Protease Nexin-1 Interacts With Thrombomodulin and Modulates Its Anticoagulant Effect

Marie-Christine Bouton, Laurence Venisse, Benjamin Richard, Cécile Pouzet, Véronique Arocas, Martine Jandrot-Perrus

Abstract—The endothelial cell membrane glycoprotein thrombomodulin (TM) plays a critical role in the regulation of coagulation. TM is an essential cofactor in protein C activation by thrombin, and a direct inhibitor of thrombin-induced platelet activation and fibrinogen clotting. Protease nexin-1 (PN-1) is a serpin synthesized and secreted by a variety of cells including endothelial cells. PN-1 binds to the cell surface through interactions with glycosaminoglycans, is an efficient inhibitor of thrombin and controls thombin-induced cell responses. An investigation of the interaction of PN-1 with TM using purified proteins and cultured human aortic endothelial cells was performed. Purified PN-1 was observed to bind to purified TM in a concentration-dependent manner. Double immunofluorescence studies indicated that PN-1 and TM were colocalized at the endothelial cell surface from which they were coprecipitated. Pretreatment of the cells with chondroitinase ABC greatly decreased the amount of the PN-1 associated to TM at the cell surface demonstrating the involvement of the TM chondroitin-sulfate chain in the formation of complexes. The inhibitory activity of the PN-1/TM complexes on the catalytic activity of thrombin, and on thrombin-induced fibrinogen clotting, was markedly enhanced when compared with the inhibitory activity of each partner. PN-1—overexpressing human aortic endothelial cells and PN-1—underexpressing human aortic endothelial cells exhibited respectively a significantly reduced ability and enhanced capacity to activate protein C. Furthermore, PN-1 decreased the cofactor activity of TM on thrombin activable fibrinolysis inhibitor activation by thrombin. These data show for the first time that PN-1 forms complexes with TM and modulates its anticoagulant activity. (Circ Res. 2007;100:1174-1181.)

Key Words: protease nexin-1 ■ thrombin ■ thrombomodulin ■ serpins ■ endothelial cells

Thrombin is the only protease in the coagulation cascade that possesses both coagulant and anticoagulant activities. The anticoagulant action of thrombin is dependent on thrombomodulin (TM), a transmembrane glycoprotein predominantly synthesized by vascular endothelial cells. The extracellular domain of TM consists of an N-terminal lectin-like domain followed by 6 EGF-like domains and a serine/threonine-rich region which contains potential sites for O-linked glycosylation supporting the attachment of a chondroitin-sulfate (CS) moiety. Thrombin/TM complex formation prevents thrombin-induced fibrinogen clotting, activation of Factor V and of platelets.1 In contrast, TM-bound thrombin activates protein C (PC) to generate the anticoagulant active protein C (APC).2 The TM/thrombin complex can also activate the latent inhibitor of fibrinolysis, Thrombin Activable Fibrinolysis Inhibitor (TAFI).3 Furthermore, TM exhibiting an attached CS moiety binds thrombin \( \approx 10 \) times tighter and accelerates inactivation of bound thrombin by antithrombin4,5 and PC inhibitor.6 The wide distribution of TM within the vascular system,7 and the marked changes in the activity pattern of thrombin on complex formation with TM, explain the major role of this protein, in the physiological anticoagulant mechanism of control in the hemostatic system.

