Binding of urokinase-type plasminogen activator–plasminogen activator inhibitor-1 complex to the endocytosis receptors α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein and very-low-density lipoprotein receptor involves basic residues in the inhibitor

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The complex of the type-1 plasminogen activator inhibitor (PAI-1) and its target proteinases, the urokinase and tissue-type plasminogen activators (uPA and tPA), but not the free components, bind with high affinity to the endocytosis receptors α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (α_2MR/LRP) and very-low-density lipoprotein receptor (VLDLR). To characterize the molecular interaction between the complexes and the receptors, alanine codons were introduced into the human PAI-1 cDNA to replace the four basic residues, Arg-78, Lys-82, Arg-120 and Lys-124, as double mutations. The purified recombinant mutant proteins, rPAI-1/R78A-K124A and rPAI-1/K82A-R120A, produced by the yeast *Pichia pastoris*, were indistinghuisable from wild-type recombinant and natural human PAI-1 with respect to inhibitory activity against uPA, stability of SDS-resistant complexes with uPA, and vitronectin binding. Radiolabelled mutant uPA·PAI-1 complexes bound with a 10- to 20-fold, and 3- to 7-fold reduced affinity to purified α_2MR/LRP and VLDLR respectively. α_2MR/LRP-mediated endocytosis of the mutant complexes by COS-1 cells was reduced to 48 and 38 % of the level of endocytosis of wild-type PAI-1. Binding of the mutant complexes to the uPA receptor was not affected. These findings suggest that the binding mode of the uPA·PAI-1 complex to both α_2MR/LRP and VLDLR is similar. The four residues are surface exposed in the region defined by α-helix D and β-strand 1A in the serine protease inhibitor (serpin) structure. Our study represents the first identification of residues in a surface region implicated in molecular recognition of protease·serpin complexes by endocytosis receptors of the low-density lipoprotein receptor family.

INTRODUCTION

The type-1 plasminogen activator inhibitor (PAI-1) is the primary inhibitor of both the urokinase-type (uPA) and tissue-type plasminogen activator (tPA). The plasminogen activators are key determinants in fibrinolysis and turnover of extracellular matrix [1–3]. PAI-1 and uPA interact to form an enzymically inactive, highly stable, and presumably covalent, complex with a stoichiometry of 1:1 [2,4–6]. PAI-1 is a member of the large protein family of serine protease inhibitors (serpins). Serpins have three characteristic β-sheets that support the exposed reactive-centre loop (RCL), which is the target site for interaction with the proteinase [7–9]. PAI-1 is one of the most extensively studied serpins, and X-ray crystal structures for two of its conformations have been solved [10,11]. Also X-ray crystal structures for some of the serine proteinases are known; however, the structure of a serpin in complex with its target protease has not been solved yet.

Complexes of plasminogen activators and PAI-1 (PA·PAI-1) are cleared from the circulation and extracellular space by binding to the endocytosis receptors α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (α_2MR/LRP), glycoprotein 330 (gp330), and the very-low-density lipoprotein receptor (VLDLR), which belong to the low-density lipoprotein receptor (LDLR) family (reviewed in [3,12–17]). Many different ligands, including lipoproteins and several serpin–proteinase complexes [18–21] are known to bind to this family of receptors [3,12–17]. Endocytosis of the uPA·PAI-1, but not the tPA·PAI-1 complex, occurs via a primary binding to the glycosylphosphatidylinositol membrane-anchored uPA receptor, uPAR [22]. *In vivo*, uPA·PAI-1 is probably formed by reaction of PAI-1 with uPAR bound uPA. Preformed uPA·PAI-1 added to cells binds primarily to uPAR, due to the higher affinity of uPA for uPAR than of the complex for the endocytosis receptors. uPAR, putatively, functions to present the complex to the receptors. uPAR is co-internalized with uPA·PAI-1 by the endocytosis receptors [23] and recycled back to the cell surface after internalization [24].

The endocytosis receptor binding of uPA·PAI-1 is determined by a combined binding area involving both PAI-1 and uPA, since the affinity for the receptors of the individual inhibitor and proteinase molecules is much lower than that of the complex. In addition, different monoclonal antibodies (Mabs) against PAI-1 or uPA prevent binding of the complex to the receptor [25–27]. The tight binding M_r 40000 receptor-associated protein (RAP) competes with uPA·PAI-1 binding to both receptors [3,12–17]. The latter finding suggests strongly that uPA·PAI-1 binds in a similar mode to α_2MR/LRP and VLDLR.

The molecular details of the ligand–receptor interactions are best known from the family member LDLR and its interaction

Abbreviations used: apoB, apolipoprotein B-100; α_2MR/LRP, α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein; DIP-uPA, di-isopropylfluorophosphate-urokinase-type plasminogen activator; LDLR, low-density lipoprotein receptor; Mab, monoclonal antibody; PAI-1, plasminogen activator inhibitor type-1; pNGase F, peptide N-glycosidase F; RAP, receptor-associated protein; RCL, reactive-centre loop; RT, room temperature; serpin, serine protease inhibitor; TCA, trichloroacetic acid; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPA·PAI-1, complex of uPA and PAI-1; uPAR, uPA receptor; VLDLR, very-low-density lipoprotein receptor

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with the apolipoproteins B-100 (ApoB) and E (ApoE) [28]. From these studies, it became apparent that basic residues in ApoE govern the ligand–receptor interaction with cysteine-rich or complement-type repeats with an acidic C-terminus, which are conserved among the family members in units of 7–11 repeats [15,29]. It is therefore likely that basic residues in ligands are involved also in the binding to the LDLR-family members, α_2MR/LRP and VLDLR, which both have units of eight complement-type repeats. This hypothesis is in agreement with the observation that heparin prevents the binding of most ligands to α_2MR/LRP, gp330 and VLDLR, including uPA·PAI-1 [12,15]. The involvement of basic residues in the ligand interaction to α_2MR/LRP has been shown for the receptor-binding domain of α_2-macroglobulin [30] and strongly suggested for lipoprotein lipase [31] and thrombospondin-1 [32].

The present study shows for the first time the involvement of basic residues in the binding of serpin–protease complexes to endocytosis receptors. It localizes at least two basic residues in PAI-1, which in uPA·PAI-1, most likely, interact directly with α_2MR/LRP. In addition, these observations are extended for the interaction of uPA·PAI-1 with VLDLR.

