Roles of the Heparin and Low Density Lipid Receptor-related Protein-binding Sites of Protease Nexin 1 (PN1) in Urokinase-PN1 Complex Catabolism

The PN1 heparin-binding site mediates complex retention and degradation but not cell surface binding or internalization.

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We have previously described thrombin (Th)-protease nexin 1 (PN1) inhibitory complex binding to cell surface heparins and subsequent low density lipoprotein receptor-related protein (LRP)-mediated internalization. Our present studies examine the catabolism of urinary plasminogen activator (uPA)-PN1 inhibitory complexes, which, unlike Th-PN1 complexes, bind almost exclusively through the uPA receptor. In addition, the binding site in PN1 required for the LRP-mediated internalization of Th-PN1 complexes is not required for the LRP-mediated internalization of uPA-PN1 complexes. Thus, the protease moeity of the complex partially determines the mechanistic route of entry. Because cell surface heparins are only minimally involved in the binding and internalization of uPA-PN1 complexes, we then predicted that complexes between uPA and the heparin binding-deficient PN1 variant, PN1(K7E), should be catastabolized at the same rate as complexes formed with native PN1. Surprisingly, the uPA-PN1(K7E) complexes were degraded at only a fraction of the rate of native complexes. Internalization studies revealed that both uPA-PN1(K7E) and native uPA-PN1 complexes were initially internalized at the same rate, but uPA-PN1(K7E) complexes were rapidly retro-endocytosed in an intact form. By examining the pH dependence of complex binding in the range of 4.0–7.0, it was determined that the uPA-PN1 inhibitory complexes must specifically bind to endosomal heparins at pH 5.5 to be retained and sorted to lysosomes. These studies are the first to document a role for heparins in the catabolism of SERPIN-protease complexes at a point further in the pathway than cell surface binding, and this role may extend to other heparin-binding LRP-internalized ligands.

Human protease nexin 1 (PN1)‡ is a 43-kDa member of the SERPIN family of protease inhibitors (1). PN1 is a potent inhibitor of thrombin (Th) and urinary plasminogen activator (uPA) (2) and is a less potent but still effective inhibitor of plasmin and trypsin. Although the precise molecular nature of the PN1-protease inhibitory complexes under native conditions remains controversial, it is most likely a 1:1 covalently linked stoichiometric complex or is at least very stable under physiological conditions (3–5). Complexes formed between PN1 and all of the proteases that it inhibits are stable to SDS-PAGE after boiling and reduction (6). Interestingly, in the presence of heparin, PN1 is a faster thrombin inhibitor than heparin-activated antithrombin III (ATIII), the most physiologically important inhibitor of Th in plasma (2). However, because of the physiological distribution of PN1, which is restricted primarily to tissues with the exception of a low abundance form in platelets (7), PN1 probably inactivates extravasated thrombin and does not participate directly in the fibrinolytic system. Because of the limited range of specificity of PN1 and the nature of the proteases it inhibits, PN1 has been implicated in neurite outgrowth (8), the protection of neuronal cells from proteolytic damage and thrombin-induced apoptosis in brain injury (9–11), and the maintenance of neuromuscular junctions (11).

An integral aspect of PN1 physiology is the catabolism of PN1-protease inhibitory complexes, because the only way to ensure the removal of protease activity is through endocytosis and intracellular degradation. In addition, the endocytosis of inhibitory complexes may act as a feedback signaling mechanism to regulate inhibitor synthesis and secretion (12). Recent progress has revealed that the low density lipoprotein receptor-related protein (LRP) is responsible for the cellular internalization of PN1-protease (12–15). In the case of Th-PN1 complexes, LRP binding is regulated by the exposure of a cryptic binding site that is rendered LRP binding-competent only when PN1 is in complex with a protease (16). Using a synthetic peptide library, this binding site has been narrowed to a 12-amino acid sequence, 43PHDNIVISPHGI96 (13). This is a predicted transition sequence between the A helix and strand 6 of sheet B in PN1, based on the projected structure of PN1 using α-1-antitrypsin coordinates as a model (1). More recent studies using an antibody directed against this sequence, as well as site-directed variants of PN1 with point, isosteric amino acid substitutions in this sequence, have confirmed this region as a structural determinant required for the LRP-mediated internalization of Th-PN1 complexes (17). Interestingly, the role of this sequence is limited to complex internalization by the LRP. The majority of the cellular binding of Th-PN1 complexes,
which plays a substantial role in the overall rate of catabolism of the complexes, is mediated by the heparin-binding site in PN1 and its interactions with cell surface heparins or heparan sulfate proteoglycans (14).