The catalytic activity of thrombin can be inhibited by a variety of serine protease inhibitors (serpins), including antithrombin (AT), heparin cofactor II (HCII), the plasminogen activator inhibitor-1 (PAI-1) and protease-nexin-1 (PN-1). PN-1, a 43 to 50 kDa glycoprotein, is a potent inhibitor of thrombin and also inhibits other serine proteases such as, u-PA (urokinase-plasminogen activator), t-PA (tissue-type-plasminogen activator) and plasmin. However, in the presence of glycosaminoglycans (GAGs), such as heparin, thrombin becomes the preferential target of PN-1.8 In the presence or absence of heparin, PN-1 is a more potent thrombin inhibitor than AT.9 In contrast to AT and HC II, PN-1 is barely detectable in plasma.10 PN-1 is synthesized and secreted by a variety of cells including vascular smooth muscle cells,11 endothelial cells,12 human foreskin fibroblasts,13 human skeletal muscle myotubes14 and glial cells or neurons.15
At the cell surface, PN-1 forms SDS-stable equimolecular complexes with target proteases.13 Once formed, these complexes are rapidly internalized and degraded as has been reported in human foreskin fibroblasts.16,17 PN-1, therefore, has been suggested to be an important specific regulator of protease activities in the pericellular environment. The aim of this study was to determine whether PN-1 could interact with TM, thereby improving the efficiency of thrombin inactivation by endothelial cells. For this purpose, an investigation of the capacity of TM and PN-1 to interact under purified conditions as well as on endothelial cells was performed. Furthermore the effects of TM/PN-1 complexes on the regulation of thrombin activity were analyzed by using wild-type endothelial cells or endothelial cells that both overexpress or underexpress PN-1.

Materials and Methods
Cell culture, immunofluorescence, cell transfections, enzymatic treatment of cells, reverse transcription and quantitative real-time polymerase chain reaction, TAFI activation, statistical analysis, antibodies and reagents are described in the online data supplement, available at http://circres.ahajournals.org.

Binding of PN-1 to Thrombomodulin
Binding of PN-1 to rabbit TM was analyzed in 96-multiwell plates (Immulon II, Dynatech, Chantilly, Va). Rabbit TM (0.5 μg/well in 50 mM/L bicarbonate buffer, pH 9.6) was used for adsorbance for 18 hours at 4°C. After saturation with 1% BSA in phosphate-buffered saline (PBS), pH 7.5, recombinant PN-1 (1.25 to 100 μg/mL) in PBS, 0.1% Tween-20, 0.1% BSA) was incubated with immobilized TM for 90 minutes at room temperature, in the absence or presence of various competitors: heparin, fucoidans, polybrene, heparin- or chondroitan-sulfates. After washing, bound PN-1 was detected using various competitors: heparin, fucoidans, polybrene, heparin- or chondroitin-sulfates. The process was allowed and immunoprecipitated proteins were solubilized in 500 μL saline (PBS), pH 7.5, containing 2.5 mmol/L CaCl2, and 0.1% PEG 8000, in absence or presence of thrombin (0.5 nmol/L). Thrombin activity was determined by measuring the rate of substrate hydrolysis at 405 nm using a microtiter plate reader and the Biolyse 2 application (Labsystem, Courtaboeuf, France). Values for k, were calculated as described previously.17 The uncatalyzed second order rate constant, ie, in the absence of thrombomodulin, was determined using the above method but with PN-1 (150 nmol/L) present in the medium.

Results
Thrombin Inhibition at the Surface of Endothelial Cells
Thrombin (0.5 nmol/L in TBS-20 mmol/L Tris, 150 mmol/L NaCl pH 7.5- containing 2.5 mmol/L CaCl2, and 0.1% PEG 8000) was incubated with confluent cells which have been or not pre-incubated with PN-1 (5 or 10 nmol/L) and rinsed before use. In other experiments, thrombin was incubated with chondroitinase-treated confluent HAEC monolayers. After 10 minutes incubation of thrombin on confluent cells, aliquots were removed and transferred into a microtiter plates containing S-2238 (0.3 mmol/L). Thrombin activity was measured as above and residual thrombin activity was calculated.

Fibrinogen Clotting
Fibrinogen (2.5 mg/mL in 10 mmol/L Imidazole, 150 mmol/L NaCl, 10 mmol/L CaCl2, 0.1% PEG 8000, pH 7.5) was mixed with different concentrations of thrombomodulin and PN-1. After 5 minutes at 37°C, clotting was initiated by the addition of 1 mmol/L thrombin. The time to clot formation was measured using a KC 10 automatic coagulometer. A standard curve (α-thrombin 0.125 mmol/L to 2 mmol/L) was used to calculate the percentage of residual thrombin activity.

