We recently identified a synthetic peptide, Pro$^{47}$–Ile$^{58}$, derived from the mature protease nexin 1 (PN1) sequence, that inhibited the low density lipoprotein receptor-related protein (LRP)-mediated internalization of thrombin-PN1 (Th-PN1) complexes. Presently, we have analyzed this sequence in Th-PN1 complex catabolism using two independent approaches: 1) An antibody was generated against Pro$^{47}$–Ile$^{58}$, which inhibited complex degradation by 70% but had no effect on the binding of the complexes to cell surface heparins. This places the structural determinant in PN1 mediating complex internalization by the LRP outside of the heparin-binding site. This is the first analysis of a structural determinant in thrombin-protease nexin 1 complexes that mediates clearance by the low density lipoprotein receptor-related protein.

Analysis of a Structural Determinant in Thrombin-Protease Nexin 1 Complexes That Mediates Clearance by the Low Density Lipoprotein Receptor-related Protein*

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Mary F. Knauer‡, Robert J. Crisp‡, Steven J. Kridel§, and Daniel J. Knauer‡¶

From the ‡Department of Developmental and Cell Biology, School of Biological Sciences, University of California, Irvine, California 92697 and the ¶Burnham Institute, La Jolla, California 92037

Protease nexin 1 (PN1) is a member of the SERPIN superfamily (1, 2), and an important physiological regulator of thrombin (Th) and urinary plasminogen activator (uPA) (2). PN1 forms stoichiometric complexes with both Th and uPA (3) that ultimately results in their removal by cellular endocytosis and degradation (4). The precise biochemical nature of the complexes is still not completely clear, but they are extremely stable and possibly covalent (5–7). When complexes are formed between the SERPIN and its target protease, there is an accompanying conformational change in the SERPIN that either unmasks or causes the formation of a new binding site in the complexed SERPIN that is not present in the free SERPIN (8, 9). The cryptic nature of the LRP-binding site in the free SERPIN makes sense biologically. It ensures that SERPINS will remain extracellular, either in plasma or in tissues near cell surfaces, until they have formed an irreversible complex with a protease.

The list of SERPINS dependent on the LRP for cellular internalization includes, protease nexin 1 (PN1) (10, 11), heparin cofactor-II (12), antithrombin III (ATIII) (12) and α-1-antitrypsin (12). It is interesting to note that although the LRP acts as the internalization receptor, other cellular components are most likely required for the efficient catabolism of the SERPIN-Protease complexes (12–15). In the case of the plasma SERPINS these components remain to be identified. However PN1, which is primarily restricted to tissues, utilizes at least two different cell surface molecules to assist LRP-mediated internalization. When PN1 forms complexes with uPA, the uPA receptor is required for efficient concentration at the cell surface of uPA-PN1 complexes and subsequent internalization via the LRP (11, 16). In contrast, when PN1 is in complex with thrombin, heparin chains present at the cell surface greatly facilitate the uptake and turnover of thrombin-PN1 complexes (15), and uPA receptor is not involved (11, 16). Thus, in the case of PN1, the nature of the target protease directly plays a role in the clearance mechanism.

In a recent study using a synthetic peptide library strategy, a putative LRP-binding site was identified in PN1 (10). The library consisted of peptides 12 amino acids in length, and spanned nearly the entire PN1 sequence. A single peptide in the library, 47PHDNIVISPHGI58 was identified as a potent inhibitor of Th-PN1 internalization and degradation. Using α-1-antitrypsin structure and sequence alignments, this sequence is predicted to be a transition sequence that occurs just after helix A and continues to form the sixth strand of sheet B (1). Consequently, this site meets the criteria of being at least partially buried in the intact SERPIN, with the potential of becoming more exposed after complex formation with thrombin. There is no direct evidence, however, to distinguish this possibility from a simple conformational change in which the exposure of this site remains constant.

