Three monoclonal antibodies against the serpin protease nexin-1 prevent protease translocation

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Summary
Protease nexin-1 (PN-1) belongs to the serpin family and is an inhibitor of thrombin, plasmin, urokinase-type plasminogen activator, and matriptase. Recent studies have suggested PN-1 to play important roles in vascular-, neuro-, and tumour-biology. The serpin inhibitory mechanism consists of the serpin presenting its so-called reactive centre loop as a substrate to its target protease, resulting in a covalent complex with the inactivated enzyme. Previously, three mechanisms have been proposed for the inactivation of serpins by monoclonal antibodies: steric blockage of protease recognition, conversion to an inactive conformation or induction of serpin substrate behaviour. Until now, no inhibitory antibodies against PN-1 have been thoroughly characterised. Here we report the development of three monoclonal antibodies binding specifically and with high affinity to human PN-1.

The antibodies all abolish the protease inhibitory activity of PN-1. In the presence of the antibodies, PN-1 does not form a complex with its target proteases, but is recovered in a reactive centre cleaved form. Using site-directed mutagenesis, we mapped the three overlapping epitopes to an area spanning the gap between the loop connecting α-helix F with β-strand 3A and the loop connecting α-helix A with β-strand 1B. We conclude that antibody binding causes a direct blockage of the final critical step of protease translocation, resulting in abortive inhibition and premature release of reactive centre cleaved PN-1. These new antibodies will provide a powerful tool to study the in vivo role of PN-1’s protease inhibitory activity.

Keywords
Cancer, glia-derived nexin, SERPIN E2, serine protease, inhibitory antibody

Introduction
Protease nexin-1 (PN-1) is a serine protease inhibitor of the serpin family (for a review, see [1]). Common to inhibitory serpins is the suicide substrate mechanism of inhibition, in which the serpin baits the protease into formation of an irreversible inhibitory complex (for reviews, see [1–4]). The bait, an ~20 amino acid solvent exposed reactive centre loop (RCL), is recognised by the protease as a potential substrate. Catalysis stops after formation of the acyl enzyme complex, when the part of RCL N-terminal to the scissile bond is free to insert as the 4th strand in the central β-sheet A and the protease, tethered to the inserting RCL, is dragged along to the opposite pole of the serpin. The final product is a stable inactive protease:serpin complex in which the covalently trapped protease is rendered inactive due to a distorted active site preventing the completion of the catalytic cycle. Under certain ex vivo conditions, changes to pH and ionic strength, the protease manages to evade the serpin’s inhibitory function by completing the catalytic cycle before distortion of the active site, resulting in the release of the active protease from an RCL-cleaved and inactive serpin. In this case, the serpin is said to exhibit substrate behaviour. The driving force of the serpin inhibitory mechanism is the built-in meta-stability by which the conversion of the serpin from a thermodynamically stressed native conformation to a thermodynamically more favourable relaxed RCL-inserted conformation provides the needed energy for protease translocation and subsequent distortion (for a review, see [5]).

PN-1 is a Mr ~50,000 globular glycoprotein of 379 amino acids (β-isof orm) with three potential N-linked glycosylation sites (Asn99, Asn140 and Asn365). PN-1 exhibits strong affinity for glycosaminoglycans such as heparin (6–9). Binding of heparin and heparin-like glycosaminoglycans accelerates the reaction between PN-1 and certain target proteases, thrombin in particular, by means of the bridging mechanism in which the same heparin molecule simultaneously binds PN-1 and thrombin thereby acts as a guide for the subsequent inhibitory mechanism (6, 9–11). By site-
directed mutagenesis, the heparin binding site of PN-1 was proposed to reside around the residues Lys$^{31}$ and Lys$^{86}$ situated in helix D and the helix D β-strand 2A loop (7, 12). Recently, two structures of PN-1 have been solved, one in complex with heparin alone and the other in complex with inactive S195A thrombin (13). Although the structures do not illuminate the location of the heparin-binding site, they give valuable structural information on PN-1 itself. The same area implicated in heparin binding has been proposed to support binding of members of the low-density lipoprotein receptor family such as low-density lipoprotein receptor-related protein (LRP) 1A (14). Apart from being responsible for the internalisation and degradation of PN-1 and PN-1:protease complexes, these receptors may mediate PN-1 initiated signalling events (for a review, see [15]).

The closest phylogenetic relative of PN-1 is plasminogen activator inhibitor-1 (PAI-1). PN-1 is thought to have overlapping specificities with PAI-1 in terms of target proteases, but while PAI-1 is the main inhibitor of the plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (for a review, see [16]), PN-1 also inhibits proteases such as thrombin, plasmin and matrinptase (for reviews, see [15, 17]).