Protein C Activation in a Fluid Phase Assay
Thrombin (0.5 nmol/L) was incubated for 10 minutes at 37°C with rabbit thrombomodulin (0.5 nmol/L) in TBS containing 10 mmol/L CaCl2, and 0.1% PEG 8000, in absence or presence of PN-1 (0.25 to 2 mmol/L). Bovine protein C (80 nmol/L) was added and the incubation continued at 37°C. At specified timed, aliquots were removed; thrombin was inactivated by 100 U/mL hirudin, and activated protein C (APC) was measured using 0.2 mmol/L S-2366 in TBS, pH 7.5.

Protein C Activation at the Endothelial Cell Surface
Thrombin (0.5 nmol/L in TBS containing 2.5 mmol/L CaCl2 and 0.1% human serum albumin) was incubated for 10 minutes with confluent cells which have been or not preincubated with PN-1 (5 or 10 nmol/L) and rinsed before further use. In other experiments, thrombin was incubated with PN-1- over or underexpressing cells or with control cells. Protein C (80 nmol/L) was, then added, and the incubation at 37°C was continued for 90 minutes. Activated protein C was quantified as described above.

Bouton et al Thrombomodulin and PN-1 Are Complexed on HAECs

Thrombin Inhibition by PN-1 in the Presence of Thrombomodulin in a Fluid Phase Assay
Progress curve kinetics were used to estimate the value of second order rate constant k, for the interaction of thrombin with PN-1, in the presence or the absence of thrombomodulin, under pseudo-first order conditions. The dependence of the k, value for the inhibition of thrombin on the concentration of thrombomodulin was determined by incubating PN-1 (5 mmol/L) with increasing concentrations of thrombomodulin (0 to 5 mmol/L in 20 mmol/L phosphate, 100 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1% PEG 8000, pH 7.5) for 5 minutes at 37°C before the addition of the chromogenic substrate S-2238 (0.3 mmol/L). The reactions were started by the addition of thrombin (0.5 mmol/L). Thrombin activity was determined by measuring the rate of substrate hydrolysis at 405 nm using a microtiter plate reader and the Biolyse 2 application (Labsystem, Courtaboeuf, France). Values for k, were calculated as described previously.17 The uncatalyzed second order rate constant, ie, in the absence of thrombomodulin, was determined using the above method but with PN-1 (150 nmol/L) present in the medium.
TM and PN-1 are colocalized on HAECs. A, Control HAECs or (B) HAECs treated with chondroitinase ABC (0.2 U/mL) for 45 minutes at 37°C were processed for double indirect immunofluorescence with the anti-PN-1 monoclonal antibody (red) and the anti-TM polyclonal antibody (green). Fluorescence labeling images were obtained by confocal laser-scanning microscopy. No staining was detected when the labeling was performed without primary antibody or with an irrelevant primary antibody (data not shown). The colocalization of the 2 antibodies was analyzed using the colocalization Zeiss LSM 510 3.2 Image Browser and shown in the Merge (white). Images are representative of 3 experiments. (Bar 10 µm).

When cells were pre-treated with chondroitinase ABC, we observed a robust decrease in the labeling with the anti-PN-1 antibody whereas the labeling with the anti-TM antibody remained unchanged (Figure 2B) leading to a disruption of the protein colocalization. This indicates that the CS chain on TM is important for its colocalization with PN-1.

To determine whether PN-1 and TM colocalization was caused by protein association, immuno-precipitation experiments were performed using either the anti-PN-1 or anti-TM monoclonal antibodies. A band at ~50 kDa, corresponding to PN-1 was detected in the samples precipitated by the anti-TM antibody (Figure 3A) and reciprocally a spread signal at ~70 kDa corresponding to TM was present in the sample precipitated by the anti-PN-1 antibody (Figure 3C) confirming that the two proteins were complexed. Pretreatment of HAEC with chondroitinase ABC resulted in a decreased intensity of the PN-1 band in the cell lysates (Figure 3B) and in the TM immuno-precipitate (Figure 3A). After the treatment with chondroitinase, TM was still present in cells but detected as a narrow band, because of the loss of the highest molecular weight species of TM after deglycosylation (Figure 3D).