In the present studies we further investigate the potential role of this putative site in LRP-mediated internalization using two different approaches. In the first approach, a polyclonal IgG was generated against Pro$^{47}$–Ile$^{58}$ with a cysteine residue added after the Ile to facilitate haptenization to ovalbumin. We demonstrate that this antibody specifically and selectively inhibits the binding of Th-PN1 complexes to the LRP, but does not affect the interaction of the complexes with cell surface heparins. In the second approach we utilize site-directed mutagenesis and baculovirus-driven expression in insect cells. Two variant forms of PN1 were expressed; one with an alanine substitution at the position of His 48 (H48A), and another with alanine substitutions at the positions of His 48 and Asp 49 (H48A,D49A). Each PN1 variant was characterized biochemi-
cally by determining the $k_{assoc}$ value for thrombin inhibition and ability to form SDS-resistant complexes with thrombin. Additionally, the PN1 variant-thrombin complexes and native PN1-thrombin complexes were assayed for their capacity to bind to cell surface heparins. While the PN1 variants were found to be very similar to native PN1 in their ability to inactivate thrombin and bind to cell surface heparins, complexes made with each of the PN1 variants showed decreased rates of catabolism. These experiments define a critical role for the structural determinant, Pro$^{47}$–Ile$^{58}$, in the LRP-mediated internalization of Th-PN1 complexes. These data also demonstrate that with the use of anti-(Pro$^{47}$–Ile$^{58}$), antibody, cell surface heparin binding and LRP-mediated internalization of Th-PN1 complexes can be studied as independent events, even though they act cooperatively to facilitate complex catabolism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media and reagents were purchased from Irvine Scientific and JRH Scientific. Cell culture plastics were from Corning. Thrombin, 3,000 NIH units/mg, was purchased from Calbiochem, and Na$^{125}$I was from Amersham Pharmacia Biotech. High-trap heparin-Sepharose and Cibacron blue-Sepharose were from Amersham Pharmacia Biotech. Recombinant enzymes were from Boehringer Mannheim. All other common shelf reagents were either from Sigma or Calbiochem. The synthesis of Pro$^{47}$–Ile$^{58}$-Cys has been described previously (10). Antibodies against this peptide were raised in rabbits, using Pro$^{47}$–Ile$^{58}$-Cys coupled to ovalbumin as described previously (17). The LRP agonist, receptor-associated protein, was expressed as a receptor-associated protein-glutathione S-transferase fusion protein (RAP-GST) consisting of an amino-terminal glutathione S-transferase sequence followed by the rat RAP sequence. The fusion protein was affinity purified on a glutathione-Sepharose column as described (15).

**Cell Culture**—Human foreskin fibroblasts (HF) were grown and maintained as described previously (8). Experimental cultures were seeded at $10^5$ cells/well in 24-well plates. When the cells reached confluence, they were changed to serum-free medium and used 48 h later.

**Preparation and Expression of Recombinant Proteins**—The methods used to prepare and express recombinant forms of PN1 in a baculovirus-driven expression system have been described in detail elsewhere (18) and are briefly summarized here. Recombinant DNA constructs of PN1 in plasmids with Als substitutions at His$^{48}$ and Asp$^{49}$ were prepared using overlapping polymerase chain reaction to introduce the desired nucleotide substitutions as described previously (19). The constructs were sequenced and cloned into the pVL baculovirus shuttle vector and driven in co-transfected into Sf9 insect cells along with Baculocold baculovirus. Recombinant viruses were purified by a single round of plaque purification. For protein expression, Sf9 cells grown in T75 flasks were infected at a multiplicity of infection of 10:1. Five to seven days later the media were harvested, and the recombinant forms of PN1 were purified by affinity chromatography on Cibacron blue-Sepharose (20).

**Determination of PN1 Activity and $k_{assoc}$ Constants**—Purified samples of PN1 and the recombinant variants of known protein concentration (21) were titrated with active thrombin to determine the percent of activity. 30 ng of thrombin were added to various amounts of the PN1 samples in a final volume of 100 $\mu$l of PBS, pH 7.2, containing 0.1% bovine serum albumin. At the end of a 30-min incubation, the reactions were chilled on ice, and a 200-fold molar excess of Chromozym-Th was added. The reactions were returned to room temperature for 30 min to allow for color development as a measure of residual thrombin activity. Absorbance measurements were taken at 405 nm to quantify color development. $k_{assoc}$ values were determined as described previously (22). Specific activities ranged from 8,000 to 15,000 cpm/mg of protein.