The catabolism of uPA-PN1 complexes is also mediated by the LRP but reportedly proceeds through a pathway that utilizes the uPA receptor (uPAR) (15, 18). The apparent mechanism is a sequential series of events that is initiated by the binding of uPA-PN1 complexes to uPAR, followed by LRP-mediated internalization as a ternary complex (18). Because the association constant of uPA-PN1 complexes for the uPAR is much greater than the association constant of uPA mediated internalization as a ternary complex (18). Because the association constant of uPA-PN1 complexes for heparin, this raises the question of the role of the heparin-binding site in PN1 in the catabolism of uPA-PN1 complexes. It also raises the question of the role of the LRP-binding site in PN1 in the catabolism of uPA-PN1 complexes. These questions have particular biological relevance for three reasons. First, the catabolic route of PN1-protease complexes may be determined by the identity of the protease present in the inhibitory complex. If indeed two different pathways exist, the pathway used may affect the cellular response to these inhibitory complexes and may augment the up- or down-regulation of inhibitor synthesis and secretion (12). Second, uPAR occupied by uPA has been shown to localize to the leading edge of migrating smooth muscle cells in a wound-healing model. The uPA-uPAR complexes at the cell surface activate the JAK/STAT signaling pathway, and removal of the complexes by LRP-mediated internalization deactivates the pathway (19). Thus, PN1 has the potential to regulate this signaling pathway, depending on the entry route of uPA-PN1 complexes into the cell. Third, little is known about the clearance mechanism of SERPIN-protease inhibitory complexes in general. Based on several reports from one group, it was initially believed that all SERPIN-enzyme complexes were cleared by a common receptor, referred to as the SERPIN-enzyme complex receptor or SEC receptor (20, 21). This receptor was thought to recognize a common structural motif in the carboxyl-terminal region of all of the SERPINs. This original hypothesis has not held true (22), and there are now several published reports showing that the LRP acts as the endocytosis receptor for nearly all of the SERPINs examined (13–15, 18, 20, 23, 24). It has also been determined that the structural site responsible for binding to LRP does not reside within the carboxyl-terminal domain (13, 17).

In the present studies we have investigated the catabolic pathways of Th-PN1 complexes and uPA-PN1 complexes using a polyclonal antibody raised against the LRP-binding site in PN1 (13, 17) and a heparin binding-deficient variant of PN1 that forms inhibitory complexes with Th and uPA but is unable to bind to cell surface heparins (14). The results of these studies demonstrate that the catabolism of uPA-PN1 complexes and Th-PN1 complexes do indeed proceed through two different pathways that converge at the point of the LRP. The cell surface binding of uPA-PN1 complexes is heparin-independent and internalization does not involve the LRP-binding site in PN1. In contrast, the cell surface binding of Th-PN1 complexes is mediated by heparins, and the internalization of Th-PN1 complexes is dependent on the LRP-binding site in PN1. We have also made the unexpected observation that although the heparin-binding site in PN1 is not involved in the cell surface-binding of uPA-PN1 complexes, it is apparently required for the endosomal retention of uPA-PN1 complexes after endocytosis.

**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, and high molecular weight urokinase (uPA), 80,000 IU/mg, were purchased from Calbiochem. Na125Iodine was from Amersham Pharmacia Biotech. Porcine mucosal heparin was from Calbiochem. Soybean trypsin inhibitor and monensin were from Sigma. Protein G-Sepharose beads, high trap heparin-Sepharose and Chaceron blue-Sepharose were all from Amersham Pharmacia Biotech. All other chemicals were of analytical grade.

**Protein Radioiodination**—125I-Thrombin (16) and 125I-uPA (27) were prepared as described previously using the Iodogen method. Specific activities for 125I-thrombin ranged from 8,000 to 15,000 cpm/ng of protein. Specific activities for the 125I-uPA ranged from 6,000 to 15,000 cpm/ng of protein.

**Complex Formation and Analysis**—Complexes were formed by combining a known amount of 125I-Thrombin or 125I-uPA with a 3-fold molar excess of either PN1 or PN1(K7E), which were then diluted to a final total volume of 80 μl in PBS, pH 7.2, containing 0.1% BSA. At the end of a 30-min incubation at 37 °C, the reactions were chilled on ice for 15 min, followed by the addition of a 200-fold molar excess of Chromozym-Th. 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.

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**Complex Formation and Analysis**—Complexes were formed by combining a known amount of 125I-Thrombin or 125I-uPA with a 3-fold molar excess of either PN1 or PN1(K7E) as determined by the active site titration described above. Reactions were carried out in 300 μl of PBS. At the end of a 30-min incubation at 37 °C, the reactions were diluted with binding medium or buffer to the appropriate concentration, typically 100–200 ng/ml. After dilution, 5-μl aliquots were removed and added to 200-μl volumes of SDS-PAGE sample buffer and analyzed by SDS-PAGE on 10% polyacrylamide gels (28). 2-μl aliquots of the complexes were also collected and quantified by gamma counting to ensure that equal concentrations of complexes were added to cells.

**Cell Binding, Internalization, and Degradation Assays**—Binding and internalization experiments were done in binding medium that consisted of serum-free, bicarbonate-free Dulbecco’s modified Eagle’s medium containing 20 mM Hapes buffer, pH 7.2, and 0.1% BSA. When binding assays were done at 4 °C, all reagents were prechilled, and the cells were placed on ice in a 4 °C cold room. Competing ligands were added simultaneously with radiolabeled ligands. Concentrations of ligands are indicated in the text and figure legends. At the completion of the incubations, unbound ligand was removed, and the cells were washed rapidly four times with 1 ml of PBS, and finally lysed with 1 ml of 10% SDS. Radioactivity in the samples was quantified by gamma counting. Nonspecific binding, which accounts for approximately 25% of total binding, was determined by incubating three wells with a 400-fold excess of unlabeled complexes. Degradation assays were done by monitoring increases in trichloroacetic acid precipitable radioactivity as described previously (13, 14).

