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Interaction of Plasminogen Activator Inhibitor Type-1 (PAI-1) with Vitronectin: Characterization of different PAI-1 mutants

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1. Introduction

1.1. The uPA-System in Tumor Invasion and Metastasis

One of the first processes eventually leading to tumor cell invasion and metastasis is degradation of the extracellular matrix (ECM) by various proteases. Plasmin is one of these proteases with broad substrate specificity. Two different plasminogen activators, urokinase (uPA) and the tissue type plasminogen activator (tPA), generate plasmin from plasminogen. Whereas tPA is mainly involved in the intravascular regulation of plasmin activity, the serine protease uPA regulates pericellular plasminogen activation. Its activity is focused to the cell surface through binding to its specific glycosyl-phosphatidyl-inositol-(GPI-) anchored receptor, the uPA receptor (uPAR, CD87). The most important inhibitors of uPA are the plasminogen activator inhibitors type 1 (PAI-1) and type 2 (PAI-2). The uPA-system is not only important for ECM degradation, but it is also engaged in angiogenesis and certain other processes of cancer cell-directed tissue remodeling, such as fibroblast proliferation and secretion of ECM proteins [Andreasen et al., 2000].

These observations are supported by clinical data, showing that tumor tissue uPA- or PAI-1-antigen levels are strong markers of disease-free survival and overall survival in patients with a variety of solid tumors, *e.g.* mammary, ovarian, cervical, colorectal, bladder, renal, and lung carcinomas [Schmitt et al., 1997] [Harbeck et al., 2001]. Elevated uPA and/or PAI-1 indicate a poor prognosis; in contrast to this, elevated PAI-2 antigen levels predict a good prognosis [Magdolen et al., 2000]. Until today, it is still not clear, why an inhibitor of pericellular proteolysis like PAI-1 should have a negative impact on patient outcome. In this scenario, several functions other than its antiproteolytic activity become important, *e.g.* in cell adhesion, neovascularization, or cell signaling. The interaction of PAI-1 with Vn seems to play an important regulatory role in these processes.

1.2. Biochemical Properties of PAI-1

PAI-1 (Fig. 1) is a 50 kDa protein of the serine protease inhibitor (serpin) family. The serpins are similar in structure and inhibit proteases via a common mechanism involving conformational changes inside the serpin-molecule [Andreasen et al., 2000].

Fig. 1. Important structural elements of active PAI-1. The three-dimensional structure of active PAI-1 (PDB 1B3K) is depicted. The central β-sheet A consisting of s1A, 2A, 3A, 5A, and 6A as well as the reactive center loop (RCL) are indicated in yellow, α-helix E (hE) in orange, and α-helix F (hF) in green. The P1-residue of PAI-1 (R346) as well as residue Q123 within the proposed Vn binding region are marked in cyan blue.

During protease inhibition, the surface-exposed reactive center loop (RCL) with the P1 site of PAI-1 interacts with the target protease and an intermediate enzyme inhibitor complex, the so-called Michaelis complex, is formed (Fig. 2, E·I). This reaction is followed by cleavage of the P1-P1' bond of the inhibitor by the protease, which leads to the formation of a covalent acyl enzyme intermediate complex (E-I), insertion of the RCL as additional β-strand 4A and translocation of the protease across the plane of β-sheet A. During this process, the conformation of the protease

is altered and therefore the protease becomes catalytically inactive in the final covalent complex (E-I†). Alternatively, during slow RCL-insertion, the cleaved inhibitor can be released from the protease before the RCL is totally inserted (Fig. 2, k_3). At a much lower rate (Fig. 2, k_5), the cleaved inhibitor can also dissociate from the covalent enzyme inhibitor complex with the inserted RCL. This cleaved form is the so-called RCL-cleaved form of PAI-1 (Fig. 2, I*; Fig. 3) [Stratikos and Gettins, 1999].

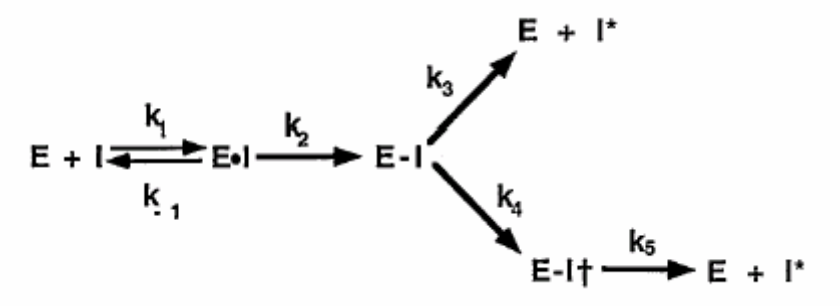

Fig. 2. Serpin enzyme inhibitor kinetics. E: enzyme, I: inhibitor, E·I: non-covalent Michaelis complex; E-I: covalent acyl enzyme intermediate, prior to RCL-insertion, E-I†: covalent enzyme inhibitor complex, I*: RCL-cleaved form of inhibitor, explanations in the text. From [Stratikos and Gettins, 1999].

A unique feature of PAI-1 among serpins is its metastability. With a half-life of one to two hours (depending on the reaction conditions) PAI-1 converts into a latent form by insertion of the (uncleaved) RCL into β-sheet A (between s3A and s5A, see Fig. 3). Latent PAI-1 does not inhibit its target serine proteases. *In vitro,* latent PAI-1 can be reactivated by chemical denaturation and subsequent refolding [Carrell et al., 1991] [Hekman and Loskutoff, 1985]. Interestingly, Berkenpas et al., 1995, generated a PAI-1 quadruple mutant (14-1b, N150H K154T Q319L M354I) with a significantly increased half-life of 145 hours by screening a PAI-1 mutant phage display library. This mutant is now widely used in *in vitro* and *in vivo* studies (see Chapters 1.6.2 and 1.6.4). Applying a similar technique, a mutant (st-44, eleven aa alterations) with an even longer half-life of more than 350 hours was produced [Stoop et al., 2001].

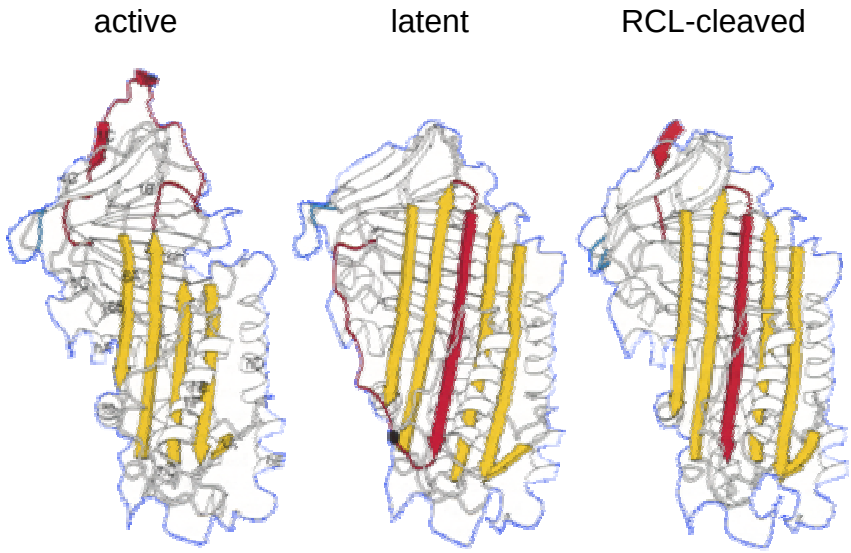

Fig. 3. Structures of active, latent, and RCL-cleaved PAI-1. In the latent and RCL-cleaved forms of the inhibitor, the whole RCL or part of it is inserted into β-sheet A as additional strand 4A (from [Sharp et al., 1999].

PAI-1 interacts with many proteins other than proteases. Most importantly, it can bind to vitronectin (Vn), an interaction with high biological relevance (see Chapter 1.5), but also to other ECM molecules such as fibrin and heparin (Hep) as well as to the acute phase protein alpha(1)-acid glycoprotein. Vn and, to a much lower extent, alpha(1)-acid glycoprotein are able to stabilize the active conformation of PAI-1 [Andreasen et al., 2000] [Boncela et al., 2001], whereas binding of Vn and Hep to PAI-1 provide it with thrombin inhibitory properties [Ehrlich et al., 1990] [Ehrlich et al., 1991].

1.3. Other Plasminogen Activator Inhibitors

The second plasminogen activator inhibitor, PAI-2 (47 kDa), reacts slower with uPA than PAI-1. It displays 55% sequence homology to PAI-1 and a similar structure. In contrast to PAI-1, it does not bind to ECM proteins such as Vn, does not convert into a latent form, and is mainly localized intracellularly [Magdolen et al., 2000]. Interestingly, PAI-2 is able to spontaneously form polymers under physiological conditions. Several studies suggested PAI-2 to play a role in inflammation, as it is induced by inflammatory mediators such as lipopolysaccharide, interleukin-1, and

tumor necrosis factor [Ny and Mikus, 1997]. One of the underlying mechanisms of this function might be its protective effect on macrophages from TNF-α induced apoptosis, which could be its main physiological role in at least some cell types [Irigoyen et al., 1999]. PAI-2 was shown to decrease tumor growth, as well as angiogenesis and metastasis of tumors [Ny and Mikus, 1997]. This is in line with results describing PAI-2 as an independent prognostic factor. Elevation of PAI-2 antigen in tumor tissue predicts a good prognosis for these cancer patients [Magdolen et al., 2000].

In addition to PAI-1 and PAI-2, there are two other serpins, which are able to inhibit plasminogen activators: proteinase nexin-1 (PN-1) and protein C inhibitor (PCI, also known as PAI-3) [Andreasen et al., 1997].

1.4. Biochemical Properties of Vn

Vn is a 75 kDa glycoprotein present in blood and in the ECM. Vn interacts with many molecules other than PAI-1, *e.g.* glycosaminoglycans, collagen, plasminogen, uPAR, heparin, components of the complement system, and thrombin/anti-thrombin III-complexes. Moreover, many integrins such as αvβ3, αvβ5, αIIbβ3, and αvβ1 can interact with Vn *via* its arginine/glycine/aspartate (RGD) motif. Vn consists of several domains. The N-terminal part encompasses the somatomedin B (SMB) domain (amino acids [aa] 1-44), followed by the RGD motif (aa 45-47), and the so-called connecting region (aa 48-131). The rest of the molecule is covered by six hemopexin repeats (aa 132-459) (for a review on Vn see [Schvartz et al., 1999]). The SMB domain is the most important domain interacting with components of the uPA system, as it contains the high affinity binding sites for PAI-1 (see Chapter 1.5.1) and uPAR [Okumura et al., 2002]. In plasma, Vn exists as a folded monomer whereas in the ECM or after binding to complement factors, thrombin/antithrombin III-complexes, or active PAI-1, it exists as a disulfide linked multimer. Most of the Vn-ligands, including PAI-1, preferentially interact with the multimeric form of Vn [Schvartz et al., 1999]. A

third form of Vn, the so-called two-chain Vn, is present in blood. Two-chain Vn results from the cleavage of full length Vn after R379 by an unidentified protease and consists of two fragments connected *via* a single disulfide bond. Due to a polymorphism at position 381 (threonine *versus* methionine), different molecular forms and also amounts of two-chain Vn may exist *in vivo*, as threonine rather than methionine at this position will favor the cleavage. However, no obvious differences with respect to the multimeric state, heparin-binding activity, and PAI-1-binding activity between full-length and two-chain vitronectin were observed [Gibson and Peterson, 2001].

1.5. Interaction of PAI-1 with Vn

Only PAI-1 in its active conformation and neither latent nor RCL-cleaved nor PAI-1 in complex with its target proteases can bind to Vn [Lawrence et al., 1997]. Addition of uPA to a PAI-1/Vn complex leads to dissociation of this complex [Loskutoff et al., 1999]. Upon binding of Vn to active PAI-1, conformational changes in PAI-1 are induced, approximately doubling its half-life [Declerck et al., 1988] and providing it with inhibitory properties towards other serine proteases, namely thrombin and activated protein C [Ehrlich et al., 1990] [Rezaie, 2001] (see also Chapter 1.5.3).