**Analysis of PN1-binding Site for LRP**

Anti-(Pro$^{47}$–Ile$^{58}$) Antibody Selectively Blocks the LRP-mediated Internalization of Th-PN1 Complexes but Does Not Affect Heparin-mediated Cell Surface Binding—A rabbit polyclonal IgG was generated against Pro$^{47}$–Ile$^{58}$, and the total IgG fraction was purified on protein G-Sepharose and brought to a final concentration of 1.0 mg/ml in PBS. To test the effect of this IgG on Th-PN1 complex degradation, $125^1$-Th-PN1 complexes were formed and pre-incubated with various dilutions of the antibody for 30 min at 37 °C. The complexes were then added to cells for 3 h at 37 °C, and release of trichloroacetic acid soluble radioactivity into the incubation medium was measured. A 1:10 dilution of the IgG, complex degradation was inhibited approximately 70% (data not shown). Because the peptide sequence, Pro$^{47}$–Ile$^{58}$, lies in close proximity to the heparin-binding site, additional experiments were done to determine whether the decrease in complex degradation was due to inhibiting the LRP-binding site in the complexes or to a steric hindrance of heparin binding. Inhibition of heparin binding could also account for the lower degradation rate in the presence of the antibody, because we have previously shown that a heparin binding deficient variant of PN1 in complex with thrombin is degraded very slowly when compared with native PN1 (15). Shown in Fig. 1 are parallel experiments done at 4 and 37 °C, where we simultaneously measured the effect of anti-(Pro$^{47}$–Ile$^{58}$) IgG on both the degradation and cell association of Th-PN1 complexes. Relative to cultures that received only $125^1$-Th-PN1 complexes or a control pre-immune IgG, experimental cultures that received anti-(Pro$^{47}$–Ile$^{58}$) IgG degraded significantly lower amounts of complexes during a 3-h incubation at 37 °C, approximately 70% less when compared with the complex only controls (Fig. 1A). As expected, degradation was reduced by 80% in the presence of the LRP agonist, RAP-GST. Insignificant quantities of trichloroacetic acid soluble radioactivity were generated at 4 °C, demonstrating that complex degradation is dependent on endocytosis and occurs intracellularly. In contrast, to the marked effect that anti-(Pro$^{47}$–Ile$^{58}$) IgG had on complex degradation, it had no effect on total cell surface binding of the Th-PN1 complexes at 4 °C or on total cell association of the complexes at 37 °C (Fig. 1B). These data indicate that the inhibitory effect of anti-(Pro$^{47}$–Ile$^{58}$) IgG on Th-PN1 complex degradation is due to the specific inhibition of binding of the complexes to the LRP and that the
binding of complexes to cell surface heparins occurs via a structural determinant that is distinct from the heparin-binding site.

To rule out the possibility that these data might be an artifact of antibody bivalency or the possibility that these complexes may be associated with another cell surface component in the presence of the antibody, we next directly demonstrated that the complexes are indeed bound to cell surface heparins in the presence of anti-(Pro47–Ile58) IgG. The 4 °C binding experiment shown in Fig. 1B was repeated, this time using complex samples. B, remaining culture supernatants were removed by aspiration, and the cells were then washed four times with PBS. The cell monolayers were solubilized in 1 ml of 10% SDS, and radioactivity was quantified by $^\gamma$ counting. Error bars indicate the standard deviation from the mean of triplicate samples in both panels.