EXPERIMENTAL

Miscellaneous materials

The following materials were obtained from the sources indicated: human α_2MR/LRP (Dr. J. Gliemann, Department of Medical Biochemistry, Aarhus University, Denmark); human recombinant RAP (Dr. L. Ellgaard, this department); human multimeric vitronectin (Dr. K. T. Preissner, Max-Planck Institute for Thrombosis and Haemostasis, Bad Neuheim, Germany); altered-sites *in vitro* DNA mutagenesis system (Promega); oligodeoxynucleotide primers (Pharmacia and DNA Technology ApS); $Na^{125}I$, [^{32}P]dATP and components for the ECL immunodetection kit (Amersham); media components for *Pichia pastoris* culturing (Invitrogen); protease inhibitor cocktail (EDTA-free; Boehringer); S-2444 (L-pyroglutamyl-glycyl-L-arginine-*p*-nitroaniline hydrochloride; Chromogenix); Centriprep 10 (Amicon); peroxidase-coupled swine-anti-mouse anti-IgG (Dako); peptide N-glycosidase F (pNGase F; Oxford Glycosystems); ultrafiltration membranes (SVLP-type; Millipore); *o*-phenylene-diamine (Kementec). All other reagents are as described elsewhere [25,27,33–36], or of the best grade commercially available.

Proteins

Human α_2MR/LRP and RAP were isolated according to the methods of Nykjær et al. [25]. The mixture of VLDLR and α_2MR/LRP was purified from cell membranes of bovine udder by affinity chromatography on a column with RAP immobilized on Sepharose 4B [27,37]. This preparation contains approximately equal molar amounts of the two receptors, determined by N-terminal amino acid sequencing of VLDLR and the α_2MR/LRP β-chain [27,37]. Natural (HT1080) PAI-1 was isolated as described by Munch et al. [34], and vitronectin by Kost et al. [38]. uPA was commercially obtained from Serono (Aubonne).

Cloning, protein expression and purification

Site-directed mutagenesis and cloning of the PAI-1 cDNA [39,40] mutants were performed essentially as described by the DNA mutagenesis system technical manual (nr. 08/94, Promega) and Sambrook et al. [41]. The following 21- and 27-mer primers, indicated below in 5′ to 3′ orientation, were used to introduce the substitutions. The substituted codon is underlined in each case: R78A, CCCGCCCTCGCGCATCTGTAC; K82A, CGGCATCTGTACGCCGAGCTCATGGGG; R120A, TTCAGGCTGTTCGCTAGCACGGTCAAG; K124A, GGAGCACGGTCGCCCAAGTGGACTTT. The basic residue codons were replaced by that of alanine. The mutations were generated in a derivative of the pAlter-1 plasmid (Promega) that contains a modified PAI-1 cDNA sequence: (1) the histidine in position 4 of the PAI-1 amino acid sequence [39,40] was deleted for convenience of cloning, leaving a *Pml*I restriction enzyme site at the start of the mature protein-encoding sequence; (2) silent codon changes at Arg-103 (CGG to CGA) and Arg-164–Leu-165 (CGGCTG to AGACTA) were introduced to result in the unique restriction enzyme sites for the endonuclease restriction enzymes *Afl*II and *Spe*I respectively (K. W. Rodenburg, unpublished work). The PAI-1 amino acid numbering is as described by Andreasen et al. [39], i.e. the first amino acid in mature PAI-1 is serine. After mutagenesis, relevant DNA fragments were sequenced to confirm the presence of the intended mutations. Subsequently, the fragments containing the double mutations R78A and K124A, and K82A and R120A, were cloned in pPAI-Δ2, a derivative of the *P. pastoris* expression vector for PAI-1, plasmid pPAI-1/wt, to yield the plasmids pPAI/R78A-K124A and pPAI/K82A-R120A. pPAI-1/wt contains the complete open reading frame of the PAI-1 cDNA cloned as a *Pml*I–*Xba*I fragment to replace the short *Sna*BI–*Avr*II fragment in plasmid pPIC9 (Invitrogen) (K. W. Rodenburg, unpublished work). pPAI-Δ2 was constructed by replacing the 161 bp *Afl*II–*Spe*I PAI-1 cDNA fragment with a 19 bp fragment that leaves the unique sites intact for further cloning (K. W. Rodenburg, unpublished work). The use of pPAI-Δ2 in construction of the plasmids encoding the mutant PAI-1 genes allowed with 100 % certainty the replacement of wild-type with mutant fragment. Plasmids pPAI-1/wt, pPAI/R78A-K124A and pPAI/K82A-R120A were introduced into the *P. pastoris* strain GS115 by electroporation. All procedures relevant to yeast manipulation were as described in the *Pichia* protein expression kit manual version 2.1 (Invitrogen). PAI-1 production by plasmid-transformed *Pichia* cells were identified by immunodetection (K. W. Rodenburg, unpublished work), using monoclonal and polyclonal antibodies and a pre-immune serum as a negative control.

The relevant yeast strains were cultured to produce the cells needed for production of the protein in methanol-containing medium. In brief, the yeast cells were cultured for 64 h at 29–30 °C in 1 litre of medium containing 1 % (v/v) glycerol, as a carbon source, in a 3 litre Erlenmeyer flask, rotating horizontally at about 300 rev./min, to a cell density of A_{600} of approximately 10. Subsequently, the cells were harvested by centrifugation, washed once with 250 ml of methanol-containing medium, and resuspended in 200 ml of methanol-containing medium to which a cocktail of protease inhibitors (EDTA-free) was added. The cultures were induced for 40 to 64 h at 29–30 °C in 1 litre Erlenmeyer flasks rotating at 300 rev./min. After the induction, the pH of the culture supernatant was set to 8.0, and it was then passed through 5.0 and 0.65 μm filters. Subsequent steps were performed at 0 °C. PAI-1 protein was purified from the filtered culture supernatants by immunoaffinity chromatography as described previously [40]. Briefly, the supernatant was loaded on a column containing anti-PAI-1 Mab from hybridoma clone 2 (Mab-2) bound to Sepharose [34]. The column was washed with at least 100 column volumes of 1 M NaCl in 0.1 M Tris, pH 8.1, after which the bound protein was eluted with 1 M NaCl in 0.1 M acetic acid, pH 2.9. Immediately after collection of the fractions, a 1/10 volume of 1 M Tris, pH 9, was added, to

bring the pH to 8. Protein-containing fractions were pooled and concentrated using Centriprep 10 devices. Finally, the PAI-1 preparation (about 1 ml) was dialysed extensively against PBS at 4 °C and stored at −80 °C. The recombinant proteins produced by the yeast will be referred to as rPAI-1/wt, rPAI-1/R78A-K124A and rPAI-1/K82A-R120A.

For some experiments, rPAI-1 and rPAI-1, in complex with ^{125}I-labelled uPA (^{125}I-uPA) (see below), were treated with the glycosidase pNGaseF according the supplier's instructions. In some cases, rPAI-1/wt, purified by affinity chromatography (see above), was re-chromatographed using gel-filtration FPLC [27], using a Superdex 200 HR10/30 column, equilibrated and eluted with 0.5 M NaCl in 10 mM $NaHPO_4$, pH 7.4.