**Internalization/surface-associated complex assays** were done in the same binding medium described above. 300-μl aliquots of complexes were added to triplicate wells at 37 °C. At the indicated times the cells were rapidly chilled to 4 °C and washed to remove free ligand. 1 ml of ice-cold trypsin solution in PBS (200 μg/ml) was then added to the cells on ice and allowed to proteolytically digest cell surface proteins for 10 min. The trypsinated cells were transferred to microcentrifuge tubes containing 200 μl of ice-cold soybean trypsin inhibitor at 5 mg/ml. Following centrifugation at 10,000 × g for 3 min, the supernatants and cells were quantified separately by gamma counting to discriminate between cell surface-associated and internalized complexes. The trypsination efficiency was determined to be 90% in control experiments where endocytosis was inhibited by incubation at 4 °C, and the ability of trypsin to release cell surface-bound complexes was tested.
To evaluate the binding of uPA-PN1 complexes at the cell surface, we used a cell line, HF cells, which have a high affinity for uPA due to the expression of PN1 receptors.

Immunoprecipitation—

To detect complexes of uPA-PN1, we used immunoprecipitation with anti-(Pro 47–Ile58-Cys) IgG or preimmune IgG overnight at 4 °C. The complexes were then precipitated with protein A-Sepharose and analyzed by SDS-PAGE on a 10% polyacrylamide gel. BSA was added to a final concentration of 0.1% prior to titration. Complexes were diluted into each of the buffers and incubated with the cells at 4 °C for 2 h.

RESULTS

The Heparin-binding Site in PN1 Does Not Play a Significant Role in the Cell Surface Binding of uPA-PN1 Complexes but Is Required for Their Efficient Catabolism—

The ability to bind heparin after SERPIN-protease complex formation is unique to PN1 (29) and plasminogen activator inhibitor-1 (30). Other heparin-binding SERPINs (such as ATIII) lose affinity for heparin after complex formation with a protease (31). This heparin binding capacity is an important feature of ThPN1 complex catabolism because it provides cells with a mechanism for concentrating ThPN1 complexes at the cell surface for subsequent internalization via the LRP (14). In recent studies we have carefully characterized the role of the PN1 heparin-binding site in Th-PN1 complex catabolism because it provides cells with a mechanism for concentrating Th-PN1 complexes at the cell surface for subsequent internalization via the LRP (14). We have previously generated and characterized a heparin-binding-deficient variant of PN1, designated PN1(K7E). PN1(K7E) reacts with thrombin and forms complexes in a manner indistinguishable from native PN1 and also binds to the catabolic pathway separate from its role in cell surface binding.

We have previously generated and characterized a heparin binding-deficient variant of PN1, designated PN1(K7E). PN1(K7E) reacts with thrombin and forms complexes in a manner indistinguishable from native PN1 and also binds to the LRP (14). The heparin-binding site in Th-PN1 complex catabolism has not been previously addressed. The fact that uPA-PN1 complexes bind to the cell surface primarily through the uPAR afforded us the unique opportunity to examine any role for the heparin-binding site in the catabolic pathway separate from its role in cell surface binding.

We have previously generated and characterized a heparin binding-deficient variant of PN1, designated PN1(K7E). PN1(K7E) reacts with thrombin and forms complexes in a manner indistinguishable from native PN1 and also binds to the LRP (14). Because this variant had not been examined for its interaction with uPA, however, its ability to form complexes with uPA was first established (Fig. 1A). Complexes of 125I-uPA
with either active PN1 and PN1(K7E) were prepared for addition to HF cells as described in the figure legend. Aliquots of 10 μl were removed and resolved by SDS-PAGE on 10% polyacrylamide gels. In the digitized image, the position of free 125I-uPA is indicated as well as the position of 125I-uPA-PN1 and 125I-uPA-PN1(K7E) complexes. Importantly, the K7E variant of PN1 formed complexes with 125I-uPA as well as native PN1, and there were only trace amounts of free 125I-uPA present in both the variant and native complexes. This is an important point, because free 125I-uPA is also able to bind to the uPAR and could lead to misleading conclusions if present in high concentrations.

These same radiolabeled complexes were then added to HF cells in binding medium at 4 °C to evaluate cell surface binding. At the end of a 3-h incubation at 4 °C, the HF cultures were washed to remove unbound ligand, lysed with 10% SDS and quantified by γ counting (Fig. 1B). As shown by the control bars, nearly identical amounts (approximately 17 fmol) of variant 125I-uPA-PN1(K7E) and native 125I-uPA-PN1 complexes bound to the cell surface. This contrasts to previous studies of PN1(K7E) in complex with a different target protease, thrombin (Th), where the cell surface binding of these variant complexes was shown to be 10-fold lower than native Th-PN1 complexes (14). Cell surface binding of the native Th-PN1 complexes was shown in the same studies to be mediated primarily by cell surface heparins, accounting for the dramatically lowered binding of the heparin-binding-deficient Th-PN1(K7E) complexes.

Unlike thrombin, uPA binds with high affinity to the uPAR present on the cell surface, and native uPA-PN1 complexes have been previously shown to bind via the uPAR (15). To demonstrate that cell surface binding of our variant uPA-PN1(K7E) complexes also proceeded via high affinity binding of the uPA moiety of the complex to the uPAR, we tested the ability of a peptide that specifically binds uPAR to compete for cell surface binding. Peptide AE78, derived from the amino-PN1(K7E) complexes also proceeded via high affinity binding site (Th), where the cell surface binding of these variant complexes was shown to be 10-fold lower than native Th-PN1 complexes (14). Cell surface binding of the native Th-PN1 complexes was shown in the same studies to be mediated primarily by cell surface heparins, accounting for the dramatically lowered binding of the heparin-binding-deficient Th-PN1(K7E) complexes.