**Inhibition of Thrombin Activity by the TM-PN-1 Complexes**

Because CS appeared to be involved in PN-1 binding to TM and are known to accelerate thrombin inhibition by PN-1, the effect of TM on the inactivation of thrombin by PN-1 was investigated in a fluid phase assay. The uncatalyzed rate constant for thrombin inhibition by PN-1 was $6.6 \times 10^5 \pm 0.2 \times 10^5$ M$^{-1}$s$^{-1}$, and is in agreement with previous findings.
thrombin inhibition by PN-1. Polybrene (50 μg/mL) accelerated approximately 20-fold thrombin inhibition by PN-1, TM (5 nmol/L) accelerated approximately 20-fold thrombin inhibition by PN-1. Polybrene (50 μg/mL) completely abolished the effect of TM on thrombin inhibition by PN-1 (data not shown) indicating that the TM-dependent acceleration of thrombin inhibition by PN-1 involves primarily glycosaminoglycan-protein interactions.

Inhibition of Fibrinoformation by TM-PN-1 Complexes

Experimental conditions were chosen in such a way as rabbit TM alone just slightly inhibited thrombin-induced fibrinogen clotting [clotting time increasing from 44 sec to 120 s respectively in the absence or presence of TM (6 nmol/L)] (Figure 4B), and as indicated by the intersection points of the different curves with the Y axis on the Figure 4B, PN-1 alone (0 to 4 nmol/L) did not prolonged the clotting time. In contrast, a progressive increase in the thrombin clotting time followed by a sharp upward tendency was observed in the presence of increasing amounts of the TM/PN-1 mixture. At a fixed concentration of TM (6 nmol/L), the fibrinogen clotting time increased from 120 sec in the absence of PN-1 to 329 sec in the presence of 1 nmol/L PN-1. In the presence of 2 nmol/L PN-1, the clotting time was further increased to 908 sec. This corresponded to a decrease in the residual activity of thrombin from 72.5%±0.7% in the absence of PN-1 to 24.0%±0.8% and 16.5%±2.9% in the presence of PN-1, 1 nmol/L and 2 nmol/L, respectively.

Inhibition of Thrombin Catalytic Activity at the Surface of Endothelial Cells

Thrombin was incubated for 10 minutes with cells which have been pretreated with PN-1. The part of unbound active thrombin was then measured (Figure 5A). Incubation of thrombin with control HAEC monolayers resulted in a decrease in thrombin catalytic activity, the residual activity being of 86.5%±1.5% (Figure 5A). When thrombin was incubated with HAECs pretreated with 5 nmol/L or 10 nmol/L PN-1, the thrombin residual activity decreased respectively to 78.8%±2.8% and 73.6%±0.4% (Figure 5A), indicating that the additional PN-1 bound to HAEC surface increased the inhibitory capacity of the cells. Interestingly, when thrombin was incubated with chondroitinase ABC-pretreated HAECs, the catalytic activity of thrombin was not blocked anymore, the residual activity being of 97.8%±4.7% (Figure 5B), indicating that the chondroitinase treatment abolished the inhibitory effect of cells on thrombin.

Inhibition of Protein C Activation by TM-PN-1 Complexes

As TM is a critical cofactor for thrombin-mediated activation of PC, an investigation of the effect of PN-1 on APC production was performed in a fluid phase assay (Figure 6A).
PC was efficiently activated by an equimolar (0.5 nmol/L) mixture of thrombin and TM, whereas in the absence of TM, thrombin failed to generate APC. PN-1 decreased the rate of protein C activation by TM/thrombin. The rate of PC activation was reduced by 2-fold in the presence of PN-1 (0.5 nmol/L), APC generation being of 6.1 ± 0.2 pmole/min in the presence of PN-1 versus 13.1 ± 1.2 pmole/min in the absence of PN-1.

**Inhibition of Protein C Activation at the Surface of Endothelial Cells**

PC activation was also performed on HAEC. In such conditions, HAECs were the source of thrombomodulin. The rate of protein C activation was reduced by 34.7% ± 4.7% on HAECs which have been incubated with 10 nmol/L PN-1 (Figure 6B). To better address the functional contribution of PN-1/TM interaction on the cell surface, PN-1 siRNA was used to transiently knock-down PN-1 expression. In another set of experiments, a vector containing the complete PN-1 coding sequence was used to overexpress PN-1 (Figure 7A). We observed that 48 hours after transfection, PN-1-underexpressing HAECs exhibited a significantly enhanced capacity to activate protein C (30% ± 18% increase) and reciprocally, PN-1-overexpressing HAECs exhibited a significantly reduced ability to activate protein C (24% ± 4% decrease) (Figure 7B).