Based on the apparent phenotype of PN-1-deficient mice and their response to externally imposed challenges, PN-1 has been implicated in important vascular and neurobiological events as well as in the maintenance of male fertility. Furthermore, PN-1 is receiving increasing attention for its potential roles in vascular diseases and cancer, which is mainly attributed to its functions as a modulator of extracellular proteolysis and tissue remodelling (for reviews, see [15, 17]). Most recently, PN-1 has been implicated in the regulation of endothelial barrier integrity (18) and angiogenesis (19). Although most studies support a pro-invasive function of PN-1 in cancer (for review, see [15]), a recent report suggest that PN-1, at least in some cancer types, may exert a protective role against tumour growth and invasion (20).

Among serpins, PAI-1 has been most thoroughly characterised with respect to inhibitory monoclonal antibodies targeting its anti-proteolytic activity. These seem to employ three different mechanisms. First, antibodies have been shown to bind PAI-1 in close proximity to the RCL thereby thought to sterically block access of the target protease to the RCL (21, 22). Second, antibodies stimulating the conversion of PAI-1 to an irreversible inactive form have been reported, some binding epitopes distant to the RCL near β-strand 1C or the helix D β-strand 2A loop (23-26). Third, antibodies have been shown to induce PAI-1 substrate behaviour. Antibodies belonging to this third class have been shown to have epitopes localised to either of two different areas on PAI-1, namely the area around helix F and the helix F β-strand 3A loop or to the helix I β-strand 5A loop (27-31). Biochemical data suggests that these antibodies induce substrate behaviour in PAI-1 by delaying RCL insertion and/or blocking the translocation of the protease across the plane of β-sheet A to the opposite pole of the serpin (32).

Here we describe the development and characterisation of three monoclonal antibodies that bind with high specificity and affinity to human PN-1. We show that binding of all three antibodies results in abolishment of PN-1’s protease inhibitory activity. By localising the respective epitopes, we propose that the antibodies share a common mechanism of inducing serpin substrate behaviour by blocking protease translocation. This work provides PN-1 inhibitors, which will be useful for investigating the physiological and pathophysiological roles of PN-1 in processes involved in haemostasis, neuronal development, and the progression of cancer, by providing the necessary tools for blocking of its anti-proteolytic activity in in vivo model systems.

**Materials and methods**

**PN-1**

Human PN-1 cDNA encoding the α-isoform (Genbank: BC042628.1) was generated from HT-1080 cell mRNA (33). In the α-isoform of human PN-1 (Genbank: BC042628.1), Arg$^{300}$ substitutes residues Thr$^{310}$, Gly$^{311}$ present in the β-isoform (Genbank: BC015663.2). Throughout this paper, we used β-isoform numbering in reference to the available structural information (13). The human PN-1 cDNA was cloned into the pTT5 vector (34), for expression in suspension growing human embryonic kidney (HEK)293–6E cells (vector and cells kindly provided by Yves Duroucher, NCR Biotechnology Research Institute, Montreal, QC, Canada). Maintenance and transfection of cells were carried out as previously described (35, 36). Glycosylated recombinant PN-1 was purified from concentrated conditioned medium by heparin affinity chromatography (HiTrap Heparin, GE Healthcare, Pittsburgh, PA, USA) followed by size exclusion chromatography on a Superdex 75 column (GE Healthcare).

For expression in E. coli, human PN-1 cDNA was cloned into the pRSET-C plasmid (Invitrogen, Carlsbad, CA, USA), previously modified to include an N-terminal extension containing hexa-His tag and a Tobacco Etch Virus protease recognition motif (MRGSHHHHHHHHNLFGQGN-Ser$^1$-His$^1$-Phe$^1$-…). Mutations in PN-1 were introduced in pTT5 and pRSET-C by PCR using specific primers and verified by DNA sequencing at Eurofins MWG Operon (Ebersberg, Germany). His-tagged recombinant PN-1, wild-type or mutant, was produced in BL21(DE3)pLysS competent cells (Novagen, Madison, WI, USA) and purified from bacterial lysate by nickel affinity chromatography (HisTrap FF, GE Healthcare) followed by size exclusion chromatography on a Superdex 75 column (GE Healthcare) as described (37). Protein concentration was determined by optical density at 280 nm using extinction coefficients calculated by the EXPASY ProtParam tool (http://web.expasy.org/protparam/; ε = 0.83 L/g, 0.84 L/g, E. coli and HEK protein, respectively. A >95% purity of the expressed PN-1 protein was verified by SDS-PAGE. The N-terminus of E. coli and HEK PN-1 was confirmed by automated Edman degradation.