Amino acid analysis and N-terminal sequencing

Amino acid analyses were performed on 0.05–0.2 nmol of protein hydrolysates prepared by incubation in 6 M HCl/0.1 % phenol at 110 °C for 16 h, using an Applied Biosystems amino acid analyser. N-terminal sequencing of protein (about 0.05 nmol) was performed using an Applied Biosystems model 470A sequencer equipped with an on-line model 120A phenylthiohydantoin analyser. The protein concentrations were determined spectrophotometrically at 280 nm or by amino acid analysis.

Activation of PAI-1 and uPA·PAI-1 formation

PAI-1 (2–10 μg), in a volume of 40 μl of 0.1 M Tris/HCl, pH 8.1, was activated by addition of SDS to a final concentration of 0.1 % and incubation for 1–2 h at room temperature (RT). The activation was stopped by the addition of 10 vol of 0.1 M Tris/HCl/1 % Triton X-100, pH 8.1. For complex formation, the volume was adjusted by the addition of 0.1 M Tris/HCl/0.2 % Triton X-100, pH 8.1, to accommodate ^{125}I-uPA at a concentration of of 0.25 μg/ml and a 10-fold excess of activated PAI-1 over ^{125}I-uPA. The uPA·PAI-1 complex was prepared as described [35]. The complex-formation reaction was allowed to proceed for 3 h at 37 °C, after which the complexes were stored overnight at 4 °C. Purification of ^{125}I-uPA·PAI-1 was achieved by affinity chromatography using, in order, Sepharose-bound anti-PAI-1 Mab-2 and anti-uPA Mab-6 (monoclonal anti-uPA antibody from hybridoma clone 6), essentially as decribed previously [25]. A specific activity of 5.5×10^{-10} Bq/μmol was achieved. A separate set of columns was used for each PAI-1 variant.

PAI-1 activity assays

The activity of the PAI-1 variants was determined by their ability to inhibit uPA, using S-2444 as substrate for the protease [34]. To determine the fraction of the active form of PAI-1 in the preparations, a titration assay was performed in a 96-well microtitre plate. A 100 μl volume of a 20 nM uPA solution was mixed with 100 μl solutions of various concentrations of SDS-activated or non-treated PAI-1 (1–500 nM) to determine the amount of active PAI-1 in the purified protein preparations. PAI-1 and uPA were allowed to form a complex by incubation at 37 °C for 30 min. To determine the remaining uPA activity in the mixtures, 20 μl of a 1.6 mM S-2444 solution was added to the mixtures and incubation was continued at 37 °C, and every third min for 1 h the absorbance at 405 nm (A_{405}) was read using a microtitre-well ELISA reader (type EL_x808, BioTek Instruments). The concentration of active PAI-1 was calculated from the amount of PAI-1 needed to inhibit half of the uPA activity. The time courses of the inhibition reactions were followed by mixing S-2444 with an amount of SDS-activated natural, recombinant wild-type or variant PAI-1; the mixture, including natural PAI-1, inhibited 75 % of the activity of a 10 nM uPA solution in a volume of 120 μl at 37 °C for 15 min. Subsequently, at time $t = 0$, 100 μl of 0.5 μg/ml uPA of 37 °C was added, and the A_{405} of the reactions was monitored at every minute for a period of 15 min.

SDS/PAGE, immunoblot and ligand-blot analyses

Protein samples were routinely analysed in 10 % or 4–16 % gradient SDS/PAGE. The gels were stained with Coomassie Brilliant Blue G250.

Immunoblot analysis was performed by standard procedures [43].

Ligand-blot analyses were performed with a mixture of bovine α_2MR/LRP and VLDLR, obtained by immunoaffinity chromatography on RAP-bound Sepharose [27,37]. The receptors were separated by SDS/PAGE and transferred by electrophoresis in standard Tris/glycine buffer to PVDF filters [27,37]. Equal amounts (60 pM, i.e. 20×10^4 c.p.m./ml) of the different ^{125}I-labelled complexes were incubated with filter pieces corresponding to parallel gel lanes in binding buffer [20 mM Hepes, pH 7.4/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgSO_4$/0.5 % (w/v) BSA]. Binding was detected using a PhosphorImager™ (SF, Molecular Dynamics) and represented using MD ImageQuant software, version 3.3 (SF, Molecular Dynamics). Complex–receptor binding was quantified by determining the radioactivity in those parts of the ligand blots that corresponded to receptor-bound complexes, using either the PhosphorImager and associated software or an auto-γ-counter (Cobra™, Packard Instruments).

Microtitre-well binding assay

The ionic strength and pH dependence of α_2MR/LRP–complex interaction were determined in a microtitre-well assay, performed in duplicate as follows. Plates with 96 microtitre wells (NUNC Maxisorb) were coated with 0.25 μg/ml of α_2MR/LRP [25] in 50 mM sodium carbonate, pH 9.6, for 2 h at RT. The amount of immobilized receptor was adjusted to result in about 10 % of the uPA·rPAI-1/wt being bound to the receptor. Subsequently, the wells were incubated for 2 h at RT with blocking solution (5 % BSA in binding buffer, i.e. 20 mM Hepes, pH 7.4, or 20 mM sodium acetate, pH 4.1/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM $MgSO_4$/0.5 % BSA) and washed thrice at 4 °C with TBS-T buffer (50 mM Tris/HCl/150 mM NaCl/0.05 % Tween-20, pH 7.4). The wells were then incubated with 20 pM ^{125}I-uPA·PAI-1 in binding buffer and varying NaCl concentrations (0–500 mM). In parallel, incubations were done with 20 pM radiolabelled complex plus 100 nM non-radioactive RAP. After incubation for 16 h at 4 °C, 90 % of the incubation mixtures was taken for determination of unbound radioactivity. After washing of the wells twice with the incubation buffer and thrice with TBS-T at 4 °C, receptor-bound radioactivity was released with 1 M NaOH and the radioactive content of these fractions was determined. The amounts of receptor-bound and unbound (i.e. free) radiolabelled complex were determined by γ-counting of the respective fractions. Binding of complex to α_2MR/LRP was expressed as the ratio of bound to free radioactivity.

