought to a concentration similar to the human foreskin fibroblast cell-purified sample, and its activity was assayed as described above.

RESULTS

PN1 Forms Complexes with Urokinase in the Presence of Collagen Type IV—Previous work indicates that in the presence of 1 μM collagen type IV, PN1 was able to form inhibitory complexes with thrombin but became refractory to formation of inhibitory complexes with either plasmin or urokinase (1). To verify this assay, PN1 (5 nM) was incubated with either 125I-thrombin (20 nM) or 125I-urokinase (20 nM) in the presence of increasing concentrations of collagen type IV, and the amount of complex formation was quantified as described under “Experimental Procedures.” At all concentrations of collagen type IV, PN1 was able to form inhibitory complexes with thrombin, a result that is consistent with previous work (Fig. 1A). However, our results with urokinase disagreed with previous results (1). We show that using PN1 purified according to the protocol described under “Experimental Procedures,” PN1 was able to form inhibitory complexes with urokinase with the same efficiency independent of the presence or absence of the...
collagen type IV (Fig. 1B). These results conflict with the data presented in the previous study, which demonstrate reduced ability of PN1 to form inhibitory complexes with urokinase in the presence of 1 $\mu$M collagen type IV. The experiment was repeated using concentrations up to 100 $\mu$M collagen type IV, but PN1 continued to form complexes with urokinase at the same levels as the control that had no collagen type IV (data not shown).

 Fibroblast-secreted Extracellular Matrix Does Not Alter PN1 Target Protease Specificity—To determine whether the commercially available collagen type IV might be inactive and thus account for the disparity in results, we turned to a different protocol using fixed extracellular matrix preparations as a source of collagen type IV. Previous studies by the same group demonstrated that in the presence of collagen type IV, PN1 retained its specificity regardless of the concentration of collagen type IV present. This important control shows, however, that the mere exposure of PN1 to acid does not affect its ability to act as a SERPIN. The rate of complex formation was shown to be independent of the concentration of collagen type IV.

The Rate of PN1-Urokinase Complex Formation Is Not Changed by the Presence of Collagen Type IV—Previous work established that the rate of urokinase inhibition by PN1 in the absence of collagen type IV was $1.50 \times 10^{-5}$ M$^{-1}$ s$^{-1}$ (1). In that study, the presence of 1 $\mu$M collagen type IV caused a 10-fold decrease in the pseudo-second-order rate constant ($k_{obs}$) to $1.50 \times 10^{-6}$ M$^{-1}$ s$^{-1}$. To examine the effect of collagen type IV on the rate of inhibitory complex formation, PN1 (5 nM) was incubated with $^{125}$I-urokinase (20 nM) in the presence and absence of 1 $\mu$M collagen type IV and assayed for complex formation as described under “Experimental Procedures.” As shown in Fig. 2, we were unable to detect any difference in the formation of inhibitory complexes between PN1 and urokinase in the presence and absence of 1 $\mu$M collagen type IV. At all time points, there are equal amounts of complexes between PN1 and $^{125}$I-urokinase independent of the presence or absence of collagen type IV.

 Transient Acid Exposure of PN1 Confers Allosteric Regulation of Inhibitory Specificity by Collagen Type IV—In an effort to understand the disparity between our data and those previously published, we turned to any potential biochemical differences between the PN1 preparations used in our present studies and those of the published studies (1, 19). It came to our attention that the PN1 used in the previous studies on target protease regulation by collagen type IV was purified using a monoclonal antibody column, whereas a heparin affinity column was used to purify PN1 for the present studies. During the elution of PN1 from the monoclonal antibody column, it was transiently exposed to a strong acid at pH 3.0. To test whether this acid exposure resulted in the altered protease specificity of PN1 in the presence of collagen type IV, we exposed a sample of PN1 purified on a heparin-Sepharose column to 100 mM glycine-HCl, pH 3.0, for 1 min, followed by neutralization by the addition of 200 mM NaPO$_3$, pH 7.4. Longer exposure of PN1 to these conditions (15–30 min) resulted in an 80% loss of PN1 inhibitory activity (data not shown). PN1 exposed to acid for 1 min was then evaluated using the linkage assay described above. We first conducted a control experiment to assess the ability of the acid-exposed PN1 to form complexes with $^{125}$I-thrombin in the presence and absence of collagen type IV. As expected, the data in Fig. 3A demonstrate no significant difference in the quantity of $^{125}$I-thrombin-PN1 complexes formed regardless of the concentration of collagen type IV present. This important control shows, however, that the mere exposure of PN1 to acid does not affect its ability to act as a SERPIN. The acid-exposed PN1 was also able to form complexes with $^{125}$I-urokinase in the absence of collagen type IV but, importantly, became refractory to complex formation in the presence of increasing concentrations of collagen type IV (Fig. 3B). A comparison of the data in Figs. 3B and 1B clearly shows that the acid exposure of PN1 confers allosteric regulation by collagen IV at the level of protease-inhibitor complex formation.