Characterization of PN1 Variants with Alanine Substitutions at His48 and Asp49—Both of the PN1 variants, PN1(H48A) and PN1(H48A,D49A), were expressed in Sf9 insect cells under control of the polyhedron promoter in baculovirus and purified using Cibacron blue-Sepharose as described previously (20). Native PN1 was purified from the serum-free conditioned medium harvested from HF cells using heparin-Sepharose affinity chromatography (8). Active site titrations using thrombin in a chromogenic substrate assay were performed on both native PN1 and each of the variants. Based on the active site titrations and the actual protein concentration, the percentage of active protein was calculated (Table I). Very little difference in the activity of the protein preparations was measured. In fact, PN1(H48A) and PN1(H48A,D49A) were 74 and 76% active, respectively, which was slightly higher than native human fibroblast PN1 that displayed 70% activity. Using the activity measurements, the concentrations of each of the PN1 variants and native PN1 were then adjusted to equivalent active concentrations, and the $k_{assoc}$ were determined for each of the forms of PN1 as described previously (24). For each variant of PN1, the $k_{assoc}$ constants for thrombin were found to be similar to the $k_{assoc}$ constant of native PN1 for thrombin. The PN1(H48A) variant had the highest measured $k_{assoc}$ (6.8 × 10$^{-5}$ M$^{-1}$ s$^{-1}$), but the PN1(H48A,D49A) variant and native
PN1 displayed very similar $k_{\text{assoc}}$ values (5.7 $\times$ $10^{-6}$ M$^{-1}$ s$^{-1}$ and 6.3 $\times$ $10^{-6}$ M$^{-1}$ s$^{-1}$, respectively).

In addition to the $k_{\text{assoc}}$ constant and activity analyses presented above, which argue strongly that the introduced substitutions do not significantly affect the biochemical activities of PN1, we also examined the ability of the variant forms of PN1 to form complexes stable to SDS-PAGE with $^{125}$I-thrombin. The characterization of the complex formation was critical, because any variation in the capacity of the PN1 variants to form stable complexes with thrombin due to the introduced substitutions could have a major effect on their LRP-mediated catabolism. Human recombinant native PN1, purified from Sf9 cells by the same method as each variant of PN1, was used for comparison. Shown in Fig. 3 is a digitized image of complexes formed between each form of PN1 and $^{125}$I-thrombin. In each lane where recombinant native PN1 or a variant form of PN1 was present, greater than 90% of the thrombin appeared in complex with the PN1 or PN1 variant, demonstrating that each of the variants formed stable complexes with $^{125}$I-thrombin at levels comparable with the recombinant native PN1. Note that some $^{125}$I-thrombin becomes inactivated and unable to form complexes, probably due to oxidation during the radioiodination procedure, but all lanes showed a similar low level of this nonreactive thrombin. Cumulatively, the close similarity in activity, $k_{\text{assoc}}$ constants for thrombin, and ability to form SDS-resistant high molecular weight complexes with $^{125}$I-thrombin, argue that the alanine substitutions introduced at the positions of His$^48$ and Asp$^49$ do not affect the stability nor thrombin inhibitory activity of either of the PN1 variants.

The Heparin-mediated Binding of $^{125}$I-Th-PN1 Variant Complexes to the Cell Surface Is Identical to Native $^{125}$I-Th-PN1 Complexes—In recent studies (15), we have shown that the binding of Th-PN1 complexes to cell surface heparins acts synergistically to enhance LRP-mediated internalization. Heparin binding plays a similar role in the catabolism of thrombomodulin 1 (25) and lipoprotein lipase (26). The aim of our current studies was to measure the ability of the PN1 variant complexes to bind to cell surface heparins, because this could also significantly affect catabolism rates. Confluent cultures of HEK293 cells in 24-well plates were incubated with 200 ng/ml of $^{125}$I-Th-PN1 (native) complexes (■), $^{125}$I-Th-PN1(H48A) complexes (○), or $^{125}$I-Th-PN1(H48A,D49A) complexes (△) in the presence and absence of the indicated concentrations of soluble heparin. The incubations were allowed to reach equilibrium at 4 °C (4 h), at which time the unbound complexes were removed, and wells were washed four times with cold PBS. The cell monolayers were lysed with 10% SDS and quantified by $^{125}$I-thrombin counting.