Vitronectin binding assay

The vitronectin binding assay is a modified version of those described earlier [38,44]. Briefly, a 96-microtitre-well plate was coated overnight at 4 °C with 50 μl per well of a 5 μg/ml

multimeric vitronectin in PBS. After washing the wells four times with water for 5 min at RT, they were sequentially incubated for 1 h under the following conditions: (1) at 37 °C with blocking solution (5 % BSA in PBS); (2) at RT with SDS-activated PAI-1 in concentrations ranging from 1 to 62.5 ng/ml (in 0.01 % Tween-20 in PBS); (3) at 37 °C with a polyclonal anti-PAI-1 antiserum (5 μg/ml in 0.01 % Tween-20 in PBS); and (4) at 37 °C with a swine-anti-rabbit peroxidase-coupled anti-IgG (1:2000). After every incubation step, the wells were washed four times for 5 min with 0.01 % Tween-20 in PBS at 37 °C. After the last wash step, the wells were rinsed with water and subsequently subjected to a peroxidase-specific colour reaction by the addition of 200 μl of 0.1 M citrate/phosphate buffer/1 mg/ml *o*-phenylene-diamine/0.01 % H_2O_2, pH 5.0, for 10 min at RT. The reaction was stopped by the addition of 100 μl of 1 M H_2SO_4, and the absorption was read at 490 nm (A_{490}). In the control experiment, it was shown that the binding of the different components in the assay was not affected by the small amounts of Triton X-100 and SDS that were present in the SDS-activated PAI-1 preparations.

Endocytosis and cell binding assays

Endocytosis of ^{125}I-uPA·PAI-1 by COS-1 cells was followed by the concomitant degradation of radiolabelled ligand to trichloroacetic acid (TCA)-soluble material. COS-1 cells were cultured and prepared for endocytosis as described [35]. Typically, 7.5 pM (25×10^3 c.p.m./ml) of radiolabelled complex in binding buffer was added to 1×10^5 cells in a total volume of 250 μl of binding buffer. The experiments were performed in triplicate. Control experiments were done where 100 nM RAP and 200 nM di-isopropylfluorophosphate (DIP) uPA (DIP-uPA), or 100 nM RAP plus 200 nM DIP-uPA (both components non-radioactive), were added to the complex in binding buffer. After 2 h at 37 °C, the total amount of radioactivity in the individual wells was determined by γ-counting the radioactivity in the TCA-precipitable and TCA-soluble fractions of culture supernatant and cells. Cell binding of the complexes was performed under similar conditions, but now the cells and ^{125}I-labelled complexes, either without further additions or with 100 nM RAP or 200 nM DIP-uPA plus 100 nM RAP (both components non-radioactive), were incubated overnight at 4 °C. The radioactivity in cell pellet and culture supernatant was determined and expressed as the ratio of the radioactivity in the bound and free fractions.

X-ray crystal structures and computer software

The coordinates (PDB-files) for the X-ray crystal structures of latent [10] and cleaved PAI-1 [11], and the model of active PAI-1 [55] were obtained from Dr. E. J. Goldsmith (University of Texas, TX, U.S.A.) and Dr. K. Aertgeerts (University of Leuven, Leuven, Belgium). To prepare the final presentation that is shown in Figure 4, a file obtained from the PhosphorImager™-associated MD ImageQuant software, version 3.3 (SF, Molecular Dynamics), was processed as a TIFF bitmap (TIF) file in the drawing program CorelDRAW™ version 6.0 (Corel Corporation).

RESULTS

Production and characterization of recombinant wild-type and mutant PAI-1

Recombinant human wild-type PAI-1 (rPAI-1/wt) and the mutants rPAI-1/R78A-K124A and rPAI-1/K82A-R120A were produced in *P. pastoris*. The yield of rPAI-1/wt, as well as of mutant rPAI-1, from the yeast culture supernatant after purification, was about 3 mg/l. This indicated that the wild-type and mutant proteins behaved similarly with respect to secretion and stability. SDS/PAGE showed a combination of a major and a minor band with M_r values of approximately 50×10^3 and 47×10^3 respectively, and a smear with M_r between 84×10^3 and 100×10^3 for both wild-type and mutant rPAI-1 (Figure 1a). The two bands and the smear reacted with polyclonal (Figure 1b) and Mabs directed against PAI-1, but not with a pre-immune serum (results not shown). A minor band of M_r 60×10^3, estimated to make up about 5 % of the isolated protein preparation, was not detected by the antibodies (Figure 1b), and thus was not PAI-1. N-terminal sequencing of the first nine residues of the different PAI-1 variants showed in all cases the sequence EAEAYVHPP, indicating that the processing upon secretion of the different recombinant proteins from the yeast had occurred as expected, leaving a five amino acid N-terminal extension in the mature recombinant proteins, i.e. EAEAY, encoded by the cloning vector (*Pichia* expression manual; Invitrogen). The preparations

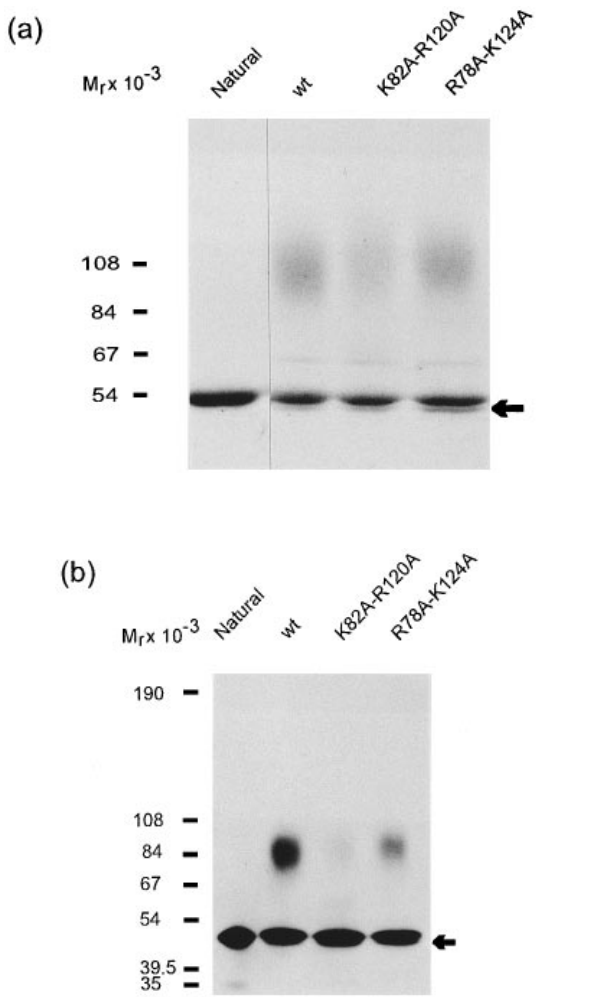

Figure 1 SDS/PAGE and immunoblot analysis of PAI-1 variants

(**A**) 2.5 μg and (**B**) 50 ng portions of the different purified PAI-1 variants were electrophoresed in 10% SDS/PAGE. (**A**) Coomassie Brilliant Blue staining of the gel: Natural, natural PAI-1; wt, rPAI-1/wt; K82A-R120A, rPAI-1/K82A-R120A; R78A-K124A, rPAI-1/R78A-K124A. The positions of M_r markers are indicated on the left of the gel, and the arrow on the right indicates the position of cleaved PAI-1. (**B**) Immunoblot of the same proteins as in (**A**) using a polyclonal rabbit anti-PAI-1 antiserum.