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Measuring no significant decrease in cell surface binding of the variant uPA-PN1(K7E) complexes when the uPAR was available, we predicted that degradation of these heparin binding-deficient complexes would also be similar to that of native uPA-PN1 complexes. Aliquots of media taken from HF cells incubated at 37 °C at 2 and 4 h were subjected to trichloroacetic acid precipitation to measure degradation. Unexpectedly, the degradation rate of 125I-uPA-PN1(K7E) complexes was approximately 3-fold lower than native 125I-uPA-PN1 complexes (Fig. 1C). The data in Fig. 1 (A and B) show that this surprising result is not due to an artifact of inefficient complex formation or cell surface binding and suggested a novel role for the heparin-binding site in complex catabolism.

The LRP-mediated Internalization of uPA-PN1 Complexes Proceeds by a Mechanism That Does Not Require the LRP-binding Site in PN1 (Pro47–Ile58)—uPA-PN1 and Th-PN1 complexes are both internalized by the LRP, and this appears to be a point of convergence in two separate pathways (14, 15, 18). uPA-PN1 complexes first bind directly to the uPAR and are subsequently internalized as part of a uPA-PN1-LRP ternary complex (15, 18). In contrast, Th-PN1 complexes first bind to cell surface heparins and are subsequently internalized by the LRP without uPAR involvement (14). Whether the Th-PN1 complexes are “transferred” to the LRP or internalized as a Th-PN1-heparin-LRP quaternary complex is not known. Based on our unexpected finding of a potential role for heparin in the catabolism of uPA-PN1 complexes, we next investigated whether the LRP-binding site in PN1, which was identified in PN1 complexed with thrombin, also participated in uPA-PN1 catabolism.

We recently developed a polyclonal antibody that inhibits the LRP-mediated internalization of Th-PN1 complexes (17). It is a synthetic peptide directed antibody generated against the PN1-derived sequence, 47PHDINIIPSHGF85, a peptide previously shown to inhibit Th-PN1 complex internalization (14). Previous studies with this antibody characterized its effect on Th-PN1 complexes, so we first wanted to determine the relative accessibility of 47PHDINIIPSHGF85 of the immune IgG in uPA-PN1 and Th-PN1 complexes. Factors such as a difference in conformation, inaccessibility of this site to uPA or differential exposure of the site after complex formation could all affect the ability of the antibody to bind to the LRP-binding site of PN1. To test this, the ability of the anti-LRP-binding site IgG to immunoprecipitate 125I-Th-PN1 and 125I-uPA-PN1 complexes was evaluated over a broad concentration range (Fig. 2A). Shown is a digitized image of the samples after resolution by SDS-PAGE. In the graph below the image, band densities were measured and normalized to the respective specific activities of the radioiodinated species. At concentrations of complexes ranging from 10 to 200 ng/ml, there was no significant difference in immunoprecipitation efficiency. Only a slight difference was seen at the complex concentration of 500 ng/ml, but the next experiments were performed within the region where the efficiency of immunoprecipitation of the antibody was equivalent for both Th-PN1 and uPA-PN1 complexes.

To test the effect of the antibody on the catabolism of uPA-PN1 complexes, HF cells were incubated with either 125I-Th-PN1 or 125I-uPA-PN1 complexes at 37 °C for 3 h in the presence and absence of immune IgG (Fig. 2B). At the end of the incubation, aliquots of the media were removed and subjected to precipitation with 10% trichloroacetic acid. The appearance of trichloroacetic acid soluble radioactivity in this assay is a direct measurement of the appearance of low molecular weight degradation products (13, 14). Over the 3-h time course, approximately 45 fmol of 125I-Th-PN1 complexes were degraded, and the presence of anti-LRP-binding site IgG had no significant effect on the rate of degradation. 20 fmol of 125I-Th-PN1 complexes were degraded during the same time period. But in sharp contrast, the degradation of Th-PN1 complexes was inhibited 80% by the anti-LRP-binding site IgG. These data suggest that the LRP-binding site in PN1 is not
required when uPA-PN1 complexes are internalized by the LRP, because the immunoprecipitation experiment shown in Fig. 2A demonstrates equal accessibility of this site in both types of complexes. The simplest interpretation of these data is that uPA-PN1 complexes and LRP interact with the PN1-binding site IgG (open bars) or preimmune IgG (closed bars) and 100 ng/ml of 125I-Th-PN1 or 125I-uPA-PN1 complexes. After the incubation, 100 μl of the cell culture supernatant was removed, and trichloroacetic acid soluble radioactivity was measured. Background trichloroacetic acid soluble radioactivity was subtracted from the mean of the triplicate samples. Each error bar indicates ± one standard deviation from the mean of the triplicate samples.