RCL-cleaved E. coli PN-1 (presumed cleavage site at Ala$^{297}$, Ala$^{398}$) was generated by incubation for 125 minutes (min) at 25°C with porcine pancreatic elastase in 150-fold molar excess in 0.3 M NaCl, PBS, pH 7.4. The reaction was quenched by the addition of 100 μM 3,4-dichloroisocoumarin and the RCL-cleaved
PN-1 was repurified by heparin affinity chromatography (1 ml HiTrap Heparin, GE Healthcare) as previously described (14).

PN-1-uPA complex was prepared by incubation of 1.7 µM uPA with three-fold molar excess of *E. coli* PN-1 for 20 min at 37°C in PBS, pH 7.4 supplemented with 0.5 M NaCl. The reaction mixture was concentrated (10 kDa spin column, Amicon, Millipore, Billerica, MA, USA) and subjected to size exclusion chromatography on a Superdex75 column (GE Healthcare) equilibrated in 0.02 M MES pH 5.5, 1 M NaCl.

Circular dichroism spectroscopy

Wild type PN-1 and selected variants were subjected to structure content analysis and thermal stability studies by circular dichroism (CD) spectroscopy as described in (38). All experiments were carried out on a JASCO-810 CD spectrometer equipped with Peltier temperature-controlled cuvette holder.

Proteases

The following proteases were acquired from the indicated sources: human uPA (Prospec, Rehovot, Israel); human matriptase catalytic domain (R&D systems, TriChem, Skanderborg, Denmark); human thrombin (John Fenton, Albany, NY, USA), porcine pancreatic elastase (Worthington Biochemical Corporation, Lakewood Township, NJ, USA), human plasmin (Molecular Innovations, Novi, MI, USA), human thrombin (John Fenton, Albany, NY, USA), human uPA (Prospec, Rehovot, Israel); human matriptase catalytic domain (R&D systems, TriChem, Skanderborg, Denmark); human thrombin (John Fenton, Albany, NY, USA), porcine pancreatic elastase (Worthington Biochemical Corporation, Lakewood Township, NJ, USA), human plasmin (Molecular Innovations, Novi, MI, USA), human matriptase catalytic domain (R&D systems, TriChem, Skanderborg, Denmark); human thrombin (John Fenton, Albany, NY, USA), porcine pancreatic elastase (Worthington Biochemical Corporation, Lakewood Township, NJ, USA), human plasmin (Molecular Innovations, Novi, MI, USA).

Antibodies

The mouse monoclonal antibodies mAb33, mAb34 and mAb39 were raised against recombinant human PN-1 expressed in *E. coli*. Also, an anti-mouse PN-1 monoclonal antibody, mAb16, was raised against murine PN-1 (Molecular Innovations) in PN-1-/- mice. Female Naval Medical Research Institute (NMRI) mice were immunised subcutaneously three times with two-week intervals with 25 µg of the antigen adsorbed onto Al(OH)₃ and administered in Gerbu adjuvant. Three days prior to fusion the mice received an intravenous injection with 25 µg of the antigen in saline administered together with adrenalin. The fusion was performed essentially as described by (39) and as modified by (40) with PEG as fusogen and with the SP2/0-AG14 myeloma cell line as fusion partner. Positive clones were selected by ELISA screening against the immunising antigen coated onto Maxisorp polystyrene plates (NUNC, Roskilde, Denmark). Cloning was performed by the limiting dilution method. Selected single clones were finally grown in culture flasks in RPMI + 10% fetal calf serum and mAbs were purified from culture supernatants by Protein A affinity chromatography using the Äkta FPLC system according to the manufacturer's instructions (Amersham Pharmacia, Uppsala, Sweden). The isotopes of mAb33, 34 and 39 were determined using the Pierce Rapid Antibody Isotyping Kit for mouse antibodies (Thermo Fisher Scientific, Rockford, IL, USA).

Anti-PAI-1 monoclonal antibody mAb5 has been described previously (41, 42).

SDS-PAGE

SDS-PAGE was performed using freshly cast 11% gels and standard Laemmli buffers. Wild type or mutant PN-1 (1.5 µg) was incubated with/without 1.5-fold excess mAb (30 min, room temperature) followed by two-fold excess protease (10 min, room temperature). In the case of thrombin, the effect of heparin was tested by the addition of heparin (cat no. 3393, Sigma Aldrich) to PN-1 prior to the addition of protease, using 1.5-fold molar excess of heparin relative to PN-1. Protein samples were reduced by the addition of loading buffer with 2% 2-mercaptoethanol and subsequent boiling for 2 min. Electrophoresis was performed in a standard Tris-glycine buffer system at 35 mA for 1 hour and Coomassie stained. Where appropriate, band intensities were quantified using Totallab Quant software package.