**Inhibition of Thrombin Activatable Fibrinolysis Inhibitor Activation**

In addition to protein C activation, the thrombin/TM complex mediates TAFI activation, a procarboxypeptidase U that

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**Figure 5.** Inhibition of thrombin catalytic activity at the surface of HAEC. A, Thrombin (0.5 nmol/L) was incubated with confluent HAEC which have been preincubated or not with PN-1 (5 or 10 nmol/L). B, Thrombin (0.5 nmol/L) was incubated with control HAEC (HAEC CTL) or with chondroitinase-treated HAEC (HAEC ChABC). Residual thrombin catalytic activity was measured as described in the Materials and Methods section by measuring the rate of S2238-hydrolysis. Results are presented as residual thrombin activity [% (thrombin activity after 10 minutes incubation on HAEC/thrombin activity at time 0) x 100] and are the means ± SD of 3 determinations. *P < 0.05 is significantly different from data obtained with control HAEC.

**Figure 6.** PN-1 inhibits protein C activation by thrombin-thrombomodulin. A, Thrombin/TM complexes were preformed by mixing the two proteins in equimolecular concentration (0.5 nmol/L). Protein C was activated by the thrombin/TM complex for 15, 30, 60, 90 and 120 minutes, in the absence ( ), or presence of PN-1 ( ) 0.25 nmol/L, ( ) 0.5 nmol/L ( ) 1 nmol/L ( ) 2 nmol/L. Activated protein C was measured using 0.2 mmol/L S-2366 in TBS, pH 7.5. Results are the means ± SD of 3 independent experiments. Standard deviations were small where deviation bars are not seen. B, Thrombin (0.5 nmol/L) was incubated with confluent cells which have been preincubated or not with PN-1 (5 or 10 nmol/L). Protein C was added and its activation was then measured as described in the Materials and Methods section. *P < 0.05 significantly different from the respective control cells in the absence of PN-1.
relatively poor activator of TAFI. In the present study, TAFI activation was completely blocked in the presence of 10 nmol/L PN-1. On 10 minutes activation of TAFI, a mixture of thrombin and TM, in the absence or the presence of 10 nmol/L PN-1 (-PN-1). The reaction was stopped by the addition of 2% SDS and the samples analyzed by SDS-PAGE followed by silver staining. The migration of TAFI (56 kDa), activated TAFIa (36 kDa) and products of degradation of TAFIa (25 kDa and 11 kDa) is indicated.

Figure 8. PN-1 inhibits TAFI activation by thrombin-thrombomodulin. TAFI (450 nmol/L) was activated by an equimolecular (10 nmol/L) complex of thrombin/TM for 0, 10 or 60 minutes at 37°C in the absence (-PN-1) or presence of 10 nmol/L PN-1 (+PN-1). The reaction was stopped by the addition of 2% SDS and the samples analyzed by SDS-PAGE followed by silver staining. The migration of TAFI (56 kDa), activated TAFIa (36 kDa) and products of degradation of TAFIa (25 kDa and 11 kDa) is indicated.

Discussion

The results of the present study demonstrate that PN-1 binds to TM and that the CS moiety of TM is critical for the interaction. These proposals are supported by the following evidences: First, PN-1 bound to immobilized TM. Second, PN-1 binding to thrombomodulin was inhibited by various polysaccharides such as CS and by the polycation, polybrene. Third, thrombin inhibition by PN-1 was accelerated in the presence of TM and polybrene reversed the effect of TM.