**Fig. 2.** The LRP-binding site of PN1 is required for the degradation of Th-PN1 complexes but not for uPA-PN1 complexes. A, complexes of 125I-Th-PN1 (top row) or 125I-uPA-PN1 (bottom row) at the indicated concentrations (ng/ml) were incubated with anti-LRP-binding site IgG at 4 °C overnight. Protein G-Sepharose beads were used to immunoprecipitate as described under "Experimental Procedures." The immunocomplexes were released from the beads and resolved by SDS-PAGE on 10% polyacrylamide gels. Shown is the digitized image after exposure of the fixed gels to a Phospho-Imager screen for 2 h. Because the specific activities of the 125I-Th (15,000 cpm/ng) and 125I-uPA (6,300 cpm/ng) differed, the density of each band was quantitated in pixel density units (PDU’s) and normalized. The graph shows the indicated concentration of complexes versus the normalized density of the corresponding band in the digitized image. Closed squares, 125I-Th-PN1; open squares, 125I-uPA-PN1. B, triplicate confluent cultures of HF cells in 24-well plates were incubated at 4 °C overnight. Protein G-Sepharose beads were used to immunoprecipitate as described under "Experimental Procedures." The immunocomplexes were released from the beads and resolved by SDS-PAGE on 10% polyacrylamide gels. Shown is the digitized image after exposure of the fixed gels to a Phospho-Imager screen for 2 h. Because the specific activities of the 125I-Th (15,000 cpm/ng) and 125I-uPA (6,300 cpm/ng) differed, the density of each band was quantitated in pixel density units (PDU’s) and normalized. The graph shows the indicated concentration of complexes versus the normalized density of the corresponding band in the digitized image. Closed squares, 125I-Th-PN1; open squares, 125I-uPA-PN1. B, triplicate confluent cultures of HF cells in 24-well plates were incubated for 3 h at 37 °C in the presence of anti-LRP-binding site IgG (open bars) or preimmune IgG (closed bars) and 100 ng/ml of 125I-Th-PN1 or 125I-uPA-PN1 complexes. After the incubation, 100 μl of the culture supernatant was removed, and trichloroacetic acid soluble radioactivity was measured. Background trichloroacetic acid soluble radioactivity was subtracted from the mean of the triplicate samples. Each error bar indicates ± one standard deviation from the mean of the triplicate samples.

**Fig. 3.** uPA-PN1 and uPA-PN1(K7E) complexes bind to the cell surface primarily via the uPAR-binding site of uPA. Triplicate confluent cultures of HF cells in 24-well plates were incubated at 4 °C with either 125I-uPA-PN1 (closed circles), 125I-uPA-PN1(K7E) (open circles), or 125I-Th-PN1 (closed squares) complexes. All of the complexes were at a final concentration of 100 ng/ml. After 3 h, excess unbound complexes were aspirated, and the cell monolayers were washed four times with ice-cold PBS. Binding medium was then added back to the cells in the presence (data shown) and absence (data not shown) of 200 nm soluble heparin. After the indicated times, medium was aspirated, and cells were washed four times with ice-cold PBS. Cell monolayers were solubilized in 1 ml of 10% SDS, and radioactivity was quantified by β counting. Each error bar indicates ± one standard deviation from the mean of the triplicate samples.

Soluble Heparin Does Not Significantly Dissociate Cell Surface-bound 125I-uPA-PN1 or 125I-uPA-PN1(K7E) Complexes—Because of the unexpected differential in the degradation rate of 125I-uPA-PN1 and 125I-uPA-PN1(K7E) complexes (Fig. 1C), the potential role of cell surface heparin in the binding of complexes was more rigorously examined. In previous studies we have shown that 125I-Th-PN1 complexes bind almost exclusively to cell surface heparins prior to LRP-mediated internalization (14, 17). In addition, because of the large number of cell surface heparins present, the Th-PN1 complexes do not dissociate significantly even when their concentration in the binding medium is effectively reduced to zero but are rapidly released by the addition of soluble heparin (14, 17). To more carefully determine whether any of the cell surface binding of uPA-PN1 complexes was heparin-mediated, 125I-uPA-PN1, 125I-uPA-PN1(K7E), and 125I-Th-PN1 complexes, each at 100 ng/ml, were bound to HF cells at 4 °C for 3 h. At the end of the incubation, the monolayers were rapidly washed four times and placed in binding media containing soluble heparin to measure dissociation (Fig. 3). In agreement with previously published data, 125I-Th-PN1 complexes were rapidly released by the addition of soluble heparin (14, 17). In contrast, 125I-uPA-PN1(K7E) heparin binding-deficient variant complexes were not released significantly, demonstrating that their binding to the cell surface is non-heparin-mediated. This is consistent with the data shown in Fig. 1B, where the cell surface binding of uPA-PN1(K7E) complexes was shown to occur primarily via the uPAR and was greatly decreased only when the uPAR was occupied by peptide AE78. In the case of native 125I-uPA-PN1 complexes, a small fraction (20%) was released by soluble heparin, suggesting that the majority of these complexes were bound to the uPAR as well. Consistent with Fig. 1B again, these native complexes can also use the heparin-binding site of PN1 as an alternative and therefore show some dissociation in the presence of soluble heparin. However, when a high affinity uPAR-binding site is available, the heparin-binding site in PN1 ap-
Catabolism of uPA-PN1 Complexes

Importantly, these data clearly show that no measurable amount of \( ^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes were bound to the cell surface by heparin, although almost equivalent amounts of both uPA-PN1 and \( ^{125}\text{I}-\text{uPA-PN1(K7E)} \) are cell surface-associated (Fig. 1B). Both native uPA-PN1 and variant uPA-PN1(K7E) complexes are bound to the cell surface predominantly via the uPAR (high affinity), although a small percentage of the native complexes are also bound via cell surface heparins. The inability of the variant complexes to bind to heparin appears to have a much greater than expected inhibitory effect on internalization (Fig. 1C), which led us to consider alternative roles for heparin in the catabolism pathway. However, because some binding of the native uPA-PN1 complexes is mediated by heparin, it was important to determine whether this small difference in the initial step in internalization could account kinetically for the difference in degradation rate observed in Fig. 1C.