Surface plasmon resonance analysis

All surface plasmon resonance (SPR) analyses were performed on a BIACORE T200 instrument at 25°C, unless otherwise stated, using standard CM5 sensor chips (GE Healthcare) and running buffer: 0.02 M Tris-HCl pH 7.4, 0.5 M NaCl, 0.005% Tween 20, 0.1% bovine serum albumin (BSA). A 0.5 M salt concentration was required to remove unspecific binding of PN-1 to the reference flow cell.

For studying the binding of different forms of PN-1 to immobilised anti-PN-1 mAbs, anti-PN-1 and anti-β2-microglobulin mAbs (GE Healthcare) were immobilised to similar level on active and reference flow cells, respectively. Chips with immobilised mAb were regenerated using short pulses of 0.01 M glycine, pH 2.5, 0.7 M NaCl.

For antibody competition experiments, *E. coli* PN-1 (5 nM) was pre-incubated with or without mAb (50 nM) and injected over mAb immobilised at 2,000 response units (RU).

To study the binding of mAbs to the different conformational forms of PN-1, sets of at least five different concentrations in the range of 1–600 nM of active, cleaved or protease-complexed PN-1 were injected over immobilised mAb (250–300 RU). Kinetic parameters were determined using a 1:1 binding model in the Biacore T200 evaluation software (GE Healthcare).

To test binding of mAbs in the presence of heparin, *E. coli* PN-1 was captured on the active flow cell (220 RU) of a heparin sensor chip (GE Healthcare) and 10 nM anti-PN-1 mAb was injected in both flow cells. Running buffer was 0.02 M Hepes, pH 7.4, 0.15 M NaCl, 0.05% Tween 20, 0.1% bovine serum albumin. A 0.5 M salt concentration was required to reduce unspecific binding of PN-1 to the reference flow cell.

To test the effect of mAbs on the binding of PN-1 to LRP, fragments of LRP produced in SF9 insects cells containing ligand binding domain cluster II and IV (a kind gift from Mingdong Huang, Fuzhou, China) were immobilised using standard amine coupling to 1,000 and 4,000 RU on flow cell 1 and 4, respectively. *E. coli* PN-1 was injected simultaneously on all four flow cells at a flow rate of 10 µl/min and a concentration of 50 nM with and without > 20 min incubation with 100 nM mAb at 15°C in the

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running buffer similar to the heparin experiment. The surface was regenerated using a pulse of 0.01 M sodium acetate, pH 2.5 followed by a pulse of a mixture of 0.25 M EDTA, pH 8.0 and 0.05% SDS.

To test the relative binding of PN-1 variants to the monoclonal antibodies, the respective mAbs were captured on the active flow cell (450–550 RU) by a polyclonal rabbit anti-mouse IgG (GE Healthcare) immobilised on both flow cells (10,000–14,000 RU). A fixed concentration of PN-1 variants was injected in both flow cells at 30 µl/min for 600 seconds. Depending on the mAb, between 5–30 nM mutant E. coli PN-1 was injected, resulting in a maximum of 70% chip surface saturation.

To test the specificity of anti-PN-1 mAbs, human E. coli PN-1 (see above), murine PN-1 (Molecular Innovations), rat PN-1 (a kind gift from D. Monard, Friedrich Miescher Institute for Bio-medical Research, Basel, Switzerland) and human PAI-1 (produced in E. coli and purified as previously described [37]) was injected at 200 nM. Besides the anti-human PN-1 mAbs, relative binding to anti-murine PN-1 mAb16 (see above) captured to the anti-mouse IgG chip was measured. Regeneration was performed by injecting short pulses of 0.01 M glycine-HCl, pH 1.7, at 10 µl/min.

Chromogenic assays with peptidylic substrates

The amidolytic activities of human uPA, plasmin, thrombin and matriptase were assayed using the chromogenic substrates S-2444 (pyro-Glu-Gly-Arg-p-nitroanilide), S-2288 (H-D-Ile-Pro-Arg-p-nitroanilide) and S-2251 (H-D-Val-Leu-Lys-p-nitroanilide) (all from Chromogenix). Assays were performed in 200 µl reaction volumes in a buffer consisting of 0.1 M Hepes, pH 7.4, 0.14 M NaCl, 0.1% BSA. The substrate turnover was monitored at 405 nm in a standard absorbance plate reader (Powerwave, BIO-TEK instruments inc.).

The apparent second order rate constant describing the reaction between E. coli PN-1 and various proteases in the absence or presence of heparin (cat no. 3393, Sigma Aldrich) were determined using previously described procedures (43) (see method section in Suppl. Material, available online at www.thrombosis-online.com).