Two other serpins, AT and the protein C inhibitor, have already been shown to bind to different sites on TM. TM has been shown to enhance the rate of thrombin inactivation by ATs.23,24 and CS is important for AT binding to TM, as deglycosylated TM lacks the AT-dependent anticoagulant activity but retains its PC activation cofactor activity. Thus, TM, thrombin and PN-1 or AT appear to associate as trimolecular complexes in which the catalytic site of thrombin remains accessible to the inhibition by serpins.23,26 Nevertheless, using our solid phase assay we neither detected direct binding of AT to TM nor observed any inhibition of PN-1 binding to TM in the presence of an excess of AT (10-fold) over PN-1 (data not shown). Furthermore, TM accelerated thrombin inhibition by PN-1 by about 20-fold whereas it has been reported to accelerate thrombin inhibition by AT by only 4 to 8-fold. Together these data suggest that PN-1 binds to TM with a higher efficacy than AT does. PN-1 has been actually reported to have a higher affinity for CS than AT.27

In contrast, PAI-1, another serpin with poor antithrombin activity does not bind to the thrombin/TM complex. Similarly, PAI-1 had no effect on PN-1 interaction with TM.

The interaction of PN-1 with TM appears to be physiologically relevant since PN-1/TM complexes were detected on HAECs. PN-1 and TM were efficiently coprecipitated from lysates of HAECs and their colocalization was observed by confocal laser-scanning microscopy. Secreted PN-1 is known to bind at the cell surface through interactions with polysaccharides and is released on cell treatment with heparin.20 The present report identified TM as a major PN-1-binding proteoglycan. The role of the CS moiety of TM in PN-1 binding to endothelial cells is demonstrated by the treatment with chondroitinase ABC, which resulted in the disruption of protein colocalization as well as in the reduction of their...
coprecipitation. In contrast, no difference in the colocalization or coprecipitation was observed after heparinase treatment (data not shown). Therefore, the efficacy of PN-1 binding to TM may be related to the TM content in CS which is variable depending on the vascular origin.30 Interestingly, in the present report, HAECs have been shown to limit the catalytic activity of thrombin by both a CS- and PN-1-dependent mechanism. Thus, TM and PN-1 variations are likely to determine the anti-thrombin activity of the endothelium. Therefore, arterial cells which express a high proportion of CS–associated TM are assumed to carry out a better protection against thrombin than venous cells which express a low proportion of CS-associated TM. The fact that we did not succeed in coprecipitating TM and PN-1 in lysates of HUVEC (data not shown) is in favor of this hypothesis. Therefore the amount of TM/PN-1 complexes at the endothelial cell surface may vary according to the vascular territory. Whether or not PN-1 expression is variable in different vascular beds remains to be established.

When TM and PN-1 at concentrations too low to prolong the thrombin clotting time were mixed, a synergistic effect on clot formation was observed as indicated by a sharp prolongation of the clotting time. This striking anticoagulant activity of the TM/PN-1 complex is explained by the observation that TM prevents fibrinogen binding to the thrombin exosite 1,31 and enhances the inhibition by PN-1 of fibrinogen proteolysis by thrombin. The TM/PN-1 complex thus inhibits clotting in a fashion similar to hirudin, with the simultaneous blockade of the thrombin exosite 1 and catalytic site.

On another hand, TM increases the rate of PC activation by thrombin by a factor ≈1 000.3 A recent study demonstrated that the affinity of PC for the thrombin/TM complex is determined in a primary way by active site dependent interactions.32 In the present report, PN-1 was shown to reduce the rate of PC activation by thrombin in the presence of thrombomodulin, not only in a fluid phase assay but also at the surface of endothelial cells. Indeed, transient knock-down or overexpression of PN-1 respectively increased or reduced PC activation. Membrane bound–PN-1 has thus a direct influence on PC activation and therefore a significant impact on the antithrombotic properties of endothelial cells.

TM is also a cofactor for the thrombin-catalyzed activation of TAFI. The active form of TAFI prevents fibrinolysis by removing lysine residues from fibrin. The present study indicates that PN-1 blocks TAFI activation by thrombin/TM and thus may favor fibrinolysis. Consequently, PN-1 can modulate the regulation of both the coagulation and fibrinolytic cascades by its interaction with TM. Nevertheless, the net effect of the PN-1/TM interaction on the different activities of thrombin remains to be determined.