The Heparin-binding Site of PN1 Is Required for the Intracellular Retention of \(^{125}\text{I}-\text{uPA-PN1} \) Complexes after Endocytosis—We next examined the initial internalization step to determine whether only the native complexes were being initially internalized, potentially because of the heparin dissociable species. The endocytosis of \( ^{125}\text{I}-\text{uPA-PN1(K7E)} \) variant complexes was directly compared with that of native \( ^{125}\text{I}-\text{uPA-PN1} \) complexes by determination of the ratio of internalized ligand to cell surface-bound ligand (34). If only heparin dissociable native complexes can be internalized effectively, the initial ratio of internalized native complexes to surface-bound native complexes (In/Sur ratio) should be much greater than the initial In/Sur ratio of variant complexes. HF cells were incubated with \( ^{125}\text{I}-\text{uPA-PN1} \) or \( ^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes at 37 °C. At the indicated times, triplicate cultures were chilled to 4 °C and subjected to trypsinization to remove cell surface-bound complexes. Internalized radiolabeled ligand in the cell pellet and surface-bound trypsin-sensitive ligand were quantified by \( \gamma \) counting. Control incubations done only at 4 °C demonstrated that the trypsinization procedure was over 90% efficient in removing cell surface-bound complexes (data not shown). Shown in Fig. 4A is the ratio of internalized/cell surface-bound complexes (In/Sur) plotted versus time (34). At the 1-min time point, the In/Sur ratios for \(^{125}\text{I}-\text{uPA-PN1} \) and \(^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes were close, 0.8 and 0.6, respectively. At the 3-min time point however, the In/Sur ratio for the \(^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes dropped to 0.5, whereas the In/Sur ratio for the \(^{125}\text{I}-\text{uPA-PN1} \) complexes climbed to 1.6. The In/Sur ratios for the \(^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes remained fairly constant throughout the remainder of the 15-min time course, whereas the In/Sur ratios for the \(^{125}\text{I}-\text{uPA-PN1} \) complexes reached a near maximum of 2.5 at 10 min and then increased at a much slower rate thereafter. The slower rate of increase after 10 min is consistent with the time expected for the earliest internalized complexes to reach the lysosomes (34).

To exclude the possibility that the \(^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes are never endocytosed, the In/Sur ratios were also determined in the presence of the LRP agonist, RAP (Fig. 4A, open symbols). In the presence of RAP, the In/Sur ratios of both \(^{125}\text{I}-\text{uPA-PN1} \) and \(^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes were markedly reduced initially and did not increase, indicating that RAP inhibited the internalization of both complexes. Importantly, the much lower initial In/Sur ratio for the variant uPA-PN1(K7E) complexes in the presence of RAP strongly suggests that, in the absence of RAP, \(^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes are internalized initially. Because the initial internalization at the 1-min time point of both normal and heparin binding-deficient complexes is very similar, the quick decrease of the In/Sur ratio of \(^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes at the 3-min time point and beyond in the absence of RAP may be caused by a lack of retention because of retro-endocytosis.

Following this line of reasoning, we noted that one of the earliest changes that occurs after internalization and endosome formation is a rapid lowering of the pH, and we decided to explore the potential role of pH in the endosomal retention of uPA-PN1 complexes. Monensin, a sodium ionophore that prevents the lowering of pH in the endosomes, was tested for its effect on the In/Sur ratio of normal uPA-PN1 complexes. Interestingly, in the presence of monensin, native \(^{125}\text{I}-\text{uPA-PN1} \) complexes displayed endocytosis kinetics nearly identical to \(^{125}\text{I}-\text{uPA-PN1(K7E)} \) complexes. This suggests a possible role for pH in the retention of the complexes in the endosomes. The endocytosis kinetics of uPA-PN1(K7E) complexes did not change significantly in the presence of monensin (data not shown).

Because the uPA-PN1(K7E) complexes are internalized, initially we next wanted to determine whether they were simply
were removed, and the cultures were washed four times to remove any residual unbound ligand. The cultures were then solubilized in 10% SDS and quantified by \( \gamma \) counting. From pH 5.75 to 5.25, the binding of \(^{125}\text{I}-\text{uPA-PN1}\) and \(^{125}\text{I}-\text{uPA-PN1(K7E)}\) complexes was remarkably similar, approximately 24 fmol. Between pH 5.25 and 5.0 there was a marked decrease in the cell surface association of \(^{125}\text{I}-\text{uPA-PN1(K7E)}\) complexes, whereas the association of \(^{125}\text{I}-\text{uPA-PN1}\) was relatively unchanged. Below pH 5.0, the association of \(^{125}\text{I}-\text{uPA-PN1}\) complexes continued to decrease and approached that of \(^{125}\text{I}-\text{uPA-PN1(K7E)}\) complexes as the pH was lowered. The large difference in cell surface complex association in the pH 5.25 to 5.0 range has physiological relevance, because this is the reported pH of late endosomes (35). The simplest interpretation of these results is that below pH 5.2, the binding of \(^{125}\text{I}-\text{uPA-PN1}\) complexes switches from the uPAR, to an unidentified component through a mechanism that involves the heparin-binding site of PN1.

To probe the identification of this entity further, we next...
examined the ability of heparin to compete for the cell surface binding of native uPA-PN1 complexes over the same pH range (Fig. 5B). Test cultures were incubated with native uPA-PN1 complexes under the same experimental conditions as shown in Fig. 5A and then were placed in binding medium alone or containing 100 nm soluble heparin for an additional 15 min. From pH 5.75 to pH 5.25, none of the bound complexes were competed for by the addition of exogenous soluble heparin. Interestingly, at pH 5.0, just over 80% of the bound native complexes suddenly became dissociable in the presence of added heparin. These data are most consistent with a mechanism that predicts a shift in the binding of native uPA-PN1 complexes from the uPAR to heparin at between pH 5.25 and pH 5.0.