The ability of mAb33, mAb34, and mAb39 to inactivate the anti-proteolytic activity of PN-1 was tested in chromogenic assays for the various proteases. The minimum concentration of E. coli PN-1 needed to completely inhibit 0.1 nM thrombin, 1 nM uPA, 2 nM plasmin or 0.2 nM matriptase was determined and used in the assays. This concentration of PN-1 was incubated with at least 1.5-fold excess mAb for 30 min at 37°C. PN-1 with or without antibody was then incubated with a target protease for 60 min at 37°C. The reaction was started by the addition of the protease specific substrate (see above) and the evolution of substrate conversion was monitored for 30–60 min at 37°C.

Results

Supplementary Figures and Tables to this article can be found online at www.thrombosis-online.com.

The isolated antibodies bind specifically to human PN-1

From a pool of monoclonal antibodies raised against human PN-1 produced in E. coli, three clones were selected for further characterisation based on their apparent high affinities from a standard antigen-ELISA in the presence of 10% fetal calf serum (data not shown). The three monoclonal antibodies, termed mAb33, mAb34 and mAb39, were found to bind specifically to human PN-1 as virtually no binding was observed at 200 nM of human PAI-1 or either of the PN-1 orthologs from mouse and rat to the individual mAbs on a surface plasmon resonance (SPR) sensor chip surface (see online Suppl. Figure 1). A positive control, mAb16 raised against murine PN-1 (C. Koch and K. Danø, unpublished data), bound both human, murine and rat PN-1 but not human PAI-1. All three antibodies were of the IgG1 isotype with κ (mAb33, mAb39) or λ light chains (mAb34) and all facilitate detection of intact human PN-1 in Western blots and direct capture of HEK PN-1 from conditioned media (data not shown).

The antibodies inactivate PN-1 by induction of substrate behaviour

Next we wanted to know whether binding of the antibodies had direct effects on the inhibitory functions of PN-1. First we determined that PN-1 is able to efficiently inhibit thrombin, uPA, plasmin and matriptase with rate constants in the order of 10^6 M^-1 s^-1 (see online Suppl. Table 1). While heparin increased the rate of
reaction of PN-1 with thrombin (>100-fold), the presence of heparin had no observable effect on the other proteases (online Suppl. Table 1). We then examined the ability of PN-1 to inhibit these proteases in the presence of our antibodies. For all proteases (see online Suppl. Figure 2), as exemplified by thrombin in Figure 1, the three antibodies abolished the inhibitory action of PN-1. This was also the case in similar assays performed in HEK conditioned media or in buffer supplemented with 2% human plasma, using HEK glycosylated PN-1 (data not shown).

When analysing the effect of the mAbs on the PN-1:protease interaction by SDS-PAGE (Figure 2 and online Suppl. Figure 3A-C), the almost complete lack of a band representing the PN-1:protease complex suggests that the antibodies inhibit complex formation. The lack of complex formation concurs with the conversion of PN-1 into a slightly faster migrating form. This was also the case in the presence of saturating concentrations of heparin (see online Suppl. Figure 3C).

Analysis of the protein from the faster migrating PN-1 band by Edman degradation confirmed an intact N-terminus, compatible with PN-1 missing a C-terminal part of the protein. To support this conclusion, a thorough mass spectrometric analysis was performed. This analysis clearly confirmed the cleavage following Arg346 in the RCL of PN-1 by the target protease in the presence of mAb (for detailed analysis and discussion, see online Suppl. Material and Suppl. Figure 4A-C). Together, these observations strongly support that binding of the mAbs efficiently renders substrate behaviour to PN-1, which upon cleavage by the protease in the RCL and subsequent exposure to the denaturing conditions of SDS-PAGE, loose the 3.5 kDa C-terminal fragment (Ser347-Pro379), resulting in the observed faster migrating form of PN-1.

The three antibodies compete for binding to PN-1

Inspired by the similarity of the PN-1 neutralising effect of the three antibodies, we next tested whether a common mechanism could be explained by similarities in their respective epitopes. The ability of the three antibodies to mutually compete for binding to PN-1 was tested by SPR analysis (Figure 3). Prior complex formation between PN-1 and mAb was found to exclude subsequent binding of the same mAb as well as the remaining two antibodies, suggesting overlapping or proximate epitopes of all three antibodies.

