Identification of a Binding Site in Protease Nexin I (PN1) Required for the Receptor Mediated Internalization of PN1-Thrombin Complexes*

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An overlapping synthetic peptide library was constructed representing most of the mature protease nexin I (PN1) sequence from the amino terminus to the reactive center. This library, along with peptides from the heparin binding domain and from the region carboxy-terminal to the P1 residue of the cleavage site, was screened for the inhibition of 125I-thrombin (Th)-PN1 complex binding and degradation. A peptide corresponding to residues Pro47–Ile58 in the PN1 sequence was identified as a potent inhibitor of 125I-Th-PN1 complex degradation, although it did not affect binding significantly. Pro47-Ile58 was shown to competitively inhibit the low density lipoprotein receptor-related protein (LRP)/α2-macroglobulin receptor-mediated endocytosis of 125I-Th-PN1 complexes in mouse embryonic fibroblasts. Pro47-Ile58 is an apparent transition sequence in PN1, separating sheet-B and helix-B. The sequence of Pro47-Ile58, PHDNIIVSPHGI, suggests that it forms a loop structure defined by the seven underlined amino acids bordered by proline residues at each end. These studies are the first to identify a putative binding site in a serine protease inhibitory domain required for LRP-mediated internalization.

**Protease nexin I (PN1)** is a member of the serine protease inhibitor (SERPIN) super-gene family that acts as an important physiological inhibitor of thrombin and urokinase (1–3). The mature form of PN1 is a 43-kDa glycoprotein that is found in several tissues throughout the body including a platelet-bonded form, although the vast majority of PN1 is found outside the vascular compartment (4, 5). PN1 forms complexes with its target proteases, acting as a suicide inhibitor, and is presumably cleaved during the inhibitory reaction (2). It is clear that a structural rearrangement of PN1 occurs as a result of complex formation that renders it endocytosis competent, because free PN1 competes 100–1,000-fold less effectively for the uptake of radiolabeled PN1-protease complexes by clearance receptors on human fibroblasts than the corresponding unlabelled complexes at 37 °C (6). The ultimate clearance receptor for complexes of PN1 with urokinase (Uk-PN1) has recently been identified as the α2-macroglobulin receptor also known as the low density lipoprotein receptor-related protein (LRP) (7). Interestingly, whereas the LRP is responsible for the endocytosis of Uk-PN1 complexes, the urinary plasminogen activator receptor is required for the initial binding of the complexes to cells, which is then dependent on the LRP for endocytosis (8). The endocytosis of Th-PN1 complexes is also LRP-dependent, but it is not dependent on the urinary plasminogen activator receptor (8).

Identification of the domain(s) in SERPINs that mediate their internalization and degradation remain controversial. A sequence carboxyl-terminal to the cleavage site in several plasma-borne SERPINs (including antithrombin III, heparin cofactor II, and α1-antitrypsin) has been implicated in the binding and internalization of these SERPINs by the SERPIN-enzyme complex receptor in liver cells (9, 10); however, these studies have recently been challenged (11). We initially investigated the homologous carboxyl-terminal sequence in PN1 to determine its potential involvement in PN1-protease complex clearance by human fibroblasts because of the large degree of sequence identity between SERPINs in this region. However, our laboratory was unable to detect any effect of peptides from this region on either the binding or internalization of Th-PN1 complexes by human fibroblasts.

In the present studies we have identified a putative structural domain near the amino-terminal region of PN1 that mediates the binding of Th-PN1 complexes to the LRP. A synthetic peptide library representing PN1 sequence was constructed containing peptides 12 amino acids in length with a 3-amino acid overlap. A peptide composed of residues Pro47–Ile58 in the mature PN1 sequence was identified that did not inhibit the binding of Th-PN1 complexes to the cell surface but specifically inhibited their internalization. By comparing the uptake and degradation of PN1-protease complexes in normal mouse embryonic fibroblasts (MEF) and PEA 13 cells (LRP-deficient MEF), it was concluded that the peptide specifically inhibited the transfer of cell surface-bound complexes to the LRP. These studies are the first to identify a narrowly defined, putative binding site in a SERPIN required for LRP-mediated internalization.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture medium was Cellgro DMEM from Mediatech Inc. Antibiotics and amino acid supplements were from Irvine Scientific. Fetal bovine serum was purchased from Gemini, Inc. Cell culture plastics and pipettes were from Corning. Normal MEF and PEA 13 cells, mouse embryonic fibroblasts deficient in the LRP, were a generous gift from Dr. J. Herz (12). Pure human thrombin, approximately 3,000 NIH units/mg was from Boehringer Mannheim. Porcine mucosal heparin was purchased from Calbiochem. Na125-Iodine was from American. Na125-Iodine was from American and Fisher. Thrombin, U1-urokinase, LRP, low density lipoprotein receptor-related protein; HP, human foreskin fibroblasts; MEF, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; SERPIN, serine protease inhibitor; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; HEM, heparin binding peptide; CT, carboxy-terminal peptide.