A Heparin-binding Peptide Derived from the Heparin-binding Domain of PN1 Specifically Inhibits the Degradation of uPA-PN1 Complexes—As an independent means of probing the potential role of the heparin-binding site in PN1 in intracellular retention and perhaps trafficking of uPA-PN1 complexes, we employed a synthetic peptide strategy. The heparin-binding site in PN1 has been narrowed to residues 70–87 in the mature protein (29). A synthetic peptide, Gly70–Asp87 (GKILKKKNK1VSKKKNKD) was synthesized and purified as described previously (13). A control peptide representing the homologous heparin-binding site of ATIII (residues 124–145, AKLNCRLYRKANKSKKLVSN) was also prepared.

The heparin binding properties of the ATIII heparin-binding peptides have been well characterized in previous studies (25, 36), but the peptide derived from the PN1 heparin-binding sequence is not as well studied. The experiment shown in Fig. 6A was done to illustrate that the PN1-derived synthetic peptide, Gly70–Asp87, does indeed bind heparin in a specific and saturable manner. 1-μg quantities of Gly70–Asp87 in individual dots on nitrocellulose were incubated in duplicate with 100 ng/ml of 125I-F-heparin in the absence and in the presence of increasing concentrations of nonlabeled soluble heparin. Relative to the control incubation with 125I-F-heparin only, increasing the concentration of nonlabeled heparin initially increased binding slightly but eventually competed for nearly all of the binding at the 500 ng/ml concentration.

Having established that the PN1 heparin-binding peptide, Gly70–Asp87, does bind heparin specifically and saturably, the effect of Gly70–Asp87 on the cell surface binding of uPA-PN1 complexes was evaluated. Confluent HF cell cultures were incubated with 100 ng/ml of uPA-PN1 complexes, in the absence and presence of Gly70–Asp87 over a concentration range of 1 to 50 μg/ml at 4 °C (Fig. 6B). Even at the highest concentration (50 μg/ml), Gly70–Asp87 was able to compete for only about 10% of uPA-PN1 complex binding. This is consistent with the data shown in Fig. 1, where it was shown that the majority of the complexes were bound to the uPAR, and in Fig. 3, where it was shown that only a small fraction of the complexes were dissociable from the cell surface by the addition of soluble heparin. It also supports a model in which the uPA-PN1 complexes do not release from high affinity binding to the uPAR at the cell surface under normal conditions but only after internalization occurs.

The effect of Gly70–Asp87 on the degradation of uPA was next examined at intervals of 1 and 2 h. Triplicate cultures of HF cells were incubated with uPA-PN1 complexes, in the presence and absence of Gly70–Asp87 (Fig. 6C). At 1- and 2-h intervals, samples were subjected to trichloroacetic acid precipitation to measure the degradation of uPA-PN1. Degradation was fairly linear, with 30 fmol degraded in the first hour and 72 fmol degraded by the end of the second hour. At the 1-h time point, at a concentration of 10 μM, Gly70–Asp87 decreased degradation by approximately 70%. At the 2-h time point, the decrease was not as prominent, approximately 50%, which might be explained by a rapid degradation of the peptide. At a concentration of 20 μM Gly70–Asp87, the effect on degradation was much more pronounced, with an 87% decrease in the degradation of uPA-PN1 at the 1-h time point. When a second addition of Gly70–Asp87 at 20 μM at the end of the 1-h time point, nearly the same magnitude of effect was observed at the 2-h time point, supporting the idea that the Gly70–Asp87 may have a relatively short half-life in presence of cells at 37 °C.

Finally, we wanted to rule out the possibility that the effect of Gly70–Asp87 might be explained by artifact because of its high positive charge density. As a control, we repeated the degradation experiment shown in Fig. 6C using Gly70–Asp87 and Ala124–Arg145, which represents the heparin-binding site of human ATIII, in parallel. The PN1-derived heparin-binding peptide, Gly70–Asp87 again markedly reduced the degradation of uPA-PN1 complexes, whereas in contrast, the ATIII heparin-binding peptide, Ala124–Arg145, had no effect (Fig. 6D). This is an important and convincing control, because the ATIII- and PN1-derived peptides are nearly identical in charge density, both bind heparin, and are predicted to have the amphipathic helical structure. These data suggest that there is a specific heparin sequence responsible for the endosomal retention of uPA-PN1 complexes.

**DISCUSSION**

The present studies were undertaken to determine whether the pathways and components involved in the cellular binding, internalization, and degradation of uPA-PN1 complexes differed significantly from that of Th-PN1 complexes. Utilizing a genetically engineered PN1 variant that is deficient in heparin binding and a recently developed polyclonal antibody specific for the LRP-binding site in PN1 that inhibits the LRP-mediated internalization of Th-PN1 complexes, we conclude that the pathways are distinct but share the LRP as a common endocytosis mechanism.