The antibodies bind active, cleaved and protease complexed PN-1 with high affinities

We then tested which conformational forms of PN-1 bind the individual mAbs. Interestingly, all three mAbs were shown by SPR analysis to bind all tested conformational forms of PN-1, namely, active, RCL-cleaved and uPA-complexed PN-1 (Figure 4). The
SPR data obtained for active and cleaved PN-1 were well explained by a 1:1 binding model. All three antibodies bound active and cleaved PN-1 tightly with dissociation constants in the range of 0.4–41 nM (Table 1). The affinities were determined at 0.5 M NaCl in order to remove unspecific binding of PN-1 to the chip surface. Although binding of the three mAbs to PN-1:uPA complex was observed, it could not be explained by a simple 1:1 binding model. Reduced rates of association of all three mAbs to the PN-1:uPA complex were observed in comparison with active and cleaved PN-1. Reduction in association rate was most pronounced for mAb34 (Figure 4). Interestingly, all three antibodies bound with increased affinity to the RCL-cleaved form of PN-1.

Figure 4: All mAbs bind all conformational forms of PN-1. Binding of the indicated forms of PN-1 (row 1: HEK PN-1, row 2: E. coli PN-1, row 3: RCL-cleaved E. coli PN-1 and row 4: E. coli PN-1:uPA complex) were tested by injection of a concentration series, as indicated, on SPR chips immobilised with the individual mAbs (column 1: mAb33, column 2: mAb34 and column 3: mAb39). mAb33, mAb34 and mAb39 were immobilised to levels of 300, 250 and 300 RU, respectively. Each insert figures represents one representative experiment out of at least three determinations. Where applicable, the corresponding fit to 1:1 binding model are shown (solid black lines).
Glycosylation does not affect mAbs binding

The majority of the experiments described above, including immunisation, were performed using recombinant E. coli expressed PN-1 protein. The three potential N-linked glycosylation sites (Asn99, Asn140, and Asn160) in PN-1, are located in surface exposed areas at the β-strand 2A-helix E loop, helix F and the loop connecting β-strand 4B to β-strand 5B, respectively (Figure 5A and B). Importantly, we showed that all three antibodies bound both glycosylated and non-glycosylated PN-1 with similar high affinity (Figure 4, Table 1). During our mutational work, we observed that S101A PN-1 from HEK migrates faster than wild type PN-1 from HEK in SDS-PAGE. This is compatible with the S101A mutation disrupting the glycosylation recognition motif (NXS) at Asn99, hence at least this glycosylation site must be utilised in the wild type protein and thus cannot be part in any of the epitopes.

mAbs do not compete with heparin or LRP for binding to PN-1

To further restrict the possible surface area on PN-1 for our epitope search, the ability of the three mAbs to compete with heparin or LRP binding was evaluated using SPR analysis. The results clearly showed that all three antibodies were still able to bind PN-1 captured on a heparin derivatised sensor chip surface (see online Suppl. Figure 5A). Furthermore, PN-1:mAb complexes were still able to bind immobilised ligand binding domain cluster II and IV from LRP (online Suppl. Figure 5B) as observed for the RU increase as a function of complex formation. Thus, we postulate that the area comprising helix D and the helix D-β-strand 2A loop, expected to contain both heparin and interaction site(s) for endocytosis receptors (7, 12, 14) could also be excluded from any of the antibody epitopes.

Initial characterisation of alanine substituted PN-1 variants

To perform the actual epitope mapping, residues in PN-1 were selected for alanine substitution (see legend Figure 5) from the available structure (13) using PyMOL Molecular Graphics System (Schrödinger, LLC) based on three criteria: 1) fully solvent exposed side chain; 2) no observable interactions between the selected side chain and surrounding residues; 3) non Pro, Gly or Ala side chain. Following pre-incubation with uPA, all PN-1 variants migrated in the SDS-PAGE either as SDS-stable complexes with the protease or as cleaved PN-1, both indicative of an overall correctly folded and protease reactive serpin (see online Suppl. Figure 6). Judged from SDS-PAGE band quantification, the majority of single substituted variants did not deviate significantly from that of the wild type protein with 80% of the total protein able to form SDS-stable complexes with uPA (analysis data not shown). A significantly increased tendency for substrate behaviour was observed for the K286A variant only, with 60 ± 10% of total PN-1 protein ending up in the band representing the substrate cleaved form vs 19 ± 10% of the wild-type (p < 0.005, Student’s t-test). To further support the validity of using the PN-1 mutants for epitope mapping purposes, the secondary structure composition and structural integrity of selected mutants was studied by CD spectroscopy. As an example, CD spectra obtained for PN-1 D153A, shown below to be involved in all epitopes, had the same overall shape as the corresponding spectra obtained from active wild type PN-1, suggesting a similar content of secondary structure elements (online Suppl. Figure 7A). The denaturing temperature (T_m) of the PN-1 D153A was only 4°C lower than that of active wild type PN-1 (T_m ~57°C) (online Suppl. Figure 7B), but 3°C higher than active wild type PAI-1 (T_m ~50°C), well within the range of expected values for an active inhibitory serpin (38, 44, 45).