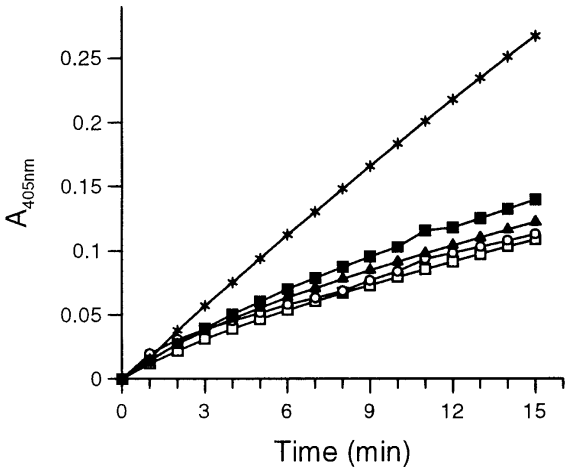

Figure 2 Time course of the inhibition reaction between PAI-1 variants and uPA

The various PAI-1 variants were activated with SDS. The concentrations of active PAI-1 in the preparations were then determined by titration against uPA, as described in the Experimental section. The time course of the reaction of each variant with uPA was then followed by taking the amount of each variant equivalent to 0.1875 mg/ml of uPA, adding S-2444 corresponding to a final concentration of 0.15 mM and starting the reaction by addition of uPA to a final concentration of 0.25 mg/ml. A_{405} was measured at the indicated time points. □, Natural PAI-1; ○, rPAI-1/wt; ▲, rPAI-1/K82A-R120A; ■, rPAI-1/R78A-K124A; *, no PAI-1. The data represent the means of six independent determinations in a typical experiment out of a total of two.

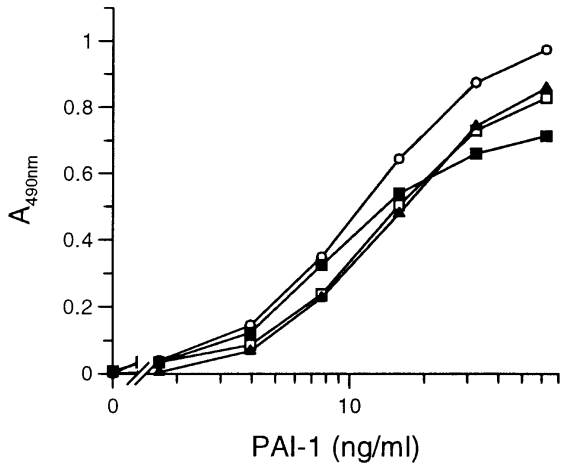

Figure 3 Binding of PAI-1 variants to vitronectin

Vitronectin was immobilized in microtitre wells and incubated with activated PAI-1 at the indicated concentrations. The concentrations of active PAI-1 in the various preparations were determined by titration against uPA, as described in the Experimental section. The bound PAI-1 was determined with a layer of polyclonal rabbit anti-PAI-1 antibodies and a peroxidase reaction. A background corresponding to the signal in wells without vitronectin was subtracted. □, Natural PAI-1; ○, rPAI-1/wt; ▲, rPAI-1/K82A-R120A; ■, rPAI-1/R78A-K124A.

contained two additional N-termini, present in equal amounts, starting at the P_1 and P_2' residues, indicating cleavage in the RCL in PAI-1. This explained the M_r 47×10^3 band in the recombinant PAI-1 preparations (Figure 1). The rPAI-1/wt preparation contained about 5% cleaved PAI-1, and those for PAI-1/K82A-R120A and PAI-1/R78A-K124A, 10 and 20% respectively (results not shown). Amino acid analysis indicated that the protein preparations were 90–95% pure in PAI-1 protein (not shown), which is in agreement with the results from SDS/PAGE. The smear in the recombinant preparations disappeared after pNGaseF treatment (results not shown), and thus was due to hyper-glycosylation of PAI-1.

As determined in a PAI-1 activity assay, more than 90% of the recombinant PAI-1 produced by *P. pastoris* was in the latent form after purification. There was no distinguishable difference between the efficiency of SDS activation of natural PAI-1 and recombinant PAI-1 (results not shown). When incubating equivalent amounts of the four proteins (natural PAI-1, rPAI-1/wt and both mutant rPAI-1s) with uPA, the time progression curves of the inhibition of uPA had the same shape for all four inhibitors (Figure 2). From the accuracy of the assays, it was estimated that the second-order rate constant for the different PAI-1 proteins varied less than 2-fold. These results indicated that the second-order rate constant for the reaction with uPA did not vary measurably among the PAI-1 variants. In addition, they showed that the hyper-glycosylation of the recombinant proteins did not affect the inhibition properties of PAI-1.

The vitronectin-binding properties of both SDS-activated PAI-1 mutants were indistinguishable from that of SDS-activated recombinant wild-type and natural PAI-1, indicating that the substituted basic residues are not involved in the binding to vitronectin (Figure 3).

Natural PAI-1, rPAI-1/wt and both mutant rPAI-1s formed stable, SDS-resistant complexes with radiolabelled uPA. The four purified complexes had equal stability during incubation at 37 °C, as judged by their migration in SDS/PAGE (Figure 4). The radioactive protein material with an M_r of 150×10^3 for the three recombinant protein complexes, represents uPA in complex with hyper-glycosylated PAI-1 (Figure 1a), since it disappeared upon treatment of the complex material with glycosidase (pNGase F). In addition, it was shown that the complex of ^{125}I-uPA and rPAI-1/wt, from which the hyper-glycosylated PAI-1 (Figure 1a) had been removed by gel filtration, migrated to the same position in SDS/PAGE as uPA · natural PAI-1 (results not shown).

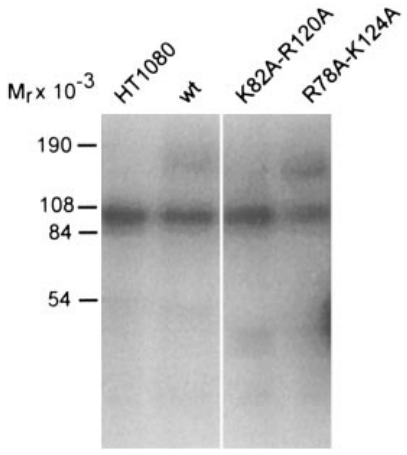

Figure 4 SDS/PAGE of ^{125}I-uPA · PAI-1

Autoradiograph of a 10% SDS/PAGE with the different ^{125}I-uPA · PAI-1 preparations. Complexes were incubated at 37 °C for 2 h. The individual samples represent 25×10^3 c.p.m. of ^{125}I-uPA in complex with the indicated PAI-1 variants (HT1080 = natural). The positions of M_r markers are indicated on the left of the gel. The autoradiograph is an imaging plate presentation (24 h exposure) of a ligand blot, as detected by the PhosphorImager (see the Experimental section for more details).