There are two major differences in how uPA-PN1 and Th-PN1 complexes are catabolized. First, the binding of Th-PN1 complexes to the cell surface is mediated primarily by heparins (14, 17). This is an important step in the catabolism of the complexes, because it serves to keep the concentration of the complexes at the cell surface relatively high, which in turn promotes interaction with the LRP, a relatively low affinity interaction. In contrast, the binding of uPA-PN1 complexes is nearly completely independent of heparins and is primarily mediated by the uPAR. The second major difference is the interaction of uPA-PN1 and Th-PN1 complexes with the LRP. In both pathways the LRP functions only as an endocytosis mechanism (14, 15). Parameters affecting the rate of transfer of protease-PN1 from heparin or from uPAR to the LRP could have a marked effect on turnover. To date it is not clear whether transfer to LRP occurs as a separate step in the pathway or whether the heparin or uPAR are co-internalized with the complex. In the case of Th-PN1 complexes, the endocytosis step requires a specific structural region in PN1, amino acid residues Pro17–Ile58 (13, 17). Amino acid substitutions in this region, as well as a polyclonal antibody generated against this sequence, markedly impair Th-PN1 complex internalization by the LRP. Interestingly, this same region is not required for the LRP-mediated internalization of uPA-PN1 complexes. There is apparently an intermediate affinity binding site in uPA that is sufficient for LRP internalization (30). Whether the LRP-binding sites in PN1 and in uPA can be used simultaneously is not known, but clearly the blockage of the PN1 site does not affect the overall rate of uPA-PN1 catabolism. Thus, although the LRP-mediated endocytosis is a common step in
the catabolism of uPA-PN1 and Th-PN1 complexes, there are major molecular differences in the details of the pathways.

One of the more interesting observations made in the present studies is the apparent involvement of the heparin-binding site in PN1 in the post-endocytic retention of uPA-PN1 complexes. After initial internalization, uPA-PN1 complexes that have a nonfunctional heparin-binding site appear to be released from the cells in an intact form. uPA-PN1 complexes made with native PN1, on the other hand, proceed on to the lysosomes where they are degraded. Examination of the binding of $^{125}$I-uPA-PN1 complexes to the cell surface at different pH levels revealed that between pH 5.25 and 5.0 the binding of $^{125}$I-uPA-PN1 complexes was relatively unchanged, whereas the binding of $^{125}$I-uPA-PN1(K7E) complexes was reduced approximately 3-fold. Interestingly, the fold difference in binding at pH 5.0 correlates well with the overall decreased rate of catabolism of $^{125}$I-uPA-PN1 complexes, which is 3-fold. The most obvious candidate molecule to participate in the binding of $^{125}$I-uPA-PN1 at pH 5.0 is heparin or a heparan sulfate proteoglycan for the following reasons. First, the only known difference between PN1 and PN1(K7E) is the inability of the latter to bind heparin. PN1(K7E) displays normal kinetics of thrombin inhibition, forms covalent complexes with both thrombin and uPA, and...
binds to the LRP (14). Second, the biochemical requirements for heparin binding are most consistent with a binding interaction that would not be adversely affected at pH 5.0. There would be no change in the status of lysine and arginine protonation from pH 7.0 to 5.0 because of the high pK of these amino acids. In addition the protonation of the sulfates on the heparin would not change until a much lower pH when the pK of the sulfate groups is reached. Heparin and heparan sulfate differ in sulfate content, although previous work has shown almost no difference in their biological activities with respect to PN1 binding and activation (29, 37). At the present level of analysis we cannot discern between the interaction of uPA-PN1 complexes with heparin compared with heparan sulfate proteoglycans on the cell surface or in the endosomes.

We directly addressed the role of heparin in the post-endocytotic retention of 125I-uPA-PN1 complexes in two different experimental paradigms. In the first, we demonstrated that the binding of 125I-uPA-PN1 complexes shifted from a state of heparin insensitivity at pH 5.0 to a state of heparin sensitivity at pH 5.0. In the second, we demonstrated that a synthetic heparin-binding peptide derived from PN1 specifically inhibited the catabolism of 125I-uPA-PN1 complexes, despite the fact that it had no effect on the cell surface binding of the complexes. A structurally similar peptide with an identical charge density derived from the ATIII heparin-binding sequence had no effect on catabolism. This ruled out a simple charge-charge interaction that would not be adversely affected at pH 5.0. There would be no change in the status of lysine and arginine protonation from pH 7.0 to a state of heparin sensitivity at pH 5.25 to a state of heparin sensitivity at pH 7.0 because of the high pK of these amino acids. In addition the protonation of the sulfates on the heparin would not change until a much lower pH when the pK of the sulfate groups is reached. Heparin and heparan sulfate differ in sulfate content, although previous work has shown almost no difference in their biological activities with respect to PN1 binding and activation (29, 37). At the present level of analysis we cannot discern between the interaction of uPA-PN1 complexes with heparin compared with heparan sulfate proteoglycans on the cell surface or in the endosomes.

Finally, the results of these studies make an important point about the clearance of SERPIN-protease complexes. There is no universal mechanism, and no structural determinant in the SERPINs that stretches across the entire SERPIN family required for cellular uptake and catabolism as has been previously proposed (21). Indeed, the data in the present report clearly illustrate that the structural requirement for the endocytosis of a single SERPIN can be determined by the protease moiety in the inhibitory complex. The larger picture that is now emerging is that the LRP acts as a common endocytosis mechanism for many of the SERPIN-protease complexes, but the delivery route to the LRP may be specific to individual SERPINs or classes of SERPINs.

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Roles of the Heparin and Low Density Lipid Receptor-related Protein-binding Sites of Protease Nexin 1 (PN1) in Urokinase-PN1 Complex Catabolism: THE PN1 HEPARIN-BINDING SITE MEDIATES COMPLEX RETENTION AND DEGRADATION BUT NOT CELL SURFACE BINDING OR INTERNALIZATION

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