PAI-1 competes with cell surface receptors like uPAR and integrins for Vn-binding (see Chapter 1.6.1) [Irigoyen et al., 1999]. Because of these important implications of the PAI-1/Vn interaction, the binding sites for PAI-1 on Vn and those for Vn on PAI-1 were mapped as will be described in this chapter.

1.5.1. The PAI-1 Binding Site on Vn

Many groups have tried to map the binding site for PAI-1 on Vn (for a summary see table 1). These studies indicate that the major high-affinity PAI-1 binding region on Vn is localized in the SMB domain and is most likely situated between aa L24 and

S30. There are indications for at least another low-affinity PAI-1 binding site in the C-terminal region of Vn (for a review see [Schroeck et al., 2002]).

Methods	**Important amino acids or regions for PAI-1-binding**
PAI-1 binding to Vn fragments resulting from acid or cyanogen bromide treatment [Seiffert and Loskutoff, 1991]	SMB
hybrid proteins containing aa 1-30, aa 1-40, and aa 1-52 of Vn and epitope mapped monoclonal antibodies [Seiffert et al., 1994]	SMB[1]
domain swapping strategy using the homologous SMB domain of the megakaryocyte-stimulating factor [Deng et al., 1996a]	aa G12-S30 and all eight cysteines in SMB[1]
alanine scanning mutants of the SMB domain of Vn [Royle et al., 2001]	aa L24-S30 in SMB[1]
two monoclonal antibodies, one directed to the SMB domain and one to the C-terminal region of Vn [Podor et al., 2000]	SMB and C-terminal domain
competition of PAI-1 with monoclonal antibodies and heparin for Vn-binding[2] [Kost et al., 1992]	aa K348 – R370
PAI-1-coated microtiter plates[2] [Gechtman et al., 1993]	aa K348 – R370
synthetic peptide inhibiting PAI-1 binding to reduced, pyridylethylated, and cleaved Vn[2] [Mimuro et al., 1993]	aa G115 – E121

Table 1. Selected studies applying different techniques to define PAI-1 binding sites on Vn. [1] In these studies, only part of Vn, *i.e.* the SMB domain and variants thereof, were used to test interaction with PAI-1. Thus, additional PAI-1 binding sites in other regions of Vn cannot be excluded. [2] Possible drawbacks of these studies have been discussed in detail by Deng *et al.*, 1995. Adapted from [Schroeck *et al.*, 2002].

1.5.2. The Vn Binding Site on PAI-1

Lawrence *et al.*, 1994, applied random and site-directed mutagenesis in order to localize the Vn binding site on PAI-1. Five point mutants showed significantly reduced affinities to Vn as compared to wild-type PAI-1 (wt-PAI-1). Three of them (Q55P, L116P, and Q123K) displayed an at least ten-fold increase in K_D values with respect

to wt-PAI-1, whereas the other two (F109S and M110T) displayed only a two- to threefold increase in their K_D values towards Vn. The authors speculated that mainly polar interactions are responsible for binding of PAI-1 to Vn as the most pronounced reduction of affinity towards Vn was obtained by mutating glutamine at position 55 or 123, respectively. The mutant Q123K is now widely used as a non-Vn-binding PAI-1 mutant (see Chapters 1.6.2 and 1.6.4).

Another approach to determine the Vn binding site on PAI-1 was used by van Meijer, *et al.*, 1994. Each of three monoclonal antibodies, which were all mapped to a target region comprising aa 110 to 145 of PAI-1, did partially inhibit PAI-1-binding to Vn.

A similar region (aa 115-130) was claimed by Padmanabhan and Sane, 1995, to be responsible for Vn-binding. Two different approaches were used to elucidate the Vn-binding region. The first one was digestion of PAI-1 with Staph V8 protease and isolation of two fragments that bound to Vn. The two fragments were predicted to overlap between residues 91-130 of PAI-1. As a second approach, four different PAI-1/PAI-2 chimeras with inhibitory activity towards tPA were expressed in an *in vitro* transcription/translation system and tested for Vn-binding using immunoprecipitation of soluble PAI/Vn complexes employing an antibody to Vn (wt-PAI-1 but not wt-PAI-2 interacts with Vn). Only wt-PAI-1 and one chimera ($PAI\text{-}1_{1\text{-}167}/PAI\text{-}2_{201\text{-}415}$) were able to form complexes with Vn, but not the other chimeras (such as $PAI\text{-}1_{1\text{-}114}/PAI\text{-}2_{147\text{-}415}$) or wt-PAI-2. This led to the conclusion that the peptide sequence between aa 115 and 167 of PAI-1 is important for Vn-binding [Padmanabhan and Sane, 1995].

Crosslinking experiments with PAI-1/Vn complexes followed by cyanogen bromide cleavage were performed by Deng *et al.*, 1995, in order to locate the Vn-binding site. Here, Vn was specifically crosslinked to PAI-1 fragments harboring aa 111-147. When the Vn-binding-deficient PAI-1 mutant Q123K was used in these experiments, no crosslinking was observed. Sui and Wiman, 1998, introduced 13 different single aa substitutions into region F113 to D138 and tested these mutants for interaction

with Vn-coated microtiter plates. None of the generated mutants displayed any significant change in affinity towards Vn.

An α-helix F (hF) deletion mutant (deletion S127-D158; [Vleugels et al., 2000]) did not display any inhibitory properties towards its target proteases nor a high affinity binding to Vn, but showed substrate behavior.

A summary of the different regions of PAI-1 implicated in Vn-binding is given in table 2. (For review and discussion of the Vn-binding site(s) see also [Schroeck et al., 2002].)

Methods	**Important amino acids or regions for Vn-binding**
PAI-1 variants with point mutations generated by random and site-directed mutagenesis [Lawrence et al., 1994]	Q55P, L116P, and Q123K (less important F109S and M110T)
monoclonal antibodies inhibiting PAI-1/Vn interaction [van Meijer et al., 1994]	aa M110 – K145
crosslinking followed by cyanogen bromide cleavage [Deng et al., 1995]	aa P111 – M147
Staph V8 protease digestion PAI-1/PAI-2 chimeras, immunoprecipitation of complexes with Vn [Padmanabhan and Sane, 1995]	aa I91 – E130 aa R115 – N167
13 single aa substitutions in the region F113 to D138 [Sui and Wiman, 1998]	None
α-helix F deletion mutant [Vleugels et al., 2000]	aa S127 – D158

Table 2. Selected Studies Defining Vn Binding Sites on PAI-1. Adapted from [Schroeck *et al.*, 2002].

1.5.3. The PAI-1/Vn Complex

Most of PAI-1 in plasma is present in a high molecular weight complex with Vn [Declerck et al., 1988]. By ultracentrifugation, the PAI-1/Vn-complex was found to have a relative molecular mass of 324,000 ± 14,000, which could be either a complex

of three PAI-1 and three Vn molecules (theoretical M_r: 345,000) or a complex of four PAI-1 and two Vn molecules (theoretical M_r: 316,000) [Podor et al., 2000]. The 4:2 complex is more likely, when considering the two possible PAI-1 binding sites on Vn (see 1.5.1). Additionally, in the ultracentrifugation experiments, free Vn was still detected after complex formation in the presence of equimolar amounts of PAI-1 and Vn. Based on these and other results, the following model for the formation of PAI-1/Vn-complexes was proposed [Podor et al., 2000] (Fig. 4): the high-affinity PAI-1 binding site within the SMB domain of Vn is not exposed in monomeric Vn [Seiffert and Smith, 1997]. Therefore, PAI-1 initially interacts with monomeric Vn *via* the C-terminally located low-affinity binding site, leading to a conformational change within the SMB domain and unmasking of the high-affinity PAI-1 binding site [Seiffert, 1997a]. Additionally, interaction of Vn with PAI-1 promotes oligo-/multimerization [Seiffert, 1997b] and thus enhanced affinity of these PAI-1/Vn complexes to the ECM and to smooth muscle cells [Minor and Peterson, 2002].

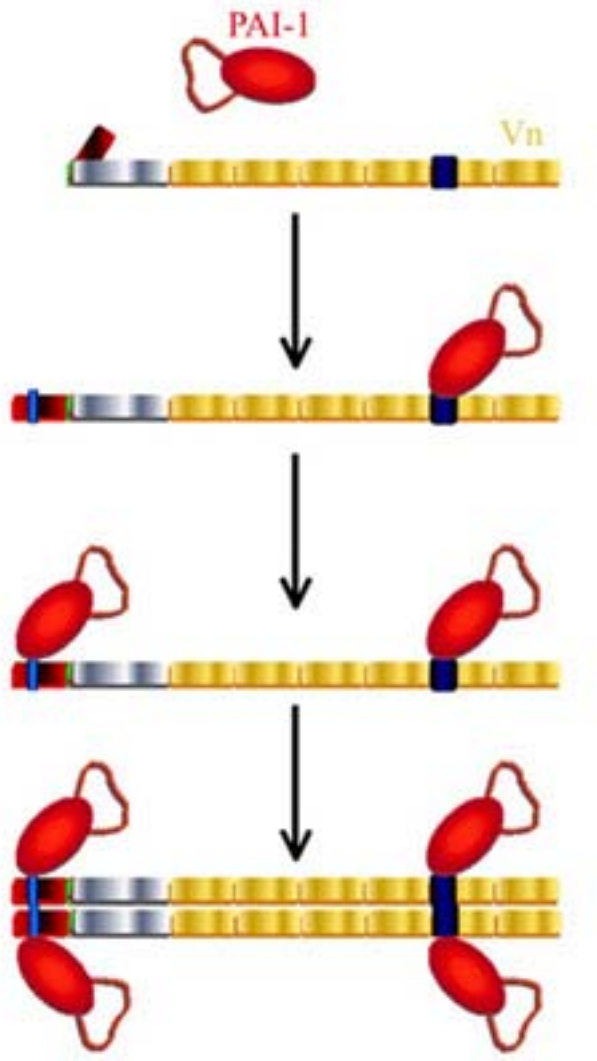

Fig. 4. Model describing the generation of PAI-1/Vn-complexes. PAI-1 initially interacts with monomeric Vn, which leads to a conformational change and to the unmasking of the high-affinity PAI-1 binding site. The PAI-1/Vn complex associates with a second complex [Podor et al., 2000].

By binding to monomeric - but not multimeric - Vn, the substrate specificity of PAI-1 is altered, because this interaction enables PAI-1 to inhibit other serine proteases, *e.g.* thrombin, in addition to uPA and tPA. Using fluorescently labeled PAI-1, it was demonstrated that binding of monomeric vitronectin to PAI-1 induces conformational changes in the vicinity of the P1-P1' bond, rather than changes affecting the whole RCL [Gibson et al., 1997]. The PAI-1/Vn-complex inhibits thrombin by formation of ternary PAI-1/Vn/thrombin-complexes as well as binary PAI-1/thrombin-complexes, as determined by surface plasmon resonance and immunoprecipitation. In contrast,

during inhibition of tPA by the PAI-1/Vn complex, only binary PAI-1/tPA-complexes are formed [van Meijer et al., 1997]. Formation of the PAI-1/Vn/thrombin-complexes allows endocytosis of thrombin via low density lipoprotein receptor-related proteins (LRP) 1 and 2 [Stefansson et al., 1996].

1.6. PAI-1 in Tumor Biology

1.6.1. PAI-1 and Tumor Cell Adhesion

As the SMB domain of Vn not only contains a PAI-1 binding site but also a binding site for uPAR (see Chapter 1.4), binding of PAI-1 to Vn can competitively inhibit uPAR-mediated binding of U937 cells to Vn [Deng et al., 1996b]. Moreover, probably due to the proximity of the PAI-1-binding SMB domain and the RGD motif in Vn, PAI-1 is able to reduce Vn-binding to $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins [Kjøller et al., 1997] and thus integrin-mediated cell adhesion to Vn [Stefansson and Lawrence, 1996]. Using three different tumor cell lines and truncated forms of Vn it could be shown that this reduction in uPAR- as well as integrin-mediated cell adhesion is due to the specific binding of PAI-1 to the SMB domain of Vn [Deng et al., 2001].