**TABLE I**

| Protein concentration Activity $k_{\text{assoc}}$ $10^5$ | % | | |
|-----------------|----|---|---|
| $^{125}$I-Th-PN1 Complexes Bound (pmoles) | | | |
| $^{125}$I-Th-PN1 (native) | 120.7 | 70 | 5.71 |
| $^{125}$I-Th-PN1(H48A) | 25.6 | 76 | 6.83 |
| $^{125}$I-Th-PN1(H48A,D49A) | 23.1 | 74 | 6.35 |

**FIG. 3.** Recombinant native PN1 and the variants PN1(H48A) and PN1(H48A,D49A) form complexes with $^{125}$I-thrombin that are stable to SDS-PAGE. 600 ng of $^{125}$I-thrombin were incubated with amounts of native PN1 and each of the recombinant variants required to achieve complete thrombin inactivation within 30 min at 37 °C in a final volume of 300 µl. At the end of the incubation, 5-µl aliquots of the reactions were removed and added to 15 µl of SDS-PAGE sample buffer. The reactions were analyzed by SDS-PAGE on 10% polyacrylamide gels to resolve free $^{125}$I-Th from $^{125}$I-Th-PN1 complexes. After drying, the gel was exposed to a Bio-Rad Phospho-Imager screen for 30 min. The digitized image was developed on a Bio-Rad GS-250 Molecular Imager.

**FIG. 4.** Substitutions of Ala at His$^48$ and Asp$^49$ do not affect the cell surface heparin binding of $^{125}$I-Th-PN1 complexes. Confluent cell cultures in 24-well plates were incubated with 200 ng/ml of $^{125}$I-Th-PN1 (native) complexes (■), $^{125}$I-Th-PN1(H48A) complexes (○), or $^{125}$I-Th-PN1(H48A,D49A) complexes (△) in the presence and absence of the indicated concentrations of soluble heparin. The incubations were allowed to reach equilibrium at 4 °C (4 h), at which time the unbound complexes were removed, and wells were washed four times with cold PBS. The cell monolayers were lysed with 10% SDS and quantified by $^{125}$I-thrombin counting.

these results we determined that any change observed in the rate of catabolism was not due to a difference in the initial binding interaction of these PN1 variant complexes to cell surface heparins.

Alanine Substitutions at His$^48$ and Asp$^49$ Markedly Impair Th-PN1 Complex Catabolism—Based on our recent studies, which identified the peptide sequence $^{47}$PHDNIVISPHGI$^{58}$ as a putative binding site in PN1 required for LRP-mediated
The degradation of $^{125}$I-Th-PN1(H48A) and $^{125}$I-Th-PN1(H48A,D49A) complexes by HF cells is markedly reduced. HF cells were incubated with 200 ng/ml of $^{125}$I-Th-PN1 (native) complexes, $^{125}$I-Th-PN1(H48A) complexes, or $^{125}$I-Th-PN1(H48A,D49A) complexes at 37 °C in binding medium. At the indicated time points, aliquots of triplicate samples were analyzed for the appearance of trichloroacetic acid nonprecipitable radioactivity. Error bars represent the standard deviation from the mean.

Internalization and catabolism of $^{125}$I-Th-PN1 complexes, we next evaluated the effect of alanine substitutions as His$^{48}$ and Asp$^{49}$ on $^{125}$I-Th-PN1 catabolism. $^{125}$I-Thrombin-PN1 complexes were formed from each variant of PN1 and from native PN1 and used at a final concentration of 200 ng/ml in binding medium as described under “Experimental Procedures.” Complexes were added to confluent HF cell cultures in 24-well plates in triplicate. At the indicated time points, trichloroacetic acid nonprecipitable radioactivity in the media, which correspond to $^{125}$I-tyrosine released from lysosomal degradation of the radiodinated thrombin (22) was determined (Fig. 5). Relative to native $^{125}$I-Th-PN1 complexes, the $^{125}$I-Th-PN1(H48A) variant complexes were degraded at about a 50% slower rate. $^{125}$I-Th-PN1(H48A,D49A) variant complexes, in which the PN1 has an additional replacement of Asp$^{49}$ with Ala, reduced the rate of degradation to about 15% of native $^{125}$I-Th-PN1 complexes. The control experiments presented in Figs. 3 and 4 and in Table I demonstrate that this differential rate of degradation is not due to a difference in PN1 cell surface binding activity, complex stability, or a differential capacity to bind to cell surface heparins. Therefore, these data would strongly suggest that there is an altered interaction of the PN1 variant complexes with their receptor, LRP, which is required for the internalization of the complexes prior to intracellular degradation.