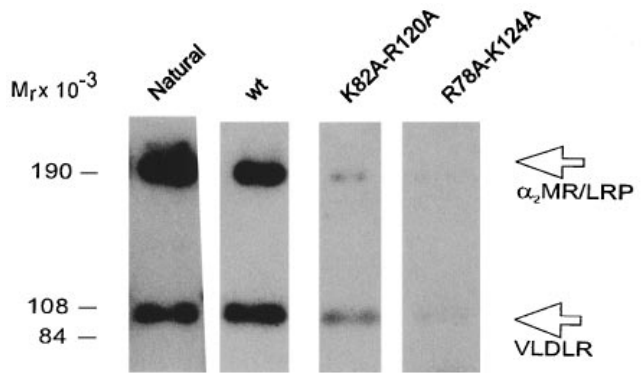

Figure 5 Ligand-blot analysis of ^{125}I-uPA · PAI-1 binding to α_2MR/LRP and VLDLR

Purified RAP-binding proteins from bovine mammary gland (65 ng per lane), containing a mixture of α_2MR/LRP and VLDLR [27,37], were resolved by SDS/PAGE in 4–16% gradient gels. Four parallel gel lanes were incubated with complexes between ^{125}I-uPA and the indicated PAI-1 variants. The positions of M_r markers are indicated on the left. The positions of α_2MR/LRP and VLDLR are indicated on the right.

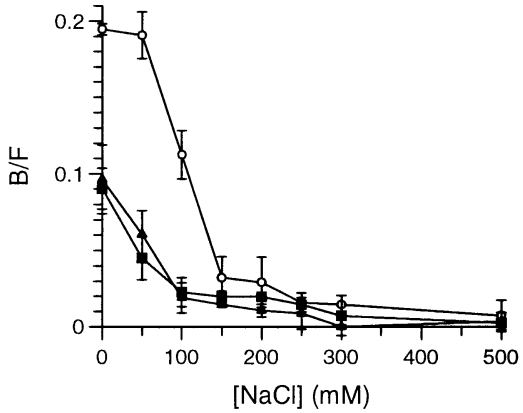

Figure 6 Effect of ionic strength on binding of ^{125}I-uPA · PAI-1 to α_2MR/LRP

Dependence of complex–receptor binding on the NaCl concentration in the binding buffer, as assayed in a microtitre-well assay. α_2MR/LRP was coated onto the wells. The binding of radiolabelled complex to receptor is expressed as the ratio of bound to free ligand (B/F). ○, rPAI-1/wt; ▲, rPAI-1/K82A-R120A; ■, rPAI-1/R78A-K124A. The data points represent means ± SD of two determinations in a typical experiment out of three.

Table 1 ^{125}I-uPA · PAI-1 binding to endocytosis receptors

The values given indicate the amount of radioactive complex that is bound to α_2MR/LRP and VLDLR, as assayed in a ligand-blot experiment for which an example is shown in Figure 5. The radioactivity in the parts of the ligand blot representing complex–receptor binding was quantified by analysis of the data from a 24 h PhosphorImager exposure of the blots. See the legend of Figure 5 for further details. Binding of the complex involving rPAI-1/wt is set to 100%, and that for the other complexes is expressed as a fraction of this. The values represent the means ± SD from three independent experiments.

	Binding (%)	
	α_2MR/LRP	VLDLR
^{125}I-uPA · PAI-1		
Natural PAI-1*	121	100
rPAI-1/wt	100	100
rPAI-1/K82A-R120A	10 ± 4	35 ± 5
rPAI-1/R78A-K124A	5 ± 4	15 ± 7

* Data from one experiment

Taken together, these results indicate that neither the mutated basic residues nor the different degrees of glycosylation in the rPAI-1 samples have detectable effects on complex formation with uPA or on vitronectin binding.

α_2MR/LRP, VLDLR and uPAR binding of uPA · PAI-1

Complexes were prepared from ^{125}I-uPA and SDS-activated mutant rPAI-1, rPAI-1/wt and natural PAI-1. In addition, complexes were prepared from ^{125}I-uPA and the M_r 50×10^3 or hyper-glycosylated rPAI-1/wt, which were separated from the original rPAI-1 preparation by gel filtration. The binding of the radiolabelled uPA · PAI-1 complexes to α_2MR/LRP and VLDLR was assessed by ligand-blot analysis. In the analysis, a mixture of α_2MR/LRP and VLDLR, purified from bovine mammary gland by immunoaffinity chromatography with RAP, was subjected to SDS/PAGE and transferred to PVDF filters. Subsequently, the filters were incubated with 60 pM radiolabelled complex. Under these conditions, the amount of radioactivity associated with each of the receptors is expected to be proportional to the binding affinity (i.e. the reciprocal of the dissociation equilibrium constant, K_d [21,27]). The amount of radioactivity in the positions in the blot corresponding to the two receptors with M_r values of 600×10^3 and 105×10^3 was determined by PhosphorImager scanning or γ-counting of relevant filter pieces. These two methods gave indistinguishable results (not shown). More than 95% of the radioactivity associated with the receptors could always be displaced by incubation with 100 nM RAP (results not shown). The binding of uPA · rPAI-1/R78A-K124A and uPA · rPAI-1/K82A-R120A to α_2MR/LRP was reduced 10- and 20-fold respectively, and the binding to VLDLR was reduced 3- and 7-fold respectively, compared with that of uPA · rPAI-1/wt and uPA · natural PAI-1 (Figure 5 and Table 1). The reduced binding of both PAI-1 mutants was neither caused by the M_r 60×10^3 contaminant in the rPAI-1 preparations nor the hyper-glycosylation of the recombinant proteins, since rPAI-1/wt and natural PAI-1 showed indistinguishable receptor-binding characteristics (Figure 5). The hyper-glycosylation of the recombinant proteins was not relevant for the receptor-binding property of their complex with uPA. This was confirmed by demonstrating in a ligand-blot assay, that complexes of rPAI-1/wt, FPLC-purified M_r 50×10^3 protein or hyper-glycosylated rPAI-1/wt with ^{125}I-uPA, bound to α_2MR/LRP with indistinguishable binding properties (results not shown).