1.6.2. PAI-1 and Tumor Cell Migration

During migration, a tumor cell needs a fine balance between enhanced adhesion at the leading edge and detachment from the ECM at the trailing edge. PAI-1, together with integrins, uPAR, uPA and the LDL receptor-related protein, has a significant influence on these processes. There are reports of a pro-migratory as well as of an anti-migratory role of PAI-1. For example, PAI-1 was reported to block smooth muscle cell migration due to inhibition of integrin $\alpha_v\beta_3$-dependent cell adhesion [Stefansson and Lawrence, 1996] as well as migration of two epithelial cell lines [Kjøller et al., 1997]. In both studies it was confirmed, that this effect is due to PAI-1 binding to Vn, as a mutant without inhibitory properties (R346A) inhibited migration

similar to wt-PAI-1. In contrast, PAI-1 enhanced uPA/uPAR-dependent migration of a melanoma cell line [Stahl and Mueller, 1997] and of U937 and 293 cells [Waltz et al., 1997] over Vn matrices. In a perfused capillary culture system, PAI-1 secreted by endothelial cells inhibited flow-induced smooth muscle cell migration. This effect was due to both the uPA-inhibiting properties as well as the Vn-binding ability of PAI-1, as tested with a stable PAI-1 mutant not binding to Vn (quadruple mutant 14-1b [Berkenpas et al., 1995] plus mutation Q123K) and with a PAI-1 mutant binding to Vn but lacking inhibitory activity (T333R and A335R) [Redmond et al., 2001].

These contradictory results may be explained by two aspects. First, the inhibitory activity of PAI-1 and the Vn-binding ability could be of different relevance in different cell types and test systems. Thus, some observations would be due to PAI-1-induced reduction of uPA activity in the test system and others due to PAI-1-binding to Vn. Second, there is the possibility that PAI-1-binding to Vn and inhibition of cell attachment is of crucial relevance in all these cell migration assays. Then, low levels of PAI-1 would lead to cells so tightly adhered that migration gets difficult (due to unopposed adhesion through integrins and uPAR) and high levels of PAI-1 would loosen cells so much that they do not have enough contact to the ECM to be able to move through it (due to almost complete blocking of cell adhesion sites by PAI-1).

Another influencing factor is the actual amount of PAI-1 attached to Vn and thus able to inhibit cell adhesion, which in turn is influenced by the amount of active uPA present (PAI-1 detaches from Vn when inhibiting uPA, see 1.5) and also by the expression of uPAR and receptors of the LDL-receptor family. Receptors of this family are able to endocytose ternary uPAR/uPA/PAI-1 complexes with subsequent degradation of uPA and PAI-1 and recycling of a substantial amount of the uPAR. There is the possibility that through this internalization process PAI-1 is also involved in the relocalization of uPAR to the leading edge of the cell during migration [Kjøller, 2002]. In fact, it was shown that any inhibition of the formation of the uPAR/uPA/PAI-1 complex as well as interference with the LRP binding to this

complex significantly reduced human muscle-satellite-cell motility [Chazaud et al., 2000].

1.6.3. PAI-1 and Tumor Cell Invasion

Tumor cell invasion is even more complicated, as it not only involves cell motion but also penetration of cells into the ECM. Similar to the results obtained with cell migration assays, there are contradictory results about the effect of PAI-1 on tumor cell invasion. *In vitro*, invasion of lung carcinoma cells into amnion membranes was inhibited by PAI-1-binding to Vn in one study [Bruckner et al., 1992], whereas in another study co-expression of PAI-1 and uPA was necessary for optimal invasion of human lung cancer cells through the experimental extracellular matrix Matrigel [Liu et al., 1995]. A third study did not detect any differences in *in vitro* invasiveness after transfection of prostate carcinoma cells with PAI-1 expression plasmids [Soff et al., 1995]. The reasons for these discrepancies might lie in the interaction of PAI-1 with uPAR, uPA and LRP, similar to those discussed above. Probably PAI-1 can have pro-invasive functions at concentrations, which allow exactly the right amount of activation of plasminogen by uPA and thus adequate ECM degradation as well as the right level of cell detachment. At higher PAI-1 concentrations, cells may be unable to invade because of a lack of ECM-degradation or the impossibility to attach to the ECM at the leading edge, whereas at very low PAI-1 concentrations cells could also be unable to invade due to unrestricted ECM degradation or too tight attachment to the ECM.

1.6.4. PAI-1 and Angiogenesis

The first ones to report a substantial role of PAI-1 in angiogenesis were Bajou *et al.* 1998. These authors provided evidence that PAI-1 deficiency of PAI-1 knockout mice prevents angiogenesis in tumors of implanted malignant keratinocytes. Angiogenesis could be restored by infection with an adenovirus carrying the human PAI-1-gene. Moreover, PAI-1 deficient mice also showed significantly reduced angiogenesis in a

fibrosarcoma model and in a bFGF induced corneal neovascularization model [Gutierrez et al., 2000]. It is important to note that these effects were due to the hosts lacking PAI-1 as wt tumor cells (with an intact PAI-1 gene) were implanted.

In contrast, tumors induced by human prostate carcinoma cells overexpressing PAI-1 showed a lower vessel density as control tumors in nude mice [Soff et al., 1995]. In another study, exogenously added stable PAI-1 (mutant 14-1b of [Berkenpas et al., 1995]) was a potent inhibitor of angiogenesis in three different *in vitro* models as well as an effective treatment for nude mice inoculated with a human prostate cancer cell line (LNCaP) [Swiercz et al., 2001]. In line with this, [Brodsky et al., 2001] reported an inhibitory effect of PAI-1 on angiogenesis in an *ex vivo* aortic ring assay. Moreover, PAI-1 was shown to inhibit FGF2 induced angiogenesis in the chicken chorioallantoic membrane. This effect was due to both inhibitory activity and Vn-binding of PAI-1 as tested with a non-Vn-binding mutant (Q123K) and a mutant binding to Vn but lacking inhibitory activity (T333R and A335R) [Stefansson et al., 2001].

Bajou *et al.*, 2001, found that PAI-1 controls tumor cell invasion and angiogenesis by interaction with proteases and not with Vn. In this study, a malignant murine keratinocyte-coated collagen gel was implanted into PAI-1 knockout mice. One day after implantation, they were infected with adenoviruses harboring expression genes for wt-PAI-1, PAI-1 mutant Q123K (not binding to Vn), or PAI-1 mutant R346M M347S (binding to Vn but not inhibiting PAs [Bajou et al., 2001]), as well as with adenoviruses containing a vector control. PAI-1 knockout mice infected with control virus or virus bearing PAI-1 mutant R346M M347S did not show any tumor cell invasion or angiogenesis, whereas in knockout mice infected with virus bearing wt-PAI-1 or PAI-1 mutant Q123K invasion and angiogenesis were restored.

Recent *in vivo* and *in vitro* studies support the idea of dose-dependent effects of PAI-1 on angiogenesis, which could explain the contradictory results described above. Implanting FGF2 containing Matrigel in mice leads to sprouting of vessels into this implant. McMahon *et al.*, 2001 added different amounts of active PAI-1 (stable mutant 14-1b [Berkenpas et al., 1995]) to these implants in addition to FGF2 prior to

implantation and then evaluated angiogenesis. They also implanted FGF2-containing Matrigels into PAI-1 knockout mice and mice overexpressing PAI-1. Implants without PAI-1 or implants in knockout mice as well as implants with high amounts of PAI-1 or implants in mice overexpressing PAI-1 induced very little angiogenesis compared to implants with an intermediate PAI-1 concentration. This study provides evidence that these effects are responsible for optimal tumor growth when intermediate concentrations of PAI-1 are present, whereas very low or very high concentrations of PAI-1 impair tumor growth. Similar results were obtained when an aortic ring assay was used to measure angiogenesis [Devy et al., 2002]. Aortic rings from PAI-1 knockout mice cultured with increasing amounts of stable recombinant PAI-1 (5 aa alterations, $t_{½}$=150h) displayed high numbers of microvessel outgrowth with intermediate PAI-1 concentrations. Lack or excess of PAI-1 led to reduced angiogenesis. In order to test whether this effect was due to inhibition of PAs or due to interaction with Vn, the mutants (in a stable background) Q123K, R346A and R346M M347S were used. Mutant Q123K was able to restore microvessel outgrowth, whereas the other two mutants were able to induce a similar effect only in very high concentrations. Thus, the authors drew the conclusion that the antiprotease activity of PAI-1 is crucial for the proangiogenic effect of PAI-1, but still the interaction with Vn could play a role at non-physiological high concentrations [Devy et al., 2002].

PAI-1 is not only essential for tumoral angiogenesis, but was also shown to be crucial for neovascularization in a mouse model of angiogenic ocular disorders such as age-related macula degeneration or diabetic proliferative retinopathy. Following retinal photocoagulation with an argon laser, PAI-1 knockout mice failed to develop significant amounts of choroidal new vessels in contrast to wt-mice or PAI-1 knockout mice infected with an adenovirus carrying PAI-1 cDNA [Lambert et al., 2001].

Taken together, a balanced proteolysis is necessary for angiogenesis to occur, as both a lack in proteolytic activity (due to knockout of plasminogen or excess of PAI-1 [Devy et al., 2002]) and unlimited proteolysis (e.g. in PAI-1 knockout mice) lead to impaired vessel formation.

1.6.5. PAI-1 and Metastasis in Animal Models

There is no clear picture of the relevance of PAI-1 for tumor cell metastasis. In one study, B16 murine melanoma cells were intravenously injected into PAI-1 knockout mice, mice overexpressing PAI-1 in several tissues, and control mice, but no difference in the number of pulmonary metastases could be detected [Eitzman et al., 1996]. Other groups provide evidence for PAI-1 reducing metastases: a prostate carcinoma cell line (PC3) stably transfected with PAI-1 cDNA induced smaller primary tumors and less lung and liver metastases than control cells [Soff et al., 1995]. In line with this, growth of prostate cancer xenografts in SCID mice could be reduced by intravenous injection of two different stable PAI-1 mutants (14-1b [Berkenpas et al., 1995] and T333R) [Jankun et al., 1997]. Moreover, liver metastases and metastatic tumor burden in nude mice with uveal melanoma could be significantly reduced by adenoviral gene transfer of PAI-1 cDNA [Ma et al., 1997]. Adenoviral transfer of PAI-1 cDNA to cultured HT-1080 (human fibrosarcoma) cells prior to subcutaneous implantation in SCID mice significantly reduced the incidence of lung metastases in these mice [Praus et al., 1999].

In contrast, production of uPA and PAI-1 by human melanoma cell lines correlated with their ability to spontaneously metastasize to the lung of nude mice after subcutaneous inoculation but not after intravenous injection [Quax et al., 1991]. A polyclonal antibody to PAI-1 was able to significantly reduce pulmonary metastases in nude mice after intravenous injection of a HT-1080 cell line selected for high metastatic potential [Tsuchiya et al., 1995]. Using the same model but a HT-1080 clone first selected for low constitutive expression of PAI-1 and then transfected with PAI-1 cDNA or control vector, this group could show that a three to five fold increase in PAI-1 levels will lead to significantly more pulmonary metastases [Tsuchiya et al., 1997].

It is tempting to speculate that the observed discrepancies in the effects of PAI-1 on tumor metastasis are due to a dose-dependent response of tumor behavior on PAI-1

levels, similar as it was shown for PAI-1's influence on angiogenesis (see Chapter 1.6.4).

1.7. Aim of this Study

Interaction of PAI-1 with the extracellular matrix protein vitronectin (Vn) plays an important role in tumor biology. If compounds were developed, which specifically inhibit one of the various functions of PAI-1, *i.e.* binding of PAI-1 to Vn, this could lead to the design of clinically applicable inhibitors of tumor migration, invasion, angiogenesis or metastasis. In order to find such compounds and to be able to interpret results from *in vitro* and *in vivo* studies with them, a profound knowledge about the structure of the Vn-binding site of PAI-1 and the biochemical interactions of PAI-1 with Vn is essential.

Several studies have been undertaken to define the Vn-binding site on PAI-1 in more detail (see Chapter 1.5.2), but there are still discrepancies between these studies. However, there is evidence pointing towards a Vn-binding region around α-helix E (hE) and/or α-helix F (hF) of PAI-1.