*The abbreviations used are: PN1, protease nexin I; Th, thrombin; Uk, urokinase; LRP, low density lipoprotein receptor-related protein; HP, human foreskin fibroblasts; MEF, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; SERPIN, serine protease inhibitor; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; HBP, heparin binding peptide; CT, carboxy-terminal peptide.*
FIG. 1. Peptide library construction and screening. A, peptides 1–28, HBP, and CT were synthesized as described under "Experimental Procedures." Peptides were purified, lyophilized, and dissolved in PBS to a final concentration of 500 μg/ml, except for the first amino acid in the mature PN1 sequence, a site of PN1 and ends at the carboxyl terminus. Shown in Fig. 1 are all of the peptides in the library, including HBP, a 17-amino acid heparin binding peptide derived from PN1 sequence (18), and CT, a 35-amino acid carboxyl-terminal peptide that begins at the P1 residue in the reactive center. All of the peptides were synthesized on Cys derivatized resin, such that all of the peptides were primed with the first amino acid in the mature PN1 sequence, a triplicate cultures of HF cells in 24-well plates were prepared as described in Fig. 1. The medium was replaced with DMEM containing 0.1% BSA and the indicated concentrations of Pro47–Ile58 (●), Pro58–Ile60 (▲), or random sequence peptide of the same amino acid composition, IVINDHIGPSFH (■) and 200 ng/ml of 125I-Th-PN1 complexes. At the end of a 3-h incubation at 37 °C, 100-μl aliquots of the medium were removed and assayed for the appearance of trichloroacetic acid soluble radioactivity. The total amount of 125I-Th-PN1 degraded is presented as fmol and is based on the radio-specific activity of the 125I-Th-PN1 complexes used in the experiment. Background has been subtracted as described in the legend to Fig. 1, and error bars indicate one standard deviation from the mean of triplicate samples.

Cell Culture—Both human fibroblasts and mouse embryo fibroblasts were grown in DMEM supplemented with penicillin/streptomycin, L-glutamine, and 10% fetal bovine serum as described previously (6). Experimental cultures were maintained in serum-free medium for 72 h prior to use.

Peptide Synthesis—Peptides in the library were synthesized on a 10-μmol scale in individual 10 cc syringes and monitored for coupling efficiency at each step as described previously (13–15). All of the peptides were purified by reverse-phase high pressure liquid chromatography on a semi-preparative C-4 column using a 0 to 60% acetonitrile gradient over a 60-min time interval. The purified peptides were weighed and dissolved to a final concentration of 500 μg/ml in PBS. Typical recoveries ranged from 1 to 3 mg for each of the peptides. Large-scale peptide synthesis was carried out similarly, using a semi-automated peptide synthesizer (15).

Protein Radioiodination—Human thrombin was radioiodinated using Iodogen as described previously (16). The specific activities ranged from 8,000 to 20,000 cpn/mg.

Preparation of 125I-Th-PN1 Complexes—PN1 was purified from the serum-free culture medium of human fibroblasts grown on Cytodex 1 microcarrier beads and was active site titrated with human thrombin (6). 1 μg of radioiodinated human thrombin (17) was incubated with two times the amount of PN1 required to inactivate the 1 μg of thrombin. The reaction mixture was then added to the culture of cell medium and assayed for thrombin activity using a colorimetric assay (data not shown).

Trichloroacetic Acid Precipitation Assay—We have previously documented that the appearance of trichloroacetic acid soluble radioactivity (primarily 125I-Tyr) in the supernatants of cells incubated with 125I-Th-PN1 complexes results from the internalization and lysosomal degradation of the complexes (16). Cell cultures were incubated with 125I-Th-PN1 complexes at a concentration of 100 or 200 ng/ml in DMEM containing 1 mg/ml of BSA and the indicated concentrations of peptides. At the indicated time points, aliquots were removed and assayed for trichloroacetic acid-soluble radioactivity (16).