These results were confirmed in a microtitre-well binding assay, where α_2MR/LRP was coated onto the wells and incubated with 10 pM radiolabelled complex in the presence or absence of 100 nM RAP (Figure 6). The RAP-displaceable binding, in all cases, was highly sensitive to the NaCl concentration of the binding buffer (Figure 6). The binding was reduced to approximately 15% at 200 mM NaCl compared with that in the absence of NaCl, indicating the involvement of charge–charge interaction in the binding. The 2- and 5-fold difference in binding between mutant and wild-type complex in the absence and presence of 100 mM NaCl (Figure 6) most likely indicated the direct involvement of the mutated basic residues in the interaction. Although in the absence of NaCl the difference in receptor binding of the complexes of mutant and wild-type rPAI-1 is only 2-fold, at 100 mM NaCl, a concentration which is

Table 2 Binding of ^{125}I-uPA·PAI-1 to uPAR

About 1×10^5 COS-1 cells were incubated with the different complexes for 16 h at 4 °C. Free and cell-bound radioactivity was determined. The ratio of bound to free ligand (B/F) for the different complexes are indicated in the absence (none) or presence of the non-radioactively labelled competitors 200 nM DIP-uPA and 100 nM RAP. The values represent the means ± SD of three determinations and are the results of one experiment out of two in each case.

	Cell binding (B/F)		
Competitor …	None	DIP-uPA	RAP
^{125}I-uPA·PAI-1			
rPAI-1/wt	0.083 ± 0.010	0 ± 0.005	0.064 ± 0.012
rPAI-1/K82A-R120A	0.057 ± 0.002	0 ± 0.015	0.050 ± 0.006
rPAI-1/R78A-K124A	0.066 ± 0.008	0.007 ± 0.015	0.059 ± 0.010

Table 3 Endocytosis of ^{125}I-uPA·PAI-1 by α_2MR/LRP

About 1×10^5 COS-1 cells were incubated with the different complexes for 2 h at 37 °C. The cell-dependent degradation of each complex (from which the degree of endocytosis can be determined) is expressed as a percentage of the degradation of uPA·rPAI-1/wt, which is set to 100%. 'Competitor' indicates the addition of non-radioactively labelled components to the incubations: none (no addition), 200 nM DIP-uPA, 100 nM RAP and 200 nM DIP-uPA plus 100 nM RAP. The values represent the means ± SD of two independent experiments.

	Endocytosis (%)			
Competitor …	None	DIP-uPA	RAP	DIP-uPA + RAP
^{125}I-uPA·PAI-1				
rPAI-1/wt	100 ± 1	41 ± 13	7 ± 7	0 ± 1
rPAI-1/K82A-R120A	48 ± 13	11 ± 7	8 ± 8	0 ± 1
rPAI-1/R78A-K124A	38 ± 1	6 ± 3	1 ± 1	0 ± 1

similar to that used in the ligand-blot assay, this difference is 5-fold. This 5-fold difference might well be in agreement with the 10- to 20-fold difference observed in the ligand-blot assay, considering the different amounts of receptor (65 ng and 250 ng) and concentrations of the complexes (60 pM and 20 pM) used in the ligand-blot and microtitre-well assays respectively.

Using ligand-blot analysis, the binding of all four complexes to VLDLR as well as α_2MR/LRP was strongly reduced by 250 mM NaCl (results not shown), demonstrating ionic-strength dependence also of the binding to VLDLR. The binding was also abolished by lowering the pH of the binding buffer to 4.1, but not to 6. Binding of ^{125}I-uPA/rPAI-1/wt to receptor protein in the microtitre well could be restored to control values by bringing the pH of the incubation mixture back to 7.4 or the NaCl concentration from 500 back to 120 mM NaCl (results not shown). This indicated susceptibility of the binding activity of the immobilized receptor to high salt concentrations and low pH, as opposed to protein instability.

The binding of the complexes to uPAR was determined by measuring the binding of the radiolabelled complexes to COS-1 cells at 4 °C (Table 2). Under these conditions, the binding was totally inhibited by 200 nM DIP-uPA, which blocks the uPAR binding but has no effect on binding of the complexes to the endocytosis receptors. There was at most a slight decrease in binding by the addition of 100 nM RAP. The preferential binding to uPAR under these conditions is in agreement with the higher affinity of the complexes for this receptor [26]. The uPAR-binding of mutant or wild-type complexes were indistinguishable, demonstrating that the mutations did not affect binding of the complexes to uPAR.

Endocytosis of uPA·PAI-1 complexes

α_2MR/LRP-mediated endocytosis of wild-type and mutant recombinant PAI-1 in complex with ^{125}I-uPA was estimated by measuring the degradation of radiolabelled complexes to TCA-soluble material by cultures of COS-1 cells. These cells express abundant α_2MR/LRP, but do not express other RAP-binding receptors [21,27]. In this assay, binding of the complexes to uPAR cannot be measured [27]. The endocytosis was performed with 7.5 pM radiolabelled complex and was inhibited by 200 nM DIP-uPA, 100 nM RAP or a combination of both components (Table 3). This is in agreement with a two-step mechanism for endocytosis of uPA·PAI-1 complexes (see the Introduction). The endocytosis of the radiolabelled complexes uPA·PAI-1/K82A-R120A and uPA·PAI-1/R78A-K124A by COS-1 cells was 48 ± 13 % (SD; $n = 2$) and 38 ± 1 % (SD; $n = 2$) respectively of the level for uPA·rPAI-1/wt. These results show that the PAI-1 substitutions affect the endocytosis of the mutant complex by α_2MR/LRP.

DISCUSSION

The present study shows that basic residues in PAI-1 are important for binding and endocytosis of uPA·PAI-1 by the endocytosis receptor α_2MR/LRP. The PAI-1 mutants used in this study involve the double mutations R78A/K124A and K82A/R120A. The substitutions in PAI-1 cause a 5–20-fold reduction in the binding of the mutant complexes to α_2MR/LRP and a 2–3-fold reduction in the endocytosis of these complexes by the receptor. At least two of the substituted residues, Arg-78 and Lys-82, Arg-120 and Lys-124, or a combination of one of the first with one of the second pair, are responsible for the mutant effects. However, since Arg-78 and Lys-82 localize to α-helix D, and Arg-120 and Lys-124 to β-strand 1A in the PAI-1 structure, it is tempting to speculate that either of the two, or both of the secondary structure elements are involved in the binding, not least because the four residues sit in one face of the molecule (Figure 7). The mutations do not affect the binding of the complex to uPAR, which confirms the absence of physical interaction between PAI-1 and uPAR in the initial step in the endocytosis pathway [12,23]. The difference in mutant effect between endocytosis (2–3-fold) and ligand-binding assays (5–20-fold) can be explained by assuming that uPAR functions as a scaffold for complex binding to the cell surface, which efficiently presents the complex to the α_2MR/LRP [12,26]. Also, the binding of the mutant complexes to VLDLR is reduced. This is in agreement with uPA·PAI-1 having the same binding mode for the two receptors.