In order to further resolve the Vn-binding site of PAI-1, PAI-1 variants with alterations mainly around hE and hF were expressed in *E. coli* and characterized concerning

- their specific inhibitory activity towards uPA,
- their ability to functionally interact with Vn, as measured with four different techniques, including the newly established surface plasmon resonance analysis,
- as well as their interaction with heparin, another non-proteolytic interaction partner of PAI-1.

Special attention was paid to precisely describing the differences in Vn-binding behavior between wt-PAI-1 and the now widely used non-Vn-binding PAI-1 mutant Q123K.

Moreover, *in vitro* assays were performed in order to define the role of PAI-1 in tumor cell invasion more closely. For this, the effect of wt-PAI-1 on Vn-dependent invasion of ovarian cancer cells through the experimental extracellular matrix Matrigel was analyzed.

2. Materials and Methods

2.1. Preparation of wt-PAI-1, wt-PAI-2 and PAI-1 Variants

2.1.1. Expression in *E. coli*

Frozen bacterial stocks with expression plasmids for wt-PAI-1, wt-PAI-2 and the PAI-1 variants were kindly provided by Nuria Arroyo de Prada from our research group. Briefly, the coding regions of the proteins were cloned in frame with an N-terminal His_6-tag into the *E. coli* expression vector pQE-30 and then transformed into the *E. coli* strain XL1 blue. The mutated sequences were verified by DNA-sequencing (for details see [Arroyo de Prada, N., Schroeck, F., et al., 2002]. For expression of recombinant protein 1 µl of bacterial stock solution was added to 5 ml of LB-medium (5 g NaCl + 5 g yeast extract [Difco, Detroit, MI, USA] + 10 g tryptone peptone [Difco] ad 1 L aqua bidest) supplemented with 100 µg/ml ampicillin (Sigma, St. Louis, Mo, USA) and incubated overnight at 37 °C on an orbital shaker at 200 r.p.m. On the next day, this pre-grown bacterial suspension was added to 250 ml of LB-medium supplemented with 100 µg/ml ampicillin and incubated at 37 °C on the orbital shaker until the optical density at 600nm (OD_{600}) reached 0.6 to 0.7. Expression of recombinant proteins was then induced by adding isopropyl thio-β-D-galactoside (IPTG) at a final concentration of 2 mM and incubation overnight at 37 °C on the orbital shaker. If a new bacterial stock solution was prepared, 800 µl of the bacterial suspension were mixed with 200 µl of glycerol and subsequently frozen at -80 ° C.

2.1.2. Purification

In order to isolate recombinant proteins from the bacterial suspension the following protocol was used: The bacterial culture was harvested by centrifugation at 5,000 x g at 10 °C for 10 min. The resulting pellet was frozen at –80 °C and subsequently resuspended in 20 mM Na-acetate, 1 M NaCl, 0.1% Tween-80 (v/v), pH 7.4,

supplemented with the protease inhibitor mix “Complete EDTA-free” (Roche, Mannheim, Germany). For PAI-2 a slightly different buffer was used (20 mM Na_2HPO_4, 1 M NaCl, 0.1% Tween-80 (v/v), pH 7.4, supplemented with “Complete EDTA-free”). Bacteria were disrupted mechanically by addition of glass beads (Sigma, St. Louis, Mo, USA) to the bacterial cell suspension and 10 subsequent cycles of vortexing and incubation on ice for 1 min each. The lysate was centrifuged for 15 min (12,000 x g, 4 °C), the supernatant recovered and subjected to Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) agarose affinity column purification.

The chromatography columns were prepared by adding 10 ml of aqua bidest for 10 min, then emptying them and adding 2 ml of Ni^{2+}-NTA agarose (Qiagen, Hilden, Germany). Subsequently, Ni^{2+}-NTA agarose was equilibrated by addition of 20 ml of equilibration buffer (20 mM Na-acetate, 1 M NaCl, 0.1% Tween-80 (v/v), pH 7.4) and the cleared bacterial lysate was added. This was followed by two washing steps, the first one with equilibration buffer, the second one with washing buffer (20 mM Na-acetate, 1 M NaCl, 0.1% Tween-80 (v/v), 20 mM imidazole, pH 5.6) until the absorption of the effluent had returned to the baseline (OD_{280}<0.001). The adsorbed recombinant proteins were eluted with a buffer containing 20 mM Na-acetate, 1 M NaCl, 0.1% Tween-80 (v/v), 200 mM imidazole, pH 5.6 (elution buffer). Five fractions with 2 ml each were collected per column and analyzed by SDS-PAGE. Then the protein solutions were dialyzed against equilibration buffer and subjected to another Ni^{2+}-NTA agarose affinity column as described above.

For PAI-2 different buffers were used: Equilibration buffer was 20 mM Na_2HPO_4, 1 M NaCl, 0.1% Tween-80, pH 7.4; washing buffer was the same buffer at pH 6.5 supplemented with 20 mM imidazole; and elution buffer was the same buffer at pH 6.0 containing 200 mM imidazole.

2.1.3. Denaturation and Refolding

The purified recombinant proteins, with exception of PAI-2, were exposed to 4 M guanidinium/HCl for 4 h at room temperature under light protection before refolding of

the proteins, which was achieved by dialysis (2 h, 4 °C) in 20 mM Na-acetate, 1 M NaCl, 0.01% Tween-80 (v/v), pH 5.6, followed by a second dialysis step (overnight, 4 °C). The proteins were subsequently concentrated in Centricon centrifugal filter devices (Millipore, Eschborn, Germany) and stored at -80 °C until use. PAI-2 was dialyzed against PBS (pH 7.4) in order to remove residual imidazole, and concentrated and stored like PAI-1.

2.2. Determination of Protein Concentration

Routinely, the Bradford Assay (Bio-Rad Protein Assay Dye Reagent Concentrate from BioRad, Krefeld, Germany) with a BSA standard (Pierce, Rockford, IL, USA) was used for the determination of the protein concentration of the purified proteins. First, 200 µl of the Bradford reagent were added to 800 µl of the appropriate dilution of standard or purified protein in aqua bidest, vortexed and incubated for 10 min at RT. Then samples were mixed again, 180 µl were transferred to a microtiter plate (Greiner, Frickenhausen, Germany) and the OD was measured at 570 nm. Protein concentrations of samples were calculated according to the standard curve. Each data point was at least measured in quadruplicate.

Because, according to the manufacturers manual, this assay was developed for determination of concentrations of protein mixtures and not of purified proteins, one series of measurements was performed in order to compare the results of the Bradford assay to the corresponding BCA micro-assay (Pierce, Rockford, IL, USA), each with three different standards (BSA, ovalbumin [kindly provided by Tobias Maurer, Institut für Medizinische Mikrobiologie, Klinikum Rechts der Isar der TUM], and bovine gamma globulin (BGG) [Pierce, Rockford, IL, USA]). Measurements with the BCA micro-assay were performed according to the manufacturer’s manual.

2.3. Measurement of Inhibitory Activity

2.3.1. Inhibitory Activity against uPA

The assay was performed in a 96-well microtiter plate. Purified recombinant proteins were diluted in „activity buffer“ (100 mM Tris-HCl, 0.05% Tween-20 (v/v), pH 7.5, and 100 µg/ml BSA [ICN, Aurora, Ohio, USA]), incubated with 10 U HMW-uPA (50 µl of a 1:500 dilution; rheotromb® 500,000, Curasan Pharma GmbH, Kleinostheim, Germany) for 15 min at RT and then 10 µl of chromogenic substrate Bz-β-Ala-Gly-Arg-pNA·AcOH (Pefachrome® uPA, Pentapharm LTD, Basel, Switzerland, concentration 2 mM) were added. Following incubation for 30 min at 37 ^{o}C, the OD was measured at 405 nm. One unit PAI activity was defined as the amount, which completely inhibited one unit of HMW-uPA activity.

2.3.2. Inhibitory Activity against Thrombin

The assay was performed in 96-well plates, too. The indicated amount of active recombinant PAI-1 was incubated in the presence or absence of Vn (Promega GmbH, Mannheim, Germany) or Hep (Liquemin® N 25000, Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany) with 0.1 units of thrombin (from human plasma; Sigma, St. Louis, Mo, USA) in a total volume of 130 µl of TST-buffer (20 mM Tris-HCl, 100 mM NaCl, 0.1% Tween-80 (v/v), pH 8.0) at 37 °C for 1 h (adapted after [Ehrlich et al., 1990]). Then, 10 µl of chromogenic substrate (Chromozym® TH, Roche, Mannheim, Germany, concentration: 2 mM) were added and the thrombin activity measured monitoring the change of OD at 405 nm. The initial slope of the OD/time curve of thrombin alone was set to 100% and the other residual thrombin activities were calculated by comparing the slope of the corresponding curve to this control value.

2.3.3. Inhibitory Activity against uPA by Measuring the Amount of Activated Plasminogen

This assay was also performed in microtiter plates. To 100 µl of the desired dilution of recombinant protein 10 units uPA were added per well and incubated for 30 min. at RT. Then 20 µl of 2.5 µM plasminogen (Roche, Mannheim) were added, followed by an incubation for 30 min at 37 °C, addition of 20µl of chromogenic substrate for plasmin (L1450, Bachem, Basel, CH), another incubation for 30 min at 37 °C, and finally the measurement of OD at 405 nm. As in the other assays, one unit PAI-1 activity was defined as the amount, which completely inhibited one unit of HMW-uPA activity. Therefore, when 10 units of PAI-1 were added, uPA was completely inhibited and thus no plasmin was generated as assessed by lack of color formation.

2.4. Determination of the Half-life of the Recombinant Proteins

At first, the activity of all recombinant PAI-1 proteins had to be tested as described in 2.3.1. For this, appropriate amounts of PAI-1 were diluted in a total volume of 50 µl PBS. For each time point and each PAI-1 variant one Eppendorf tube was prepared and stored at 4 °C until incubation at 37 °C started. After incubation, the samples were diluted to a concentration of 10 U per 100 µl (reference was made to the known activity as tested previously) and then 100 µl were subjected to an activity test as described in 2.3.1. Half-life of the recombinant proteins was determined graphically in plots of relative inhibitory activity *versus* time. Each experiment was performed in duplicate.

2.5. SDS-PAGE

2.5.1. Preparation of Polyacrylamide Gels

12% resolving polyacrylamide gels were prepared by mixing (for one gel) 4.35 ml aqua bidest., 2.5 ml 1.5 M Tris-HCl pH 8.8, 100 µl of a 10% SDS solution, and 3 ml of 40% acrylamide stock solution (Roth, Karlsruhe, Germany). Polymerization was

started by addition of 100 µl of a 10% (w/v) ammoniumpersulfate-solution and 10 µl of TEMED (Sigma, St. Louis, Mo, USA).

12% polyacrylamide stacking gels were prepared by mixing (for one or two gels) 6.4 ml aqua bidest., 2.5 ml 0.5 M Tris-HCl pH 6.8, 100 µl of a 10% SDS solution, and 1 ml of 40% acrylamide stock solution. Polymerization was started by addition of 100 µl of a 10% (w/v) ammoniumpersulfate-solution and 10 µl of TEMED.

When necessary, the samples were diluted, and the appropriate amount of a 3x Laemmli buffer (1.5 g SDS, 1.5 ml 1 M Tris-HCl pH 6.8, 4.5 g glycerol, 1 mg bromophenol blue *ad* 10 ml aqua bidest.) with or without mercaptoethanol (600 µl were added to the 10 ml stock) was added. The samples were incubated for 5 min at 95 °C and subsequently pipetted into the gel chambers.

The electrophoresis buffer was composed of 1.4 g/L glycine, 3 g/L Tris, and 1 g/L SDS.