Because we could now reproduce the allosteric regulation of PN1 by collagen type IV after transient acid exposure using a complex formation assay, we proceeded to a more quantitative assessment of the kinetics of the inhibitory reaction between PN1 and urokinase using a fluorometric substrate approach.
Substrate cleavage progression curves for urokinase preincubated with PN1 from 2 to 20 min were measured using the quenched fluorogenic urokinase substrate as described above. The $k_{\text{assoc}}$ rates for PN1 and urokinase were nearly identical in the absence and presence of collagen type IV, $2.13 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \pm 0.12$ and $2.04 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \pm 0.16$, respectively (progression curve data not shown; the $k_{\text{assoc}}$ values are in Table 1). These results are in close agreement with previously published values (1, 3). The same experiment was done after PN1 was transiently exposed to acidic conditions. The $k_{\text{assoc}}$ rate in the absence of collagen type IV was not changed significantly, $2.16 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \pm 0.22$. In contrast, the inclusion of collagen type IV in the reaction containing acid-exposed PN1 markedly slowed the $k_{\text{assoc}}$ rate by a factor of nearly $10, 2.83 \times 10^2 \text{ M}^{-1} \text{s}^{-1} \pm 0.21$ (progression curve data not shown; the $k_{\text{assoc}}$ values are in Table 1). This magnitude of decrease is very close to the previously published report demonstrating the allosteric regulation of PN1 by collagen type IV (1).

The potential biological significance of this data is best appreciated by the $V/V_0$ plot shown in Fig. 4. This is a plot of the data used to obtain the $k_{\text{assoc}}$ values and shows the fraction of active urokinase remaining at times after the addition of PN1. The fraction of active urokinase is reduced by 50% in the presence of native PN1 and acid-exposed PN1 in as little as 7 min. In contrast, urokinase activity was still above 80% after 30 min of incubation with acid-exposed PN1 in the presence of collagen type IV. Finally, these kinetic data also demonstrate that the effect of collagen type IV on the acid-exposed PN1 is manifest early in the mechanism of the inhibitory reaction, at the level of $k_{\text{assoc}}$.

The Lack of PN1 Allosteric Regulation by Collagen Type IV Cannot Be Attributed to Heparin Exposure during Purification.—Because the native PN1 used in the present study was purified by heparin-Sepharose affinity chromatography, we wanted to rule out the possibility that the absence of allosteric regulation by collagen type IV might be the explained by a heparin-induced “conformational lock” of the PN1 during purification. The approach we used was expression of PN1 in Sf9 insect cells to high levels, followed by concentration of the medium that contained the secreted PN1 as previously described (25). The rationale behind this approach was that the PN1 would not be exposed to either a heparin purification step or an acid exposure under these conditions. It should also be noted that the insect cell culture medium does not contain heparin or collagen type IV. Sf9 cells were induced to generate recombinant PN1 through baculovirus infection, and medium was collected and concentrated to use in the collagen type IV assays. Control experiments indicated that there was no significant biochemical or kinetic difference between the baculovirus-expressed PN1 samples and the fibroblast-secreted PN1 that was purified using the heparin-Sepharose column protocol (data not shown).

We first examined the effect of transient acidification on the baculovirus-expressed PN1 using the linkage assay. 125I-Thrombin and 125I-urokinase were incubated with transiently acidified baculovirus-expressed PN1 in the presence of increasing concentrations of collagen type IV (Fig. 5). As expected, PN1 was able to form inhibitory complexes with thrombin at the same quantitative level independent of the presence of collagen type IV (Fig. 5A). Interestingly, the acid-exposed PN1 became refractory to formation of inhibitory complexes with urokinase in the presence of increasing concentrations of collagen type IV (Fig. 5B). The magnitude of the collagen-induced decrease in linkage formation was nearly identical to that seen in Fig. 3B using PN1 purified on heparin-Sepharose. These data clearly rule out the possibility that purification on hepa-
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the presence of collagen type IV after transient acid exposure (Table I). Also included in Table I are the $k_{\text{assoc}}$ rates for urokinase and untreated baculovirus-expressed PN1 for comparison. The progression curves used to obtain the values are not shown.