The $^{125}$I-Th-PN1(H48A) and $^{125}$I-Th-PN1(H48A,D49A) Complexes Are Sharply Lowered—The results shown in Fig. 5 measure the intracellular degradation of native and variant $^{125}$I-Th-PN1 complexes as judged by an increase in trichloroacetic acid soluble radioactivity. The diminished degradation rates of $^{125}$I-Th-PN1(H48A) and $^{125}$I-Th-PN1(H48A,D49A) suggest an impaired interaction with the LRP but do not demonstrate this directly. To address this, we measured the internalization rates of $^{125}$I-Th-PN1(H48A) and $^{125}$I-Th-PN1(H48A,D49A) relative to native $^{125}$I-Th-PN1 complexes (Fig. 6). Confluent cultures of HF cells in 24-well plates were incubated with native $^{125}$I-Th-PN1 complexes, $^{125}$I-Th-PN1(H48A) and $^{125}$I-Th-PN1(H48A,D49A), each at a concentration of 200 ng/ml at 37 °C. At the indicated times, ranging from 5 to 30 min, triplicates wells were rapidly chilled to 4 °C and rinsed four times with PBS to remove unbound complexes. Cell surface bound complexes were stripped at 4 °C with a solution of EDTA and heparin as described previously (8). The remaining monolayers were solubilized in 10% SDS to determine the amount of internalized complexes. In both cases the radioactivity was quantified by γ counting. Error bars represent the standard deviation from the mean.

**Discussion**

The present studies were undertaken to further probe the potential role of the PN1 peptide sequence, Pro$^{47}$–Ile$^{58}$, in the LRP-mediated clearance of Th-PN1 complexes and to determine what structural features of this sequence might be important in this process. The potential importance of this sequence was discovered using a synthetic peptide library of PN1 sequences and screening for peptides that inhibited the catabolism of complexes by HF cells (10). The requirement for heparin-mediated binding of the complexes to the cell surface to promote an efficient interaction between the complexes and the LRP was precluded more detailed studies of this sequence using a synthetic peptide approach, because the sites in PN1 that interact with heparin and the LRP are distinct. In the present studies, two different approaches have been used to more clearly define the role of the structural determinant, Pro$^{47}$–Ile$^{58}$, in Th-PN1 complex catabolism and to identify critical residues within this determinant.
To independently confirm and more narrowly define the role of this structural determinant in Th-PN1 catabolism, an anti-(Pro$^{47}$–Ile$^{58}$) polyclonal IgG was generated. This antibody specifically inhibited the internalization and subsequent degradation of Th-PN1 complexes but had no effect on the binding of complexes to cell surface heparins. These data independently confirm the role of Pro$^{47}$–Ile$^{58}$ in LRP-mediated internalization, which had previously been based solely on synthetic peptide competition studies (10). In addition, they strongly suggest that the LRP and heparin-binding determinants in PN1 are structurally distinct, despite the fact that they act cooperatively to promote efficient complex catabolism (15).

In addition to the antibody studies, a genetic approach was used in which alanine substitutions were introduced at positions His$^{48}$ and Asp$^{49}$ of the intact PN1 protein. The rationale for these choices is derived from the predicted structural location of 47PHDNIVISPHGI$^{58}$ in PN1 based on its homology to α-1-antitrypsin (1). This sequence is predicted to be at least partially interior to the protein surface. Pro$^{47}$ most likely represents the beginning of the transition sequence between helix A and strand 6B(1). His$^{48}$ and Asp$^{49}$ are transition amino acids, and Asp$^{49}$ through Pro$^{55}$ become strand 6 of sheet B. Given this structural information, we hypothesized that the charged residues, His$^{48}$ and Asp$^{49}$, might be important for either direct interaction with the LRP receptor or assisting in the attainment of the active conformation of this structural determinant when fully exposed to the hydrophilic exterior of the molecule.