2.5.2. Silver-staining of Proteins

After electrophoresis, gels were fixed in ethanol:aqua bidest.:acidic acid (5:5:1) solution for 20 min, washed two times in water for 5 min each, and incubated for 5 min in aqua bidest supplemented with a spatulas tip of “farmers reducer“ (mixture of 16 g $Na_2S_2O_3$ x $5H_2O$ and 10 g $K_3(Fe(CN)_6)$). Subsequently, gels were washed three times in aqua bidest. for 20 min each, incubated for 30 min in 0.1% (w/v) $AgNO_3$ solution, shortly rinsed in aqua bidest, and finally developed in 2.5% (w/v) Na_2CO_3 solution supplemented with approximately 100 µl formaldehyde solution (Merck, Darmstadt, Germany) per 100 ml. Development was stopped by adding acidic acid (10% (v/v) solution).

2.5.3. Complex Formation of Recombinant PAI-1 Proteins with HMW-uPA

For complex formation, 100 U (approximately 0.7 µg) of HMW-uPA were incubated with the recombinant PAI-1 proteins at room temperature for 10 min. Samples were then subjected to SDS-PAGE. Gels were silver-stained (see 2.5.2) or Coomassie-

stained (Bio-Safe™, BioRad, Hercules, CA, USA) according to the manufacturer's manual.

2.5.4. Complex Formation of Recombinant PAI-1 Proteins with Thrombin

Fifty units of PAI-1 (variant) in the presence or absence of 600 nM Vn or 1 U/ml Hep were incubated with 0.5 U of thrombin in a total volume of 30 µl TST buffer (1 h, 37 °C) and then subjected to non-reducing SDS-PAGE followed by Western blotting (see 2.6).

2.6. Western Blotting

Gels were blotted using a semi dry technique. From bottom to top, the following layers were put in a blotting apparatus: three layers of Whatman filter paper soaked with 20% methanol in 50 mM boric acid pH 9.0, followed by the nitrocellulose membrane, the gel, and three layers of Whatman paper soaked in 5% methanol in 50 mM boric acid pH 9.0. Special attention was paid to eliminate all air bubbles between the layers. Electroblotting occurred for 2.5 h at 100 V and approximately 160 mA per gel. Afterwards, membranes were incubated in NET-gelatin (0.15 M NaCl, 5 mM EDTA [from a solution with pH 8.0], 25 mM Tris-HCl [from a solution with pH 7.5], 0.05% (w/v) Triton X-100, 0.25% gelatin [Merck, Darmstadt, Germany]) overnight at 4 °C, then incubated with a polyclonal chicken antibody to PAI-1 or a polyclonal chicken antibody to uPA diluted to a concentration of approximately 10 µg/ml in NET-gelatin (both antibodies were kindly provided by Dr. N. Grebenschikov, Institute for Chemical Endocrinology, University of Nijmegen, The Netherlands), washed three times (10 min each) with PBS, incubated with the peroxidase-labelled polyclonal rabbit antibody to chicken (Sigma, St. Louis, Mo, USA; No.A9046) diluted 1:10,000 in NET-gelatin, and finally washed three times (10 min each) with PBS. Antibodies bound to the proteins on the membrane were detected using the "ECL Western Blotting Detection Reagent" (Amersham Pharmacia, Freiburg, Germany), according to the manufacturer's manual.

2.7. Blotting of Proteins for Peptide Sequence Analysis

In order to analyze the sequence of protein samples, the proteins were separated by non-reducing SDS-PAGE (see 2.5.1) and blotted on nitrocellulose (see 2.6) as described above. Subsequently, the membrane was Coomassie stained for 15 seconds (in a solution containing 0.1% (w/v) Coomassie Brilliant Blue R250, [Merck, Darmstadt, Germany], 10% acidic acid, and 40% methanol), washed in 30% methanol, 10% acidic acid, dried and sent to Dr. Kellermann, Dept. for Protein Analysis, Max-Planck-Institute for Biochemistry, Martinsried, who kindly performed the sequence analyses.

2.8. Binding of PAI-1 Variants to ECM Proteins

2.8.1. Binding of PAI-1 Variants to Vn-Coated Microtiter Plates

Vn or collagen type IV (Sigma, Taufkirchen, Germany) was diluted to a concentration of 10 µg/ml in a buffer containing 100 mM Na_2CO_3, pH 9.6. For coating, 50 µl of the Vn or collagen type IV dilutions were poured into wells of a NuncMaxiSorp microtiter plate (Nunc GmbH & Co. KG, Wiesbaden, Germany) and incubated overnight at 4 °C. After three washes with PBS/Tween (PBS containing 0.05% Tween-20 (v/v)), the wells were blocked by addition of 200 µl per well of PBS supplemented with 2% BSA (w/v) and incubation at room temperature for 2 h. The wells were washed three times with PBS/Tween. Afterwards 100 µl per well of (mutant) PAI-1 at different concentrations were added at RT for 1 h. Following three additional washing steps, 200 µl per of well horseradish peroxidase-labelled Ni^{2+}-nitrilotriacetic acid (Qiagen, Hilden, Germany) at a dilution of 1:1000 in PBS/Tween containing 0.2% BSA (w/v) were added (1 h at RT). After another four rounds of washing, binding of PAI-1 to the solid phase was visualized by addition of 100 µl per well of a TMB substrate mix (KPL, Gaithersburg, Maryland, USA). The reaction was stopped after color development with 50 µl per well of 0.5 M H_2SO_4 and the optical density was measured at 450 nm.

In some experiments different buffers with higher salt concentrations (58 mM Na_2HPO_4 x 2 H_2O, 17 mM $NaHPO_4$ x H_2O, 250 mM NaCl or the same buffer with 500 mM NaCl) were used for washing.

2.8.2. Surface Plasmon Resonance Spectroscopy

Surface plasmon resonance (SPR) spectroscopy is a highly sensitive technique to measure protein/protein interactions on a gold surface. In this study, the BIACORE 2000 system was used, either with CM5 chips or with SA chips (research grade, BIACORE AB, Uppsala, Sweden).

The principle setup of a CM5 sensor chip with immobilized Vn is shown in Fig. 5. Polarized light of a laser (black arrow in Fig. 5) is coupled through a prism to the gold layer of the chip. At most angles of incident light, the major part of the light is reflected. Only near or around the angle of resonance (θ) plasmon resonance occurs: nearly all the incoming light energy leads to oscillations of the electron cloud of the 48 nm thick gold surface of the chip (red arrow in Fig. 5). Thus, the energy of the light is trapped in the plasmon and only a minimal amount of reflected light can be detected. If you now detect the amount of reflected light (reflectivity) in dependence of the angle θ of the incoming laser beam, you get a plot with a minimal reflectivity at the angle of resonance (Fig. 5).

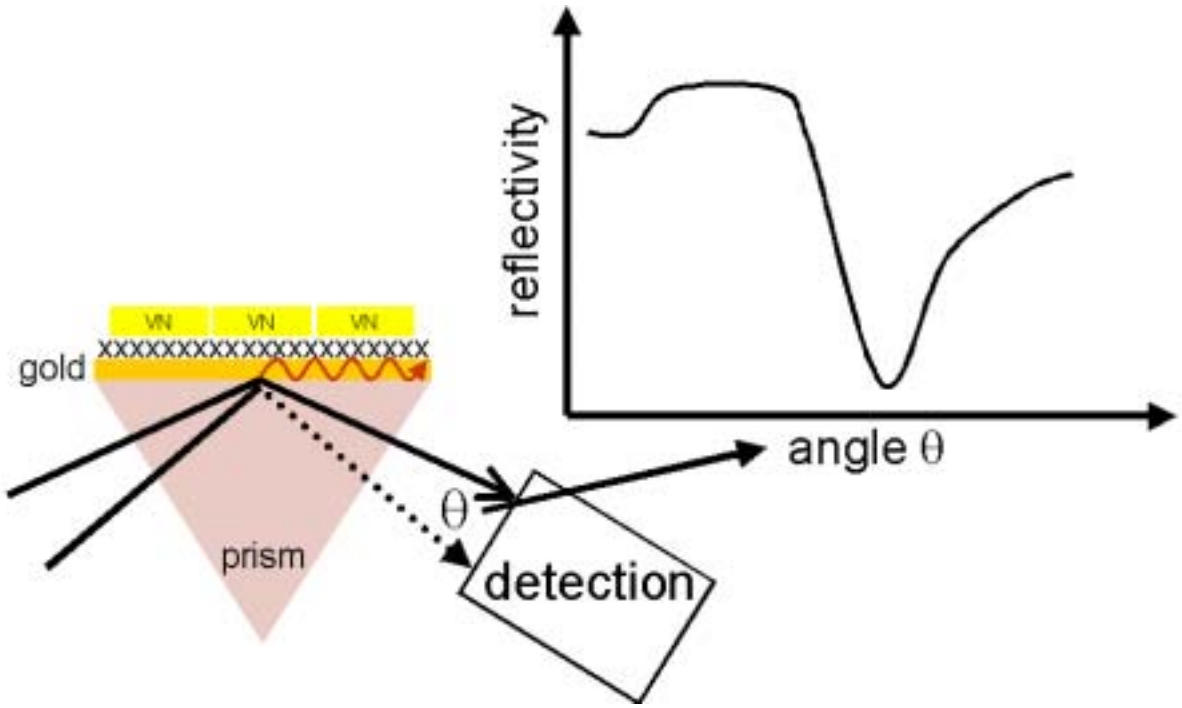

Fig. 5. Principle of surface plasmon resonance analysis. On the left side, the setup of the CM5 chip with immobilized Vn is shown and on the right side the reflectivity is plotted against the angle of the incoming light θ. For details see text.

What makes this physical phenomenon useful for detection of biochemical interactions on surfaces is the fact that the energy of the evanescent electromagnetic field of the plasmon reaches out into the area above the gold layer but decays in an exponential way with increasing distance to it. So only changes on or near the surface (in a distance of less than 300 nm to the surface) influence this electromagnetic field. Thus, if proteins (or other molecules) bind to the surface, the SPR spectroscopy plot is shifted to the right (Fig. 6) [Biacore AB, 2002].

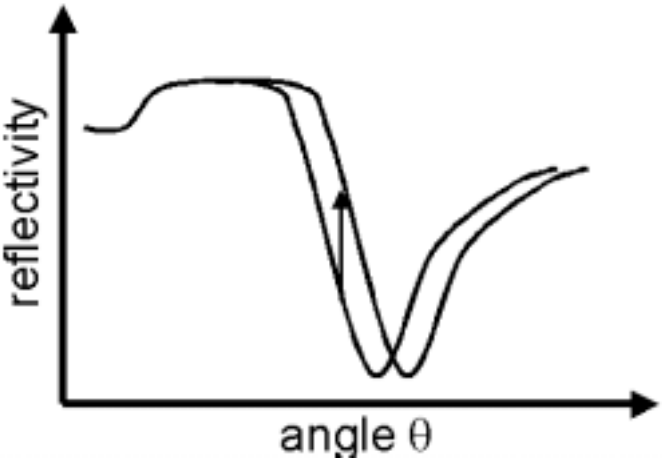

Fig. 6. The reflectivity plot shifts to the right upon binding of additional molecules to the surface. If you plot the reflectivity at an angle where the curve is linear (arrow) against the time, you get the binding profiles shown in the result section

Another possibility to measure the changes in the plasmon is to record the reflectivity at a fixed angle (where the reflectivity is falling linear with increasing angles, arrow in Fig. 6) and to plot it versus time (such plots are shown in the result section). If you now measure binding to the surface (in this study *e.g.* binding of PAI-1 to immobilized Vn or Vn bound to the mab VN7), you get time-dependent binding profiles. Still, in order to get the binding profiles shown in the result section, you have to subtract binding profiles from a control flow cell, as also changes in buffer or protein concentration in the solution running over the chip surface influence the angle of plasmon resonance, producing so-called bulk effects [Prein, 2002].