RESULTS

Construction and screening of a PN1 peptide library. Beginning with the first amino acid in the mature PN1 sequence, a synthetic peptide library was constructed that extended to the P1 residue in the reactive center. All of the peptides were synthesized on Cys derivatized resin, such that all of the peptides were 13 amino acids in length and contained a Cys at the carboxyl terminus. Shown in Fig. 1A are the sequences of all peptides in the library, including HBP, a 17-amino acid heparin binding peptide derived from PN1 sequence (18); and CT, a 35-amino acid carboxyl-terminal peptide that begins at the P1 site of PN1 and ends at the carboxyl terminus.

Initially, the peptides in the PN1 library were screened for the inhibition of cell surface binding. None of the peptides had a measurable effect (data not shown). The peptides were then
complexes were degraded in 2 h (Fig. 1). The medium was removed and replaced with DMEM containing 0.1% BSA and 100 ng/ml of 125I-Th-PN1 complexes alone (■) or in the presence of 25 μg/ml of Pro47–Ile58-Cys (●). At the indicated time points, triplicate cultures were rapidly chilled on ice and washed four times with PBS, and the cell surface-bound complexes were stripped using EOTA, heparin (1 mg/ml), pH 9.0, and quantified by γ counting (6). Nonspecific binding, approximately 30% of total, was determined by incubating three wells with a 400-fold molar excess of unlabeled complexes. The nonspecific binding has been subtracted in the graph shown. Error bars indicate one standard deviation from the mean of triplicate samples.

Screened for their effect on 125I-Th-PN1 complex degradation by human foreskin fibroblasts (HF cells) using a trichloroacetic acid soluble radioactivity assay (Fig. 1). Each peptide was added to triplicate wells of HF cell monolayers at a concentration of 25 μg/ml, in medium containing 100 ng/ml of 125I-Th-PN1 complexes. After a 2-h incubation at 37 °C, aliquots of the medium were removed and precipitated in 10% trichloroacetic acid. Of the peptides tested, only peptide 4 (Pro47–Ile58-Cys) inhibited the degradation of 125I-Th-PN1 complexes, whereas all of the peptides in the library, Pro 47–Ile58, inhibited by 60% and progressed to 90% at 5.0 μg/ml (Fig. 2). Higher concentrations of Pro47–Ile58-Cys did not result in further inhibition. The dose dependence of the inhibition of 125I-Th-PN1 complex degradation by Pro47–Ile58-Cys suggests that it acts as a competitive inhibitor for a saturable cellular component. The residual, small amount of degradation at the optimal dose of Pro47–Ile58-Cys is most likely due to adsorptive phase endocytosis.

Because Pro47–Ile58-Cys contains a carboxyl-terminal Cys, as do all of the peptides in the library, Pro47–Ile58 was synthesized without the Cys to rule out any contribution of the carboxyl-terminal Cys to the inhibitory activity. The inhibitory activity of Pro47–Ile58 without the Cys was identical to Pro47–Ile58-Cys (Fig. 2). Additionally, a randomized peptide of the same amino acid composition of Pro47–Ile58 did not inhibit 125I-Th-PN1 complex degradation, indicating that the effect of Pro47–Ile58-Cys is sequence-specific (Fig. 2).

The Inhibition of 125I-Th-PN1 Complex Degradation by Pro47–Ile58 Is Dose-dependent and Peptide Sequence-specific. To assess the relative effectiveness and specificity of Pro47–Ile58-Cys in inhibiting the degradation of 125I-Th-PN1 complexes, the dose dependence of inhibition was determined. Also tested were Pro47–Ile58 without a carboxyl-terminal Cys and a random sequence peptide. The inhibition of 125I-Th-PN1 complex turnover was found to be dependent on Pro47–Ile58-Cys concentration. At a concentration of 2.5 μg/ml, degradation was inhibited by 60% and progressed to 90% at 5.0 μg/ml (Fig. 2). Higher concentrations of Pro47–Ile58-Cys did not result in further inhibition. The dose dependence of the inhibition of 125I-Th-PN1 complex degradation by Pro47–Ile58-Cys suggests that it acts as a competitive inhibitor for a saturable cellular component. The residual, small amount of degradation at the optimal dose of Pro47–Ile58-Cys is most likely due to adsorptive phase endocytosis.