DISCUSSION

The physiological role of PN1 in biological processes that are driven by proteases depends on three primary factors: protease specificity, protease affinity, and the site of localization. Protease specificity and affinity are closely related and have the additional complication that they may be augmented by other biological molecules depending on the location. A clear example of this is the inhibition of thrombin by PN1, which is accelerated to a diffusion-limited rate in the presence of heparin found in high concentrations in the extracellular matrix (2). What remains unclear, however, is what percentage of PN1 in a tissue is bound to heparin versus that which is free in solution. It is also not clear whether heparin is the only extracellular matrix molecule that may sequester PN1 and augment its activity. It was originally reported that the specificity of PN1 was allosterically regulated by binding to components in the extracellular matrix of human fibroblasts (19). In those studies it was shown that matrix-bound PN1 was a potent thrombin inhibitor but became refractory as an inhibitor of urokinase and plasmin (19). Subsequent studies identified collagen type IV in the matrix as the molecule responsible for the allosteric regulation of PN1 (1). As previously reported, this apparent regulation is manifest in a lowering of the $k_{\text{assoc}}$ between PN1 and urokinase from $1.5 \times 10^8$ M$^{-1}$ s$^{-1}$ to $1.5 \times 10^7$ M$^{-1}$ s$^{-1}$ (1). This is a very important concept as to how the biological functions of PN1 are perceived. This apparent loss of inhibitory activity toward urokinase in the extracellular matrix would suggest a less important role for PN1 in the regulation of cell migration, in matrix turnover, and in the overall process of tissue development and remodeling where urokinase and plasmin are involved. The results of the present studies demonstrate that in fact the inhibitory activity of PN1 toward urokinase is not altered by binding to collagen type IV under normal physiological conditions and reaffirm the potentially important role for PN1 in processes where the proteolytic activity of urokinase is involved.

The original goal of the present study was to identify the domain in PN1 that mediates binding to collagen type IV and to understand the mechanism underlying the allosteric regulation. Our inability to reproduce the original results demonstrating the allosteric regulation of PN1 by collagen type IV led to a re-examination of every component of the experimental system including protein sources and purification procedures. The PN1 used in the previous studies on PN1-collagen interactions was affinity purified using a monoclonal antibody column and eluted with transient exposure to low pH (1, 19). The PN1 used in the present studies, however, was purified by heparin-Sepharose chromatography and eluted with 0.6 M NaCl (24). Three independent lines of evidence obtained in the present studies demonstrate that the transient exposure of PN1 to acid pH is required for allosteric regulation by collagen type IV. First, a 1-min acid exposure of PN1 purified on heparin-Sepharose, which was unaffected by the addition of collagen type IV prior to acid exposure, conferred the ability of the PN1 to be allosterically regulated by collagen type IV. This was determined using the same linkage assay previously described, and the collagen dose-response curves were nearly identical to those previously published (1). Second, in kinetic assays using a quenched fluorogenic peptide substrate, we determined quantitatively that the effect of the collagen type IV on the inhibition of urokinase by PN1 was due to a decrease in the association rate. This was also shown in the previous studies, and the rate constants we determined were nearly identical to theirs (1). Finally, and perhaps most importantly, we show that purification of PN1 using heparin-Sepharose does not play a role in the PN1-collagen type IV interactions. To remove the possibility that exposure to immobilized heparin during our purification protocol imposes a "conformational lock" on PN1 that is removed by acid exposure, we obtained the same data in experiments using recombinantly expressed PN1 that had never been subjected to purification. These three lines of evidence provide compelling support for the idea that indeed it is the acid exposure of PN1 that confers susceptibility to collagen type IV allosteric regulation.

This study does not rule out the possibility that the allosteric regulation of PN1 by collagen type IV might occur under specific sets of circumstance in vivo. PN1 is abundantly produced by kidney tubule epithelial cells, where it is exposed to the acid content of urine (27). In addition, little is known about extracellular changes in pH during ischemia in neuronal tissue.
where PN1 is also very abundant (3, 4). It may also be that the exposure of PN1 to acidic conditions mediates a conformational change in PN1 normally mediated by another component of the extracellular matrix, although no such components have been described. The present studies do suggest that under most physiological circumstances PN1 is a potent inhibitor of urokinase and plasmin and not subject to allosteric regulation by physiological circumstances PN1 is a potent inhibitor of urokinase bound to the urokinase receptor is the cell surface by binding to heparin sulfate proteoglycans and collagen type IV. Because PN1 is known to be concentrated at the action of urokinase bound to the urokinase receptor is directly tied to cell migration in certain cell types (28–31), the role of PN1 in the regulation of this process could be crucial. PN1 should once again be considered as a potentially important factor in cellular processes in which the proteolytic activity of urokinase and plasmin play a role.

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Robert J. Crisp, Mary F. Knauer and Daniel J. Knauer

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