2.8.2.1. Surface Plasmon Resonance Analysis of Binding of Recombinant PAI-1 Proteins to Immobilized Vn

SPR studies were conducted with a BIACORE 2000 (Biacore AB, Uppsala, Sweden). Approximately 2000 resonance units (RU) of collagen type IV (10 µg/ml in 10 mM Na-acetate, pH 4.0) (lane 1) and Vn (10 µg/ml in 10 mM Na-formiate, pH 4.0 [Ehnebom et al., 2000]) (lanes 2 to 4) were immobilized to a CM5 sensor chip (research grade, Biacore AB, Uppsala, Sweden) using the amino coupling kit according to the manufacturer's recommendation. All experiments were performed in

HBS-EP (10 mM Hepes,150 mM NaCl, 3 mM EDTA, 0.005% Tween-20 (v/v), pH 7.4) at a flow rate of 20 µl/min. HMW-uPA was used in a concentration of 400 U/ml. Regeneration of the surface was achieved by injection of 10 mM HCl for 8 min. In order to check for reproducibility during the measurement, at first 80 µl of a 200 U/ml dilution of wild-type PAI-1 were injected for two subsequent experiments, followed by two subsequent measurements of 80 µl of a 200 U/ml dilution of a PAI-1 mutant. Then, wild-type PAI-1 was measured a third time in duplicate, followed by a measurement in duplicate of the next mutant and so on. Thus, for each PAI-1 variant at least two binding profiles were recorded. The kinetics obtained in the collagen type IV-coated flow cell were subtracted from the kinetics derived from the Vn-coated flow cell in order to obtain binding profiles without bulk effects.

2.8.2.2. Surface Plasmon Resonance Analysis of Binding of Recombinant PAI-1 Proteins to Native and Denatured Vn

In order to check for differences in binding of wt-PAI-1 and Q123K to native Vn (Promega GmbH, Mannheim, Germany) and to denatured Vn (3 min. at 95 °C) approximately 6,000 RU of a monoclonal antibody to Vn (VN7, kindly provided by Dr. K. Preissner, Institute of Biochemistry, Justus Liebig University, Giessen; 10 µg/ml in 10 mM Na-acetate, pH 4.0) were immobilized on a CM5 sensor chip (research grade, Biacore AB, Uppsala, Sweden) applying the EDC/NHS procedure. This antibody binds both native and denatured Vn [Stockmann et al., 1993]. 3,800 RU of collagen type IV (10 µg/ml in 10 mM Na-acetate, pH 4.0) were immobilized on the control flow cell. All experiments were performed in HBS-EP at a flow rate of 20 µl/min. HMW-uPA was used in a concentration of 400 U/ml.

At first, 140 µl of native or denatured Vn (10 µg/ml in HBS-EP) were allowed to bind to mab VN7. Then, 140 µl of the appropriate wt-PAI-1 or Q123K dilution (in HBS-EP) were injected, followed by 60 µl of HMW-uPA. Regeneration of the surface was achieved by injection of 10 mM HCl for 8 min. In order to be able to calculate binding constants, different concentrations (100 U/ml to 800 U/ml) of (variant) PAI-1 were

measured. Again, the binding curves from a collagen type IV coated flow cell were subtracted from the VN7 coated flow cell in order to eliminate bulk effects.

In order to check for the interaction of PAI-1 (and Q123K) with Vn in solution, the following experiment was performed: Vn (10 µg/ml in HBS-EP) was incubated with a threefold molar excess of PAI-1 or Q123K (equivalent to 533 U/ml) for 15 min at RT, followed by injection and binding to VN7. Then 60 µl of HMW-uPA were injected. This should lead to the dissociation of PAI-1 from the bound Vn and subsequently only Vn is bound to VN7. The conformational form of this Vn still bound to VN7 should then be assessed. Therefore, the mab 13H1 (injection of 140 µl of 10 µg/ml in HBS-EP) was used. This mab recognizes Vn in the multimeric state with a higher affinity than native Vn [Stockmann et al., 1993]. Binding profiles obtained after injection of 13H1 to the Vn, which was previously in complex with PAI-1 (or Q123K), were compared to the binding profiles obtained in a pretest. In this pretest, the binding profiles of 13H1 binding to native or denatured Vn, which was bound to VN7, were obtained.

2.8.2.3. Calculation of Binding Constants

Binding constants for association and dissociation kinetics were calculated using the BiaEvaluation 3.1 software. For this, the obtained binding profiles were fit with a 1:1 Langmuir kinetic. Sometimes the association kinetics had to be fit separately from the dissociation kinetics. In these cases, first the whole binding profile was fit, providing an approximate Ka and a rather reliable Kd, followed by a separate fitting of just the association using the Kd values obtained in the first procedure. From these values K_D was calculated. Ka, Kd, and K_D were calculated using the total protein concentration of PAI-1 (variants) in the preparation. As only portions (and for the mutants also unknown portions) of the total protein were in the active conformation, the constants calculated do not give absolute values of PAI-1/Vn affinity. They rather can be used for comparison of the different PAI-1 variants, as the relation of active to inactive

protein as well as the total protein concentration in the preparations were similar (see also discussion).

2.8.2.4. Surface Plasmon Resonance Analysis of Recombinant PAI-1 Proteins Binding to Hep

In order to measure binding of (mutant) PAI-1 to Vn, a streptavidin-coated SA sensor chip (Biacore AB, Uppsala, Sweden) was used. Prior to coupling of the ligand, the chip was rinsed three times with 20 µl of 50 mM NaOH, 1 M NaCl at a flow rate of 20 µl/min. Then biotinylated heparin (from porcine mucosa, Calbiochem-Novabiochem, Bad Soden, Germany) was injected in a concentration of 10 µg/ml (flow rate 5 µl/min) and allowed to bind to the streptavidin-coated surface. All experiments were performed in HBS-EP at a flow rate of 20 µl/min. In order to measure binding of the recombinant proteins, 160 µl of (mutant) PAI-1 in a concentration of 200 U/ml were allowed to bind, followed by buffer and an injection of 40 µl of uPA (concentration 400 U/ml). The surface could be regenerated by injection of 160 µl 0.01 M HCl followed by 60 µl 1 M NaCl. The kinetics obtained in a flow cell without immobilized ligand were subtracted from the kinetics derived from the heparin-coated flow cell in order to obtain binding profiles without bulk effects.

2.9. Cell Invasion Assays

2.9.1. Cell Lines

The human ovarian carcinoma cell lines OV-MZ-6 and OV-MZ-19 were established from patients with advanced serous cystadenocarcinomas of the ovary [Möbus et. al, 1992]. OV-MZ-6 was chosen because of its relatively high invasive capacity and OV-MZ-19 because of its very low constitutional expression of PAI-1. Both cell lines did not differ significantly in the expression of other components of the plasminogen activation system [Will et al., 1994].

2.9.2. Cell Culture

Cells were cultured at 37 °C and 5% CO_2 in Falcon tissue culture flasks (Becton-Dickinson, Heidelberg, Germany) in Dulbecco's modified Eagles Medium (DMEM) in the presence of 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES (all from Gibco, Karlsruhe, Germany), 0.27 mM asparagine, and 0.55 mM arginine (both aa from Sigma, St. Louis, Mo, USA). This medium was called DMEM complete. Cells were detached for subcultivation with 0.05% EDTA in PBS (PBS/EDTA; Gibco, Karlsruhe, Germany).

2.9.3. Cell Invasion Assays

Cell invasion assays were performed using a two chamber assay. The two chambers were formed by placing inserts (Becton-Dickinson, Heidelberg, Germany) especially designed for this purpose into 24 well tissue culture plates (Becton-Dickinson, Heidelberg, Germany). Thus an upper and a lower chamber were formed. As the bottom of the inserts is covered with a membrane with a defined pore size of 8 µm, cell migration through this membrane is possible.

On the first day of the experiment, the membranes of the inserts were covered with Matrigel (Becton-Dickinson, Heidelberg, Germany). All steps had to be performed on ice, as Matrigel is fluid only at approximately 4 °C. In each insert, 60 µl of a Matrigel solution (568 µg/ml in PBS) with or without 28 µg/ml vitronectin (*i.e.* 5% w/w) were pipetted, followed by incubation of the inserts for 3 h at 37 °C and by overnight incubation at RT.

The second day, the dried Matrigel was rehydrated by addition of DMEM complete without FCS but supplemented with 0.1% BSA (DMEM/BSA) and incubation for 2 h at RT. Then the cells were detached (60 to 80 percent confluent) with PBS/EDTA, washed two times with DMEM/BSA, and seeded in a number of 50,000 cells per 0.5 ml DMEM/BSA per insert. The wells around the insert (*i.e.* the lower chamber) were filled with 0.75 ml of DMEM complete. As DMEM complete but not DMEM/BSA contains FCS, cells migrate along a chemoattractant through the Matrigel and the

membrane over a period of 72 h at 37 °C and 5% CO_2. The indicated amount of PAI-1 was repeatedly added every 24 hours for a total of 3 days.

Two inserts per experiment were used for quality control of the Matrigel. Therefore, these inserts were rehydrated with PBS only and subsequently the Matrigel was stained by addition of 200 µl of “Solution B” (0.5% (w/v) Coomassie Blue in “Solution A”) for 30 min. Then these inserts were washed twice with 200 µl “Solution A” (20% 2-propranol, 5% acidic acid in aqua bidest.), dried, and subsequently evaluated under a microscope.

When the three days allowed for the invasion assay were over, the inserts were removed from the wells, the medium was aspirated, the Matrigel and the cells that were still in the upper chamber were removed by wiping the inserts extensively with tissue paper (Roth, Karlsruhe, Germany), and cells attached to the lower side of the membrane were stained with the DiffQuick® Staining Set (Dade Behring, Düdingen, Switzerland). Subsequently, the number of cells which migrated through the Matrigel was determined under a microscope (Axiovert25, Zeiss, Jena, Germany) by counting the total number of cells present on the lower side of the membrane.

3. Results

3.1. Preparation of wt-PAI-1, wt-PAI-2 and PAI-1 Variants

Following the expression and purification procedures described in the Material and Methods section, recombinant proteins were obtained with a more than 95% purity as evaluated by SDS-PAGE and silver or Coomassie staining. A Coomassie stained gel of the bacterial lysates as well as the first and second eluates of the preparation of wt-PAI-1 and Q123K is shown in Fig. 7. Similar results were obtained with the other mutants (data not shown).

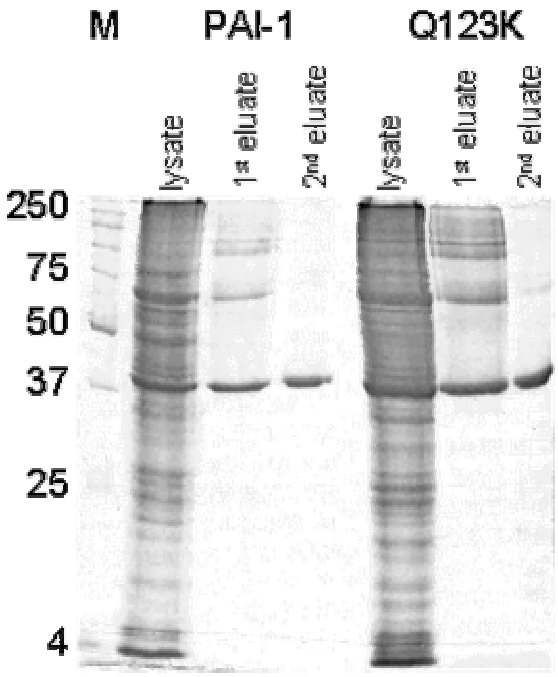

Fig. 7. Purification of wt-PAI-1 and Q123K. For each protein, the bacterial lysate as well as the eluate of the first and second Ni^{2+}-NTA affinity column were subjected to SDS-PAGE and subsequent Coomassie staining. M: Marker, apparent weight in kDa.

3.2. Determination of the Protein Concentration of the Recombinant Proteins

For routine measurement, the protein concentrations of the purified PAI-1 (variant) solutions were determined using the Bradford assay with BSA as the protein

standard. As this assay was developed for the determination of protein concentrations in crude extracts and here a purified protein was measured, experiments were performed in order to check for possible differences in protein concentrations depending on the assay and standards which were used. Besides BSA and bovine gamma globulin (BGG), ovalbumin was selected as a standard, because it belongs to the serpin family, just like PAI-1 [Huntington and Stein, 2001] [Andreasen et al., 2000]. When using BSA as a standard, the Bradford and the BCA assay yielded similar results. Moreover, the BCA assay led to the same results regardless of the standard used (BSA, BGG, or ovalbumin), whereas - just like described in the manufacturer's manual [BioRad, 2002] - the concentrations determined with the Bradford assay were falsely elevated, when BGG was used as a standard. Results did not differ using BSA or ovalbumin as the protein standard. In summary, the only results that did differ from all the others were those determined with the Bradford assay and a BGG standard. Thus, the Bradford assay with BSA as a standard was assumed to be reliable to measure the PAI-1 concentration in purified samples and was chosen for routine testing for practical reasons. The protein concentrations of the different PAI-1 (variant) preparations are summarized in Table 3 (see 3.3.1).