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Pro47–Ile58-Cys Inhibits the Internalization of 125I-Th-PN1 Complexes by Human Fibroblasts but Does Not Inhibit Cell Surface Binding. Because it has been previously shown that the LRP is not involved in the initial binding of Uk-PN1 complexes to the cell surface but is required for internalization, we next examined the effect of Pro47–Ile58-Cys on Th-PN1 complex internalization and binding. The fate of 125I-Th-PN1 complexes was followed over a 30-min time course in the presence and the absence of Pro47–Ile58-Cys (Fig. 3). At the indicated times, the cultures were rapidly chilled on ice, and cell surface-bound complexes were stripped to discriminate between internalized and cell surface-bound complexes (6). Pro47–Ile58-Cys did not have a significant effect on the cell surface binding of 125I-Th-PN1 complexes when compared with the no peptide control (Fig. 3A). In contrast, Pro47–Ile58-Cys inhibited complex internalization by 70–80% throughout the 30-min time course (Fig. 3B).
These data demonstrate that Pro$^{47}$-Ile$^{58}$-Cys potently inhibits the internalization of 125$I$-Th-PN1 complexes but apparently does not significantly inhibit cell surface binding. These data are most consistent with a model in which PN1 initially binds to an unidentified cell surface component and are subsequently internalized by the LRP. To demonstrate this directly, experiments were next done using normal MEF and PEA 13 cells (MEF in which the LRP gene is interrupted) (12). The present studies were undertaken to identify the receptors involved in the clearance of SERPIN-envelope complexes began using an in vivo model (19), and only recently have some of the components involved in this process been identified at the molecular level. The term clearance may be an understatement of the importance of the process, because in the case of some SERPINs, clearance is tied to a positive feedback signaling loop that results in SERPIN up-regulation at the level of transcription, translation, and secretion (20). In addition, SERPIN clearance has also been linked to cytokine regulation (21). The control point in the feedback loop apparently resides within the SERPIN-protease complex structure. The receptors responsible for SERPIN-envelope complex clearance are not known in all cases, but several, including proteases complexed to plasminogen activator inhibitor-1 (22–24), PN1 (7), and α-antitrypsin (25), are known to be cleared ultimately by the LRP/β2-microglobulin receptor. α$\_1$-Antichymotrypsin complexes, although closely related, are not cleared by the LRP but may be cleared by a LRP-related receptor, gp330 (25). It also was demonstrated in a recent study that the LRP is responsible for the endocytosis of heparin co-factor II-thrombin complexes and antithrombin III-thrombin complexes in HepG2 cells and normal MEF (26).

The present studies were undertaken to identify the region(s) of PN1 involved in HF cell complex clearance other than the previously implicated carboxy-terminal region (10). The approach taken here was to construct a peptide library representing virtually the entire sequence of mature PN1 excluding residues P9-P1 of the reactive loop. Peptides in the library were initially screened for their ability to inhibit the turnover of 125$I$-Th-PN1 complexes by HF cells. A single peptide, Pro$^{47}$-Ile$^{58}$-Cys, was found to be a potent inhibitor of 125$I$-Th-PN1 complex turnover. This peptide was found to inhibit endocytosis of 125$I$-Th-PN1 complexes rather than initial cell surface binding. The identity of the LRP as the receptor responsible for the endocytosis of the 125$I$-Th-PN1 complexes was rigorously verified using normal MEF (LRP$^+$) and PEA 13 mouse embryo fibroblast (LRP$^-$) cell lines (12). PEA 13 cells are unable to efficiently internalize and degrade 125$I$-Th-PN1 complexes, whereas the LRP$^+$ cells internalized and degraded large quantities of the complexes. Degradation of the complexes by the LRP$^-$ cells was markedly inhibited by Pro$^{47}$-Ile$^{58}$-Cys. Further experiments verified that Pro$^{47}$-Ile$^{58}$-Cys did not affect complex formation and did not induce aggregation of the complexes (data not shown).

SERPINs, in general, are not taken up by clearance receptors in their native conformations. Only SERPIN-envelope complexes are endocytosed efficiently, apparently due to the exposure or generation of a binding site in the complexed form (20). Previous studies have focused on residues in the P' side of the reactive loop, because of the dramatic structural shift undergone by this region upon complex formation (27). However, the present studies strongly indicate that the sequence, Pro$^{47}$-Ile$^{58}$-Cys, that resides near the amino-terminal end of PN1, plays an important role in the LRP-mediated endocytosis of PN1-Th complexes. This sequence is apparently unordered and exists as a loop structure that is bounded by sheet-6B on the amino-terminal side and by helix-B on the carboxyl-terminal side. The minimal loop most likely consists of the seven underlined amino acid residues, PHDNIVISP, set off by the two proline residues, one at each end. Because there is no crystal structure data available for PN1, it is not possible to say with certainty whether this site is masked or partially masked in uncomplexed PN1.

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