Table 1: Kinetic parameters measured by SPR-analysis. Presented are kinetic parameters describing the binding of various forms of PN-1 to mAb33, mAb34 and mAb39 determined by SPR-analysis (see Figure 4 for experimental details). The presented values are based on three independent determinations resulting in data, which could be explained by a 1:1 binding model. N.D. refers to “not determined” and represents data, which could not be explained by a 1:1 binding model.

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<th>Antibody</th>
<th>Antigen</th>
<th>k_on (10^4 M^-1 s^-1)</th>
<th>k_off (10^4 s^-1)</th>
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Epitopes mapped by site-directed mutagenesis

To map the epitopes, we had to determine the relative importance of each mutated position in PN-1 for the binding of the individual mAbs. The method of choice was to test the effect of the single alanine substitutions on the inactivation of PN-1 by the monoclonal antibodies in a functional assay, monitoring thrombin inhibition by PN-1. The ability of the mAbs to neutralise the inhibitory activity of mutant PN-1 relative to wild type PN-1 is presented in ▶ Figure 6.

The R27A mutation almost completely rescued the activity of PN-1 in the presence of both mAb33 and mAb39, whereas, only a small although statistically significant effect was observed for mAb34. Vice versa, D153A or D156A almost completely abolished binding of mAb34, whereas the effect of D153A was more moderate for mAb33. Based on the chromogenic assays, residue Arg27 appear to be directly involved in the binding of both mAb33 and mAb39. Although residues Asp153 and Lys286 appear to be important for binding of both mAb33 and mAb34, the epitope of mAb34 differs from that of mAb33 and mAb39 by including Asp156 as a major determinant of binding. Many surrounding residue substitutions had no effects (see legend ▶ Figure 5). Thus, based on the effects in chromogenic assays, we have identified a number of residues, which appear to be crucial for binding of all three mAbs.

The results from the chromogenic assays were supported by direct measurements of binding between mAbs and selected PN-1 variants by the SPR technique (▶ Figure 7). In this setup, the relative binding of wild-type and mutant PN-1 to the individual mAbs pre-captured by a polyclonal rabbit anti-mouse IgG on the sensor chip was monitored. The obtained SPR data corresponded well to the results from the chromogenic assays with only a few exceptions: while minor or insignificant effects were detected in the chromogenic assay, the binding of PN-1 R27A, N148A and K290A to mAb34 as well as PN-1 K286A and K290A to mAb39 appeared compromised when monitored by the binding kinetic sensitive SPR technique (▶ Figure 7), thus variations in $k_{on}$ and $k_{off}$ will better be picked up by SPR than by the steady-state binding chromogenic assay. Based on SPR analysis, Arg27 and Asn148 were included in the epitope of mAb34 and Lys290 was up-graded from medium to high importance for mAb34 binding. Also based on the SPR data, residues Lys286 and Lys290 were included as part of the mAb39 epitope.

![Figure 5: Structural presentation of the antibody epitopes on PN-1.](image-url) A) Ribbon presentation of PN-1 (PDBID 4DY0) in the standard orientation with the localisation of potential N-linked glycosylation sites indicated by purple spheres at the corresponding Cβ positions. The proposed heparin binding region in helix D (7, 12) is shown in cyan. A broken red line sketches the non-observable RCL. For reference, β-sheet A is highlighted in blue and helix A, helix F and helix I are indicated. B) As in (A), but in an alternative orientation (turned around the longer axis of the molecule) which is used in (C-E). C, D and E) Surface presentation of PN-1 summarising the effects of Ala-substitution on the binding of mAb33 (C), mAb34 (D) and mAb39 (E). Based on functional chromogenic assays, mutated residues resulting in abolished or reduced ability of the mAbs to neutralise PN-1 were scored to be of high (>50% reduction, indicated in red) or medium (50–20% reduction, indicated in yellow) importance for binding of the respective mAb. In blue: residues for which we observed no effect of Ala-substitution on binding of mAb34 (Ser101, Glu102, Asn123, Glu125, Asp126, Ser129, Cys131, Asp132, Asp144, Asp147, Ser151, Val158, Leu159, Glu282, Asp284, Glu287, Asp299, Ser300, His311, Ser313, His319) mAb33 and mAb39 (as for mAb34 plus Asn146 and Asp156). Residues not suited for alanine substitution (explained in text) are indicated in cyan.
Discussion

We here described the development and characterisation of three monoclonal antibodies directed against human PN-1: mAb33, mAb34 and mAb39. We showed that all three antibodies bind with high specificity and affinity to all conformational forms of human PN-1. Binding of any of these antibodies to active PN-1 resulted in abolishment of its protease inhibitory activity by what appears to be a common mechanism of induction of serpin substrate behaviour.