3.3. Measurement of Inhibitory Activity of the Recombinant Proteins against Different Proteases

3.3.1. Inhibitory Activity of the Recombinant Proteins against uPA

The inhibitory activity against uPA of the different PAI-1 variant preparations as well as their protein content is summarized in Table 3 (for a summary of all characterized mutants and their corresponding aa alterations see chapter 9.1):

preparation of 09/22/00:		[U/ml]	[µg/ml]	[U/mg]
	Δ109-112	500	41	12195
	P73A	2000	104	19231
	A114-118	0	51,2	0
	M8	0	53,5	0

preparation of 10/04/00:		[U/ml]	[µg/ml]	[U/mg]
	Δ109-112	400	142	2817
	P73A	3000	171	17544
	M8	0	152	0

preparation of 11/08/00:		[U/ml]	[µg/ml]	[U/mg]
	PAI-1	700	n. d.	n. d.
	Q55P	0	202	0
	Q123K	500	431	1160
	A114-118	400	240	1667
	M8 new	100	194	515
	M9	0	208	0
	M3/1	0	141	0
	M3/2	0	99	0
	M4/1	0	66	0
	M4/2	0	108	0

preparation of 11/20/00:		[U/ml]	[µg/ml]	[U/mg]
	PAI-1/12	10000	105	95238
	PAI-1/345	22500	235	95745
	Q55P/12	0	379	0
	Q55P/345	0	103	0
	Q123K/12	8000	136	58824
	Q123K/345	11000	231	47619

preparation of 04/30/01:		[U/ml]	[µg/ml]	[U/mg]
	PAI-1/1	27000	691	39074
	PAI-1/2	18000	518	34749
	Q123K	16000	881	18161

preparation of 06/26/01:		[U/ml]	[µg/ml]	[U/mg]
	wt PAI-2	1000	95	10526
	del PAI-2	2000	92	21739

Table 3. Summary of recombinant protein preparations. For each protein the activity in U/ml, the protein concentration in µg/ml and the specific activity in U/mg is given. Numbers behind slashes indicate different fractions of the same preparation. “del PAI-2” is a deletion mutant of PAI-2 (deletion of aa P66-P98 [Harrop et al., 1999]).

3.3.2. Inhibitory Activity of the Recombinant Proteins against Thrombin

Inhibitory activity against 0.1 U thrombin was measured for wt-PAI-1, P73A, A114-118, and Q123K as a means of testing the interaction of (variant) PAI-1 with Vn or Hep.

50 nM wt-PAI-1 alone inhibited thrombin activity only by ten percent, whereas addition of Vn led to a dose-dependent increase in thrombin inhibition. More than 50% of thrombin activity was inhibited with 50 nM wt-PAI-1 in complex with 125 nM Vn. Addition of even more Vn (up to 500 nM) did not lead to a significant increase in inhibition of thrombin. As expected, inhibition of thrombin did not only depend on the amount of Vn but also on the amount of PAI-1 used. Increasing PAI-1 concentrations (between 5 and 50 nM) together with a two-fold molar excess of Vn led to a dose-dependent increase in thrombin inhibition: A concentration of 5 nM PAI-1 inhibited 2% of thrombin, 12.5 nM PAI-1 inhibited 20% of thrombin, 25 nM PAI-1 inhibited 45% of thrombin, and 50 nM PAI-1 inhibited 70% of thrombin.

Wt-PAI-1 (50 nM) together with Hep was also able to inhibit thrombin. Addition of only 0.01 U/ml of Hep already led to a significant inhibition of thrombin activity (more than 50% inhibition). The optimal Hep dose was determined as one U/ml. At this concentration, virtually no active thrombin was left. In contrast to the effect of Vn on the ability of PAI-1 to inhibit thrombin, a surplus of Hep led to a decrease of thrombin inhibition by PAI-1 (Fig. 8).

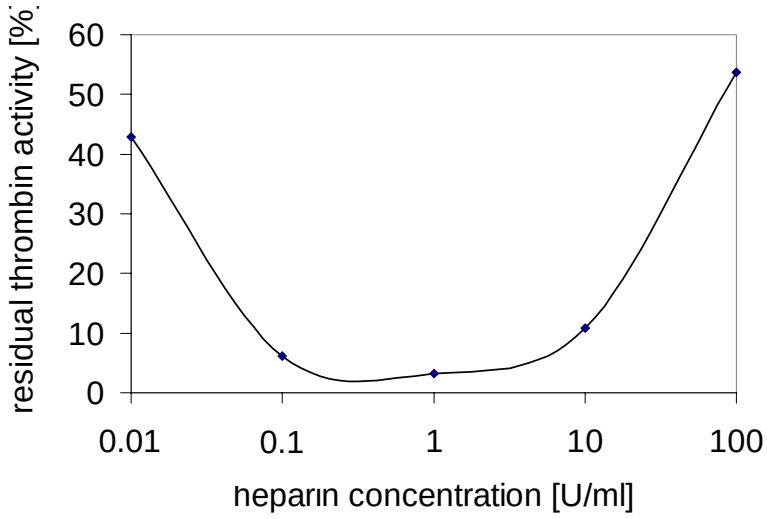

Fig. 8. Effect of heparin on thrombin inhibition by 50 nM wt-PAI-1. The activity of thrombin without inhibitor was set to 100%. Then residual thrombin activity was calculated accordingly. Residual thrombin activity of 50 nM wt-PAI-1 alone was 92%.

In order to be able to compare the results of thrombin inhibition by the different PAI-1 mutants, the same amount of active PAI-1 (fifty units, *i.e.* ~35 to ~70 nM protein concentration) was used. As the minimal Vn concentration for inhibition of thrombin by wt-PAI-1 was determined to be at least two times the amount of PAI-1, the mutants were tested at a Vn concentration of 140 nM. The ability of the mutants to inhibit thrombin together with Hep was tested with the ideal Hep concentration of one U/ml.

P73A did not differ from wt-PAI-1 in respect to thrombin inhibition. A114-118 was able to inhibit thrombin in a similar way as wt-PAI-1 together with Vn, but it displayed significantly less thrombin inhibitory properties in the presence of Hep compared to the wild-type. In contrast, Q123K did not differ from wt-PAI-1 when inhibition of thrombin was measured in the presence of Hep, but it nearly failed to inhibit thrombin in the presence of Vn. 140nM Vn or one U/ml Hep alone did not reduce thrombin activity (Fig. 9).

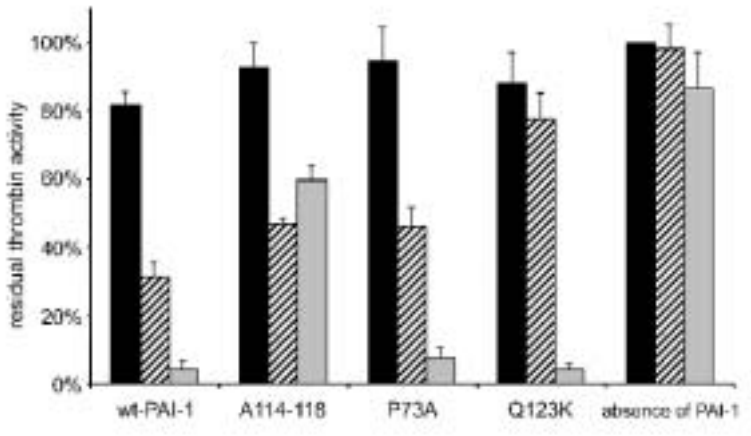

Fig. 9. Inhibition of thrombin by (mutant) PAI-1. Fifty units of recombinant PAI-1 (~ 35 to ~70 nM) in the presence or absence of 140 nM Vn or one U/ml Hep were incubated with 0.1 U of thrombin at 37 °C for 1 h. Then thrombin activity was measured. The activity of thrombin in the absence of inhibitor was set to 100%; the other activities were calculated accordingly. Data shown are from three independent experiments, each measured in duplicate (±SD). As a control, the effect of 140 nM Vn or one U/ml Hep without inhibitor was measured. Black bars, buffer, only; hatched bars, plus Vn (140 nM); grey bars, plus Hep (1 U/ml).

3.3.3. Inhibitory Activity of the Recombinant Proteins against uPA by Measuring the Amount of Activated Plasminogen

In order to test the possibility, that there still was a physiological relevant inhibition of uPA by some of the “inactive“ mutants which could not be detected with the chromogenic substrate for uPA, a plasminogen activation assay was performed. In this assay, the amount of uPA activity was measured indirectly by determining the amount of plasmin generated from plasminogen by uPA. Mutant P73A was able to completely inhibit plasminogen activation by uPA, whereas the inactive mutant M4 could not interfere with this process. Thus, similar results were obtained in this plasminogen activation assay compared to the assay directly measuring inhibition of uPA activity.

3.4. Determination of the Half-life of the Recombinant Proteins

All tested mutants displayed a similar half-life at pH 7.4, with the exception of A114-118, which displayed a reduced half-life of only 20 to 25 minutes. The following half-lives were measured (n=2): PAI-1: 1 h 15 min., Q123K: 1 h 15 min., P73A: 1 h 50 min.

3.5. SDS-PAGE and Western Blots

3.5.1. Complex Formation of PAI-1 (Variants) with HMW-uPA

Only those mutants that inhibited uPA in the chromogenic assay were able to form SDS-stable complexes. An example showing a silver-stained gel with SDS-stable complexes of wt-PAI1, Q123K, P73A, and A114-118 with uPA is shown in Fig. 10. The inactive mutant M4 did not form such complexes after incubation with uPA for 0, 10, 30, or 60 minutes at RT (Fig. 10).

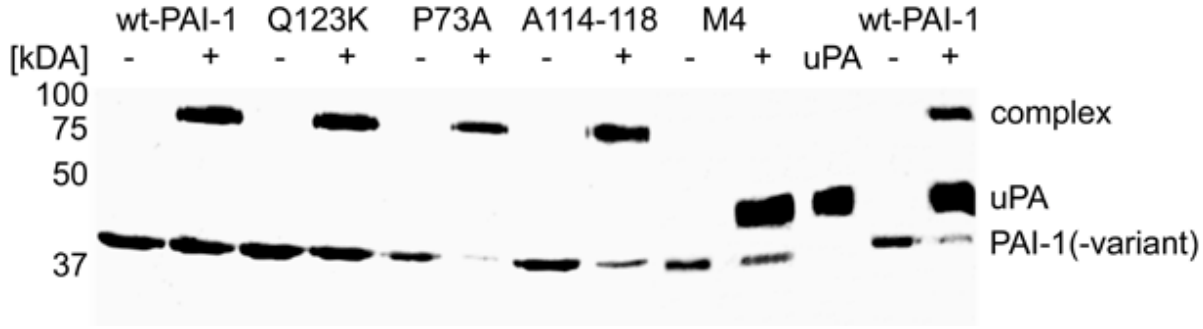

Fig. 10. Formation of SDS-stable complexes with uPA. Wt-PAI-1 or variants thereof in the presence (+ uPA) or absence (- uPA) of 100 U (~ 0.7 µg) HMW-uPA were incubated for 10 min at RT and then subjected to nonreducing SDS-PAGE. Subsequently, the gels were silver-stained. 200 U of PAI-1, 110 U of Q123K, 50 U of P73A and of A114-118 were applied; alternatively 1.4 µg of (inactive) M4 and 10 U of PAI-1 were used.

In order to exclude the possibility that minimal amounts of Δ109-123, M3, M4, or M9 were able to form complexes with uPA, Western blots with polyclonal antibodies to PAI-1 and to uPA were performed. A blot with the polyclonal antibody to PAI-1 is shown in Fig. 11. Complexes can only be seen with wt-PAI-1 (arrow) and A114-115

(arrow head). Δ109-123, M4, and M9 did not form such complexes. The polyclonal antibody to PAI-1 did not react with PAI-2, which was used as a control protein.