In summary, PN-1 binds to endothelial cell TM. This interaction appears to account for an important improvement in the reactivity of the serpin with thrombin. The coordinated action of TM with PN-1 has direct consequences on thrombin activity among which a dramatic enhancement in the inhibition of fibrinolysis. The net effect of thrombin on endothelial cells might thus be regulated by the concentration of the TM/PN-1 complexes. Because TM and PN-1 are both expressed in the vasculature, local variations of expression of PN-1 and/or TM, are likely determinants for the regulation of thrombin activity in vivo.

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Disclosures
None

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Materials

Human α-thrombin was purified as previously described \(^1\). Recombinant thrombin activable fibrinolysis inhibitor, TAFI-AT (\text{Ala}^{147}\text{-Thr}^{325}) \(^2\) was a generous gift from Dr A. Gils (Laboratory for Pharmaceutical Biology and Phytopharmacology, Leuven, Belgium). Recombinant rat PN-1 (a generous gift from Dr D. Monard, Friedrich Miescher Institute, Basel, Switzerland) was produced in yeast as previously described \(^3\). The specific siRNA duplexes targeted against human PN-1 were purchased from Ambion (Austin, TX, USA). The monoclonal antibody (1F6) directed against human PN-1 sequence was obtained by immunizing mice with the cDNA encoding human PN-1 in collaboration with Agrobio (La Ferté St Aubin, France). Anti-human PN-1 IgGs were selected for their ability to bind PN-1 in ELISA assays. IgGs were purified from ascites by chromatography on a HiTrap-protein A Sepharose (Amersham Biosciences, Uppsala, Sweden). The polyclonal rabbit anti-PN-1 antibody was a gift from Dr D. Hantaï (Paris, France). Bovine protein C was from Enzyme Research Laboratories (South Bend, IN, USA). Rabbit TM and the goat anti-rabbit TM IgG were from American Diagnostica (Greenwich, CT, USA). The monoclonal anti-human TM IgG was from Abcam (Cambridge, UK). Alexa 568-conjugated goat anti-mouse IgG, Alexa 488-conjugated rabbit anti-goat IgG and Oligofectamine were from Invitrogen (Cergy Pontoise, France). Heparin was from Sanofi-Aventis (Paris, France). Bovine serum albumin (BSA), polybrene (Hexadimethrine bromide), a synthetic quaternary polyamine used to neutralized glycosaminoglycans, high molecular weight fucoidans, human plasma fibrinogen, heparan- and chondroitin-sulfate, protease inhibitor cocktail for mammalian tissues, chondroitinase ABC, O-Phenylenediamine Dihydrochloride (OPD) were from Sigma-Aldrich (Saint Quentin-Fallavier, France). Hirudin was from Serbio (France). The chromogenic
Thrombomodulin and PN-1 are complexed on HAEC substrates S-2238 (H-D-Phe-pipecolyl-Arg-p-nitroanilide) and S-2366 (pyro-Glu-Pro-Arg-p-nitroanilide) were purchased from Biogenic (Mauguio, France). Protein A/G-coated magnetic beads were from Ademtech (Pessac, France), horseradish peroxidase coupled secondary antibodies from Jackson ImmunoResearch (West Grove, PA, USA) and ECL from Amersham Bioscience (Uppsala, Sweden).

Methods

Cell culture.

HAECs (human aortic endothelial cells, pooled donors) were purchased from Cambrex (Rockland, ME, USA) and cultured according to manufacturer’s procedures. All experiments were carried out by the 2nd to 5th cell passage.

Enzymatic treatment of cells.

Chondroitinase ABC was used to specifically cleave chondroitin-sulfate from the endothelial cell surface. The enzyme was used at a concentration of 0.2 U/mL for 45 min in the cell incubator. The efficacy of TM deglycosylation was assessed by western blot using a lectin (*Bandeiraea simplicifolia*) that binds specifically to the N-acetyl-D-galactosamine residue present on chondroitin-sulfate.

Immunocytochemical analysis.