To evaluate the role of these specific amino acids we generated two different PN1 variants: one in which only His$^{48}$ was replaced by Ala and another where both His$^{48}$ and Asp$^{49}$ were replaced by Ala. Replacement of the first charged residue, His$^{48}$ by Ala, had a significant effect on the catabolism of PN1(H48A) complexes, reducing it by 50%. The additional substitution of Ala for Asp$^{49}$, reduced complex catabolism by 85%. In both cases this was demonstrated to be due to a decreased rate of LRP-mediated internalization. Control studies revealed that the substitutions had no effect on the heparin-mediated binding of the complexes to the cell surface nor on the biochemical characteristics of the PN1 variant complexes with thrombin. Taken together, it is clear that both His$^{48}$ and Asp$^{49}$ play very important roles in the interaction of the PN1 structural determinant, 47PHDNIVISPHGI$^{58}$, with the LRP. Whether this is due to a direct interaction of these residues with the LRP or because these residues are required to establish a particular structural conformation of this determinant is presently unknown. Given the overall hydrophobic character of Pro$^{47}$–Ile$^{58}$ (PHDNIVISPHGI), it is likely that removal of the charged residues affects the solubility of the sequence. The genetic evidence using PN1 variants with point substitutions and the anti-(Pro$^{47}$–Ile$^{58}$), antibody experiments represent two important and independent lines of evidence that support and extend the original observation that the sequence Pro$^{47}$–Ile$^{58}$ in PN1 is required for the LRP-mediated clearance of Th-PN1 complexes.

A common pathway for many LRP internalized ligands seems to be emerging that involves cell surface proteoglycans and perhaps other accessory proteins in many cases. Several of the ligands first bind to cell surface heparin sulfate proteoglycans and are subsequently internalized by the LRP (15, 25–27). Even Th-ATIII complexes, which display a negligible affinity for heparin, use hepatic heparin sulfate proteoglycans as a part of their clearance mechanism but do so by an association with vitronectin (Vn) (14). Th-ATIII complexes first bind to Vn and then utilize the heparin-binding site in Vn, which is exposed only after it binds to Th-ATIII complexes (14). Recently, another accessory molecule, cytokeratin 18, has been shown to play an important role in the clearance of Th-ATIII-Vn ternary complexes (13). Antibodies specific for cytokeratin 18 were shown to markedly reduce the LRP-mediated internalization of Th-ATIII-Vn ternary complexes. Although the heparin-mediated pathway appears to be common for many LRP internalized ligands, there is at least one example where the involvement of heparin has not yet been documented (27). The uPA receptor binds complexes between high molecular weight urokinase and plasminogen activator inhibitor 1 or PN1 prior to LRP-mediated internalization (27). There are data suggesting that the uPA receptor, as well as its bound ligand, is co-internalized along with the LRP (16). Because plasminogen activator inhibitor 1 does contain a heparin-binding site, however, the potential involvement of heparin in this pathway should be examined more carefully.

Structural information on binding sites in ligands that interact with the LRP is, however, limited. The highest resolution studies have been done on the LRP-binding site in activated α2-macroglobulin, which identified lysine residues 1370 and 1374 as essential for binding to the LRP (28). The LRP-binding site in thrombospondin 1 has been localized to the same amino-terminal fragment that contains the heparin-binding domain (25), and the carboxyl-terminal folding domain of lipoprotein lipase, which also contains the heparin-binding domain, has been implicated in binding to the LRP (26). Most recently, studies on the binding of plasminogen activator inhibitor 1-protease complexes to the LRP have implicated two residues located in the heparin-binding site in LRP binding (27). These data may be subject to alternate interpretations, because the opposite charge nature of the amino acid substitutions in plasminogen activator inhibitor 1 variants that resulted in a decreased LRP affinity may impart structural changes in the general region that are not necessarily part of the heparin-binding site (27). In addition, data supporting any type of a universal structural motif shared by all LRP ligands are not very compelling, because the ligands are diverse, and many of the ligands do not cross-compete for binding (28). Although the binding of several LRP ligands to cell surface heparins has been shown to play an important role in their clearance by the LRP, it remains to be determined whether the heparin and the LRP-binding sites overlap in some cases. The data in the present report, along with previously published studies using synthetic peptides strongly argue against this in the case of PN1.

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Analysis of PN1-binding Site for LRP

281

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