rPAI-1/K82A-R120A and rPAI-1/R78A-K124A were indistinguishable from rPAI-1/wt and natural PAI-1 with respect to protein stability, inhibitory activity towards uPA, uPA·PAI-1 formation and stability, and binding to vitronectin. This indicates that the mutant proteins assume the wild-type PAI-1 conformation. That the double substitutions do not affect the inhibitory activity towards uPA is in good agreement with results by others showing that Arg-78, Lys-82 [45], Asp-104 and Lys-106 [46] are not involved in PA inhibition. The last two residues also localize to the same structural region as Arg-78, Lys-82, Arg-120 and Lys-124 [7,10,11]. Mutations in PAI-1 that reduce its vitronectin binding, map to the same secondary structural

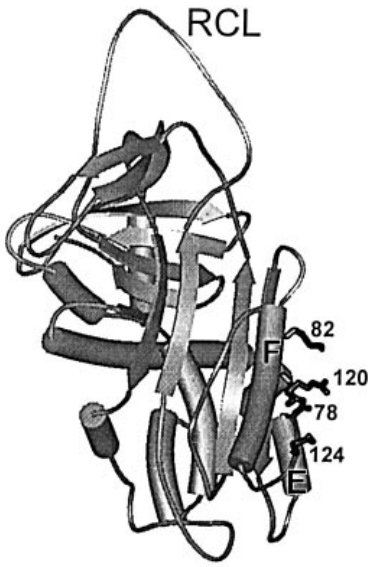

Figure 7 Representation of the substituted residues in the PAI-1 structure

Side-chains of the residues Arg-78, Lys-82, Arg-120 and Lys-124 are shown by 'stick' representation in the Cα-trace for the three-dimensional model of the active conformation of the PAI-1 serpin structure [55]. RCL, E and F indicate the RCL, and the α-helices E and F [56]. Note that we modelled an arginine residue in position 120 (see the Discussion section).

elements where Arg-120 and Lys-124 reside, i.e. helix E and β-strand 1A [47–49]. However, while the substitution Q125K strongly reduces vitronectin binding of PAI-1 [47], the substitutions presented in this study exhibit wild-type binding. The rationale for this observation must be that Arg-120 and Lys-124 lie outside the vitronectin-binding region in PAI-1.

Inspection of the X-ray crystal structures of the different conformations of PAI-1 [10,11] indicated that the residues Lys-82, Arg-120 and Lys-124 in PAI-1 are free to be involved in intermolecular interactions, since these side-chains are not involved in intramolecular interactions (Figure 7). The side-chain of Arg-78, however, makes contact with the backbone of Arg-115 in the latent as well as in the cleaved conformation [10,11]. The results presented here indicate that this contact is not relevant for the assayed functions of PAI-1. Moreover, the involvement of the implicated basic residues in uPA·PAI-1 in making direct contact with the receptor via salt bridges, is in agreement with the strong sensitivity of the receptor-binding to ionic strength. The abolished receptor-binding at pH 4.1 would be in good agreement with the involvement of carboxyl groups in the interaction, titrating between pH 4 and 6. The latter observation is in line with previous findings [28] and the working model for endocytosis receptor–ligand interaction [3,12–17]. These observations allow us to state confidently that the implicated basic residues make direct contact with the receptors.

In the X-ray crystal structures for latent [10] and cleaved PAI-1 [11], an alanine, but not an arginine, residue is seen in position 120. However, all available PAI-1 gene sequences in the EMBL database and our own nucleotide sequence records (results not shown) indicate an arginine codon in the wild-type cDNA sequence in this position. The high B-factor (relative mobility of an ion in a protein in a crystal structure) for 'Ala-120' and the absence of intramolecular interactions of the arginine side-chain could explain why it is not seen in the structures [10,11]. The structures were analysed and the alanine residue in position 120 in the PAI-1 structure was replaced by arginine, using the PC-based version of the protein modelling program WHATIF [50].

The rationale for introduction of the particular substitutions R120A and K124A came from binding-competition results showing that Fab fragment (i.e. the fragment of the Mab having the antigen binding site) of the anti-PAI-1 Mab-2 [51], with an epitope localized to the PAI-1 residues 102–145 [49], competes with the complex for binding to α_2MR/LRP [25,26]. Arg-78 and Lys-82 were substituted, since they are involved in the binding of heparin [52], and heparin prevents the binding of uPA·PAI-1 to α_2MR/LRP [26]. Since Nykjær et al. [26] showed that the multiple independent binding areas of the complex contribute to the receptor-binding, double- instead of single-site substitutions of the same nature (basic to alanine) were chosen to maximize a possible mutant effect on receptor binding. Moreover, since the selected basic residues are not involved in intramolecular interactions in the PAI-1 structures, it was considered safe to construct the double mutations. Furthermore, basic residues are often involved in protein–protein interactions [28,30,53].

The region in PAI-1 containing the residues Arg-78, Lys-82, Arg-120 and Lys-124 is part of the flexible-joint region, covering the α-helices D, E and F, and β-strands 2A and 1A, as discussed by Stein and Chothia [7]. These authors argue that this region in serpins is flexible relative to the rest of the molecule, to accommodate the transition from the active to the cleaved conformation, which involves strand insertion of the exposed RCL into the central β-sheet A after cleavage in the RCL. The flexible-joint region in PAI-1, besides the binding of heparin [52] and vitronectin [47], is also involved in binding to endocytosis receptors, suggesting that it acts as a conformation-dependent multi-protein docking area. The flexibility of this region in PAI-1 is supported by observed differences in proteolytic susceptibility of the latent, cleaved and active conformations to a variety of non-target proteases [54].

α_2MR/LRP and VLDLR belong to the LDLR family of endocytosis receptors, which includes a total of six presently known members [12–17]. α_2MR/LRP and VLDLR contain a cluster of eight so-called complement-type repeats, which in α_2MR/LRP have been shown to be ligand-binding [3,12–17]. α_2MR/LRP and VLDLR bind a number of serine protease–serpin complexes, with overlapping, but not identical, binding specificity [21], and both receptors bind RAP, whereas only α_2MR/LRP binds α_2-macroglobulin–proteinase complexes [27,37]. This is in good agreement with the observation that reduction in VLDLR binding of the mutant complexes is 3–7-fold, which is less than the 5–20-fold reduction for binding to α_2MR/LRP. This difference in mutant complex binding may reflect the sequence difference between the two receptors [12,15].

The study presented here forms a good starting point to examine whether the new structure–function relationship identified here in PAI-1 is a general feature in endocytosis receptor-binding serpins complexed to their associated proteases [18–21].

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