Human aortic endothelial cells (HAECs) were seeded on gelatine-coated glass coverslips and enzymatically treated or not as described above. Cells fixation was performed in 2 % paraformaldehyde at room temperature for 10 minutes followed by PBS containing 5 % BSA for 1 h. After washings in PBS, cells were incubated overnight at 4°C with the anti-human PN-1 monoclonal antibody and the anti-rabbit TM polyclonal antibody (20 μg/mL and 50 μg/mL respectively in PBS containing 0.5 % BSA). After washings in PBS, cells were incubated with Alexa 568-conjugated goat anti-mouse IgG and Alexa 488-conjugated rabbit
anti-goat IgG for 2 h at room temperature, mounted and visualized with a confocal laser-scanning microscope (LSM-510-META, Zeiss, Mannheim, Germany) equipped with a x63 oil-immersion objective. Simultaneous two-channel recording was performed with a pinhole size of 1.00 Airy Units by using excitation wavelengths of 488 and 588 nm. The specificity of the labelling was proved by the absence of signal when the primary antibody was omitted or when using an irrelevant antibody. The colocalization of the two antibodies was analyzed using the co-localization Zeiss LSM 510 3.2 Image Browser software.

**Expression plasmids of PN-1 and transfection.**

The cDNA coding sequence for human PN-1 preceded by a Kozak consensus translation initiation site was inserted as a KpnI/EcoRV fragment into a pcDNA3 expression vector (Invitrogen, Cergy Pontoise, France). The pcDNA3 vector containing the PN-1 coding sequence or an empty pcDNA3 vector (mock) were transfected into HAECs by using FuGENE 6 (Roche Applied Science, Meylan, France) according to the manufacturer’s instructions. Cells treated with the transfection reagents alone are referred to as “control cells”. Cells were assayed after 48 hours of transfection.

**siRNA and cell transfection.**

The following pre-designed annealed siRNA was chosen for PN-1 silencing: sense sequence 5’-GGUUUUUCAAUCAGAUUGUGtt-3’ and antisense sequence 5’-CACAAUCUGAUUGAAAACCtg-3’. The pre-designed annealed irrelevant siRNA from Eurogentec (Searing, Belgium) was used as negative control. The duplexes (150 pmol per well of 12-well cell culture plates) were introduced into subconfluent (~ 80-85 %) cultured HAECs using Oligofectamine reagents according to the manufacturer’s instructions. Cells
treated with the transfection reagents alone are referred to as “control cells”. Cells were assayed after 48 hours of transfection. To verify PN-1 extinction, RNA were collected 48 h post-transfection and analyzed by quantitative RT-PCR.

**Reverse transcription and quantitative real-time polymerase chain reaction.**

Total RNA was extracted with Trizol (Invitrogen, Cergy Pontoise, France) and was reverse-transcribed using the Superscript II Reverse transcriptase (Invitrogen, Cergy Pontoise, France) as previously described. The resulting cDNA was used as a template for quantitative PCR analysis of PN-1 and GAPDH mRNA expression in a LightCycler system with SYBR Green detection (Roche Applied Science, Mannheim, Germany). PN-1 primers were: forward 5’-CCGCTGAAAGTTCTTGGCA-3’ and reverse 5’-CAGCACCTGTAGGATTATGTCG-3’. The following run protocol for PN-1 was used: denaturation: 95°C, 10 min; amplification and quantification (40 cycles): 60°C, 10 sec; 72°C, 20 sec. The lightCycler run protocol for GAPDH was as follows: denaturation: 95°C, 10 min; amplification and quantification (40 cycles): 65°C, 10 sec; 72°C, 20 sec. GAPDH primers were: forward 5’-GGGCACCCTGGGCTAAACTGA-3’ and reverse 5’-TGCTCTTGCTGGGGCTGGT-3’. The level of mRNA encoding PN-1 was normalized relative to GAPDH mRNA level.

**Activation of TAFI.**

Recombinant TAFI (450 nmol/L) was incubated with 10 nmol/L thrombin and 10 nmol/L thrombomodulin in 30 µl TBS containing 5 mmol/L CaCl₂ and 0.1 % Tween 80 at 37°C for 0, 10, or 60 min, in the presence or absence of 10 nmol/L PN-1. The reactions were stopped at the indicated time points by the addition of 2% SDS and used for SDS-PAGE followed by silver staining.

**Statistical analysis.**
Results are shown as means ± SD. Statistical evaluation was performed using a student’s t-test. *P* values < 0.05 were considered statistically significant.

References