Initially, we were faced with the daunting task of mapping the epitopes using conventional site-directed alanine mutagenesis. Unlike in the case of PAI-1 for which mapping studies would be assisted by a vast repertoire of mutated variants found in our and collaborating laboratories (46), no vast library of PN-1 mutants was available; therefore, we needed as much indirect information on the location of the epitopes before actual mutagenesis could ensue. We were able to convincingly show binding of the antibodies to all forms of PN-1: active, RCL-inserted, glycosylated or heparin-bound. The fact that all antibodies bound tighter to the RCL-cleaved form of PN-1 as compared to active PN-1 suggested that the RCL insertion in the absence of tethered protease is associated with a beneficial epitope rearrangement. Due to the lack of structural information on RCL-cleaved PN-1 forms, we can...
This is the first description of inhibitors targeting PN-1’s anti-proteolytic activity. These combined observations allowed us to focus our search of epitopes to areas in the vicinity to where the protease domain is thought to localise in the covalent complex.

Based on the proposed epitopes of monoclonal antibodies inhibiting PAI-1 by similar induction of substrate behaviour, we therefore decided to focus on mutating surface exposed residues between helix F and the following helix F β-strand 3A loop, and the loop connecting helix I and β-strand 5A. Due to fact that the mAbs still bound PN-1:protease complexes, we could exclude most of the loop following helix I as this area must be buried by the protease domain in the complex.

Our mutagenesis work suggests that mAb33 and mAb39 bind a slightly different surface area as compared to mAb34, including Arg27 on the loop connecting helix A and helix B as a main determinant for binding (Figure 5). Even so, several hot-spot residues of the mAb34 epitope, namely Asp275, Lys286 and Lys290, are shared in binding of mAb33 and mAb39. Indeed, additive effects on mAb33 and mAb39 binding were observed when mutating both Asp275 and Lys286 (Figure 6). We found that many of the residues in immediate proximity of the hot spot residues (residues labelled red in Figure 5) were not suitable for alanine substitution based on their side chain being identical (Ala) or important for main chain conformation (Gly, Pro), solvent accessibility or involvement in maintaining the main chain conformation via hydrogen bonding, electrostatic or Van der Waals interactions (shown as cyan in Figure 5).

When comparing the identified epitopes, the location of the mAb34 epitope slightly further towards the bottom of the PN-1 molecule (standard orientation, see Figure 5A), corresponds well with the observation that mAb34 in particular, is sensitive to the presence of the protease domain in the complex with PN-1 (Figure 4).

In summary, we are confident that we have mapped all three epitopes, and base our conclusion on several facts: Identification of several hot spot residues, of which individual removal almost completely abolish antibody binding; the mappings of the epitopes are mutually supportive due to the extensive overlap in agreement with competition experiments; the distance between the two residues (Arg27, Asp275) furthest apart in the epitopes of mAb33 and mAb39 (17 Å) is bordering what is observed for an average width of a general antibody epitope (15 Å) (50); the major hot-spot residues are all located in a slightly convex if not planar geometric plane bordered by helix F on one side and the receding edge of the serpin molecule on the opposite side (as seen in Figure 5). Limited by our restriction from mutating additional residues within the area covered by the epitopes, we cannot exclude a differential importance of additional side chains as well as main chain interactions. Thus the observation that mAb39 binds with apparently higher affinity than mAb33 and mAb34 must stem from such additional favourable interactions. To obtain more details on the relative binding interactions between the three antibodies and PN-1, the structure of the individual PN-1:mAb complexes must be resolved by protein X-ray crystallography.

By identification of the epitopes of the three antibodies, we can now propose the mechanism by which the three antibodies inhibit
the anti-proteolytic activity of PN-1 (see model ▶ Figure 8). By binding across the area through which the RCL has to pass during insertion into β-sheet A, the antibody sterically delays the insertion of the RCL by acting as a simple “road block”. This allows the protease enough time to complete its catalytic cycle, resulting in the release of the active protease from the cleaved and inactive serpin.

In conclusion, the presented study demonstrates that monoclonal antibodies can inhibit PN-1 by a mechanism similar to that of PAI-1 inhibiting antibodies through the induction of substrate behaviour.

Furthermore, our new antibodies will provide the necessary tool for controlling PN-1 inhibitory activity in in vivo model systems with the purpose of studying its role in normal physiology and disease.

Acknowledgements
Christine R. Schar is thanked for careful reading of the manuscript.

Conflicts of interest
None declared.

References