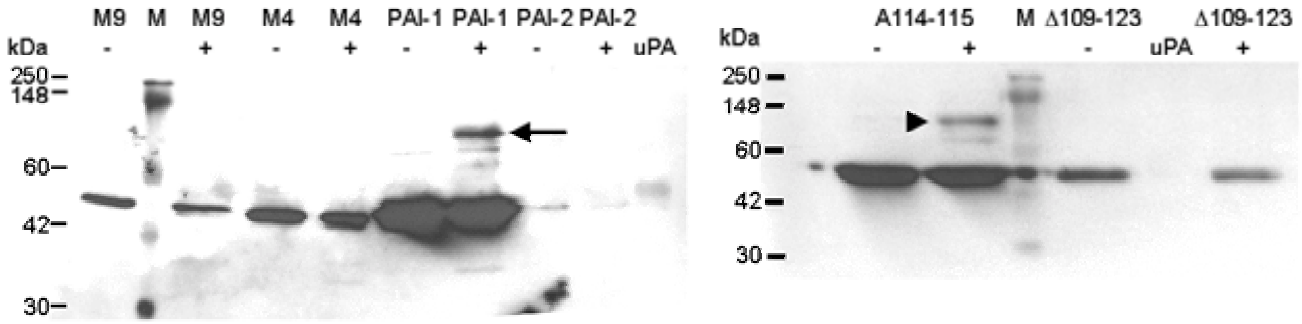

Fig. 11. Western blots showing SDS-stable complexes with uPA. (Mutant) PAI-1 was incubated with or without 100 U (~0.7 µg) of HMW-uPA for 10 min at RT and then subjected to non-reducing SDS-PAGE and subsequent Western blotting with a polyclonal antibody to PAI-1.

Fig. 12 demonstrates that M3 was also not able to form complexes with uPA. In addition, the complexes formed by PAI-1 and uPA were not only detected with an antibody to PAI-1 but also with an antibody to uPA. M9 is most probably cleaved by uPA, as an additional band containing PAI-1 antigen appears at the level of approximately 35 kDa. The bands around 60 kDa in the lanes with wt-PAI-1 and M9 are not complexes with uPA as they are not detected by the antibody to uPA in the lower gel.

In summary, the active PAI-1 variants formed complexes with uPA, whereas not even minimal amounts of those complexes were detected in Western blots when the inactive mutants were incubated with uPA.

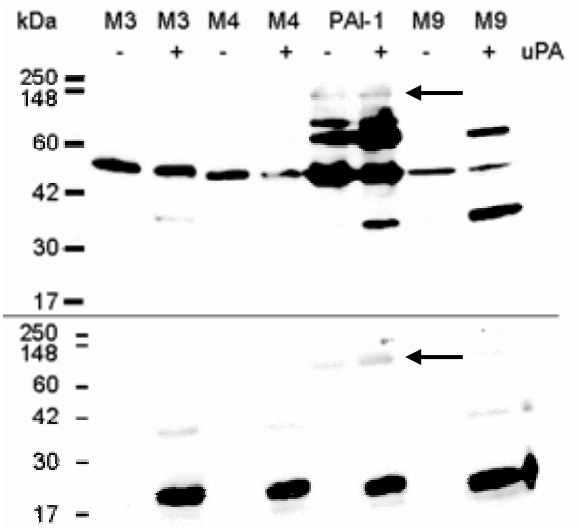

Fig. 12. Western blots showing SDS-stable complexes with uPA. (Mutant) PAI-1 was incubated with or without 233 U (~1.6 µg) of HMW-uPA for 50 min at 37 °C and then subjected to reducing SDS-PAGE and subsequent Western blotting with a polyclonal antibody to PAI-1 (upper blot) and a polyclonal antibody to uPA (lower blot). The SDS-stable complex of wt-PAI-1 with HMW-uPA is shown by an arrow. Please note that due to the reducing conditions uPA is present as the ~20 kDa A-chain and the ~35 kDa B-chain and that the polyclonal antibody reacts stronger with the A-chain (lower blot).

3.5.2. Complex Formation of PAI-1 (Variants) with Thrombin

Thrombin inhibitory properties of wt-PAI-1 and the variants P73A, A114-118, and Q123K were not only measured in thrombin activity assays (see 3.3.2) but also visualized in Western Blots showing SDS-stable complexes of PAI-1 (variants) with thrombin. Wt-PAI-1 and P73A were equally able to form SDS-stable complexes with thrombin in the presence of Vn or Hep. Interestingly, during thrombin inhibition a new band with an apparent molecular weight of approximately 35 kDa is formed. The appearance of this new band exactly mirrored the results obtained with the thrombin inhibition assays: Only incubation of A114-118 with thrombin in the presence of Vn, but not heparin, led to a significant 35 kDa band. Thrombin incubated with Q123K was also able to produce this band, but only in the presence of Hep (Fig. 13). P73A behaved similar to wt-PAI-1 (data not shown). Control lanes containing Hep, Vn, and thrombin in different combinations were not stained by the antibody to PAI-1.

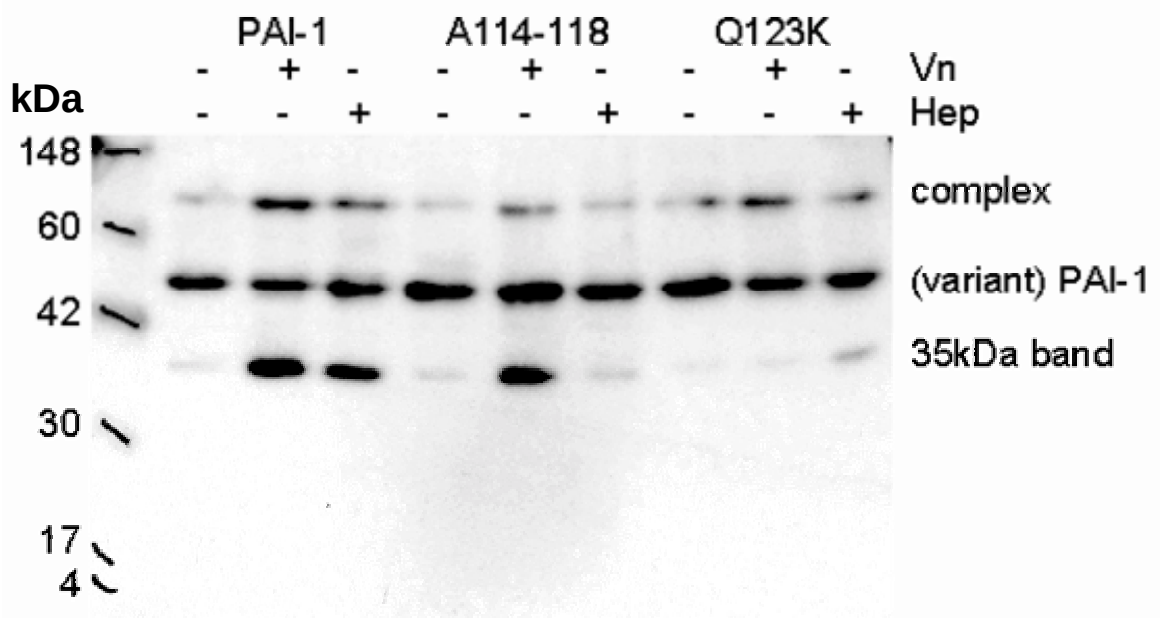

Fig. 13. Western blot of SDS-stable complexes with thrombin. (Mutant) PAI-1 was incubated with 0.1 U of thrombin with or without 530 nM Vn or one U/ml of Hep for 1 h at 37 °C and then subjected to non-reducing SDS-PAGE and subsequent Western blotting with a polyclonal antibody to PAI-1.

3.6. Blotting of Proteins for Peptide Sequence Analysis

As the new 35 kDa band mentioned above displayed PAI-1 antigen and was not only seen during inhibition of thrombin but also during inhibition of uPA by PAI-1, the N-terminal aa sequence of this fragment was determined. For this, SDS-stable complexes of PAI-1 with uPA and thrombin were formed and blotted onto a membrane (Fig. 14).

With PAI-1 and uPA, the ~90 kDa complex, HMW-uPA, free wt-PAI-1, and RCL-cleaved PAI-1 (from top to bottom, Fig. 14) could be identified. Incubation of wt-PAI-1 with Hep and thrombin led to the ~70 kDa complex, free wt-PAI-1, and free thrombin (from top to bottom, Fig. 14).

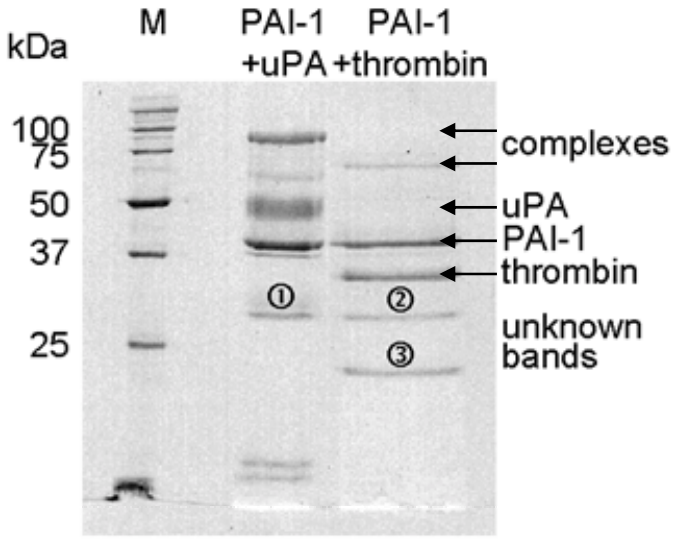

Fig. 14. Blot for sequence analysis. 3.6 µM PAI-1 was incubated with 500 U HMW-uPA (~3.5 µg) in PBS for 10 min at RT (left lane), whereas 1.8 µM PAI-1 was incubated with 2,5 U of thrombin in the presence of 1 U/ml Hep in TST for 1 h at 37 °C (right lane). Subsequently, the protein solutions were subjected to non-reducing SDS-PAGE and blotted onto a membrane. The membrane was Coomassie-stained as described in the Materials and Methods section.

The amino terminal sequence of both fragment number one and number two was MAPEEIIMDYPFL (one letter aa code). This sequence corresponds to the N-terminus of the so called C-terminal PAI-1 peptide, which is released after cleavage of PAI-1 at the P1 site. Thus, the appearance of this 35 kDa band in Fig. 13 reflects either cleavage of (variant) PAI-1 by thrombin or complex formation of (variant) PAI-1 with thrombin, as inhibition of a protease by PAI-1 always involves cleavage of PAI-1 (see 1.2). Theoretically, this C-terminal PAI-1 peptide has a molecular mass of 3.7 kDa, so it very likely displays an unusual apparent weight in the gel. Interestingly, a similar band has already been detected as containing PAI-1 antigen before, which is in line with my data [Patston and Schapira, 1994].

Fragment number three had two different amino-terminal sequences: TFGSGEADCGLRP and IVEGSDAEIGMSP. The first one is the amino terminal sequence of the thrombin A-chain (4 kDa) and the second one is that of the thrombin B-chain (29 kDa). These chains are linked via a disulfide bond in thrombin [Fenton, 1986]. Active thrombin runs in the gel at approximately 30 kDa, which is in line with its theoretical mass of 33 kDa. Therefore, band number three (apparent molecular

weight of less than 25 kDa) must be a degradation product of thrombin. As the fragment is even smaller than the B-chain alone and both the N-termini of the A- and B-chain are intact, the fragment consists most likely of the A-chain, which is disulfide linked to a B-chain lacking its C-terminus.

3.7. Binding of PAI-1 Variants to ECM Proteins

3.7.1. Binding to Vn-coated Microtiter Plates

The mutants M2, M3, M4, M8, M9, ∆109-123, ∆109-112, A114-115, A114-118, and P73A had already been tested for binding to Vn-coated microtiter plates by Nuria Arroyo de Prada, another member of our research group [Arroyo de Prada, N., Schroeck, F., et al., 2002]. The inactive mutants M2, M3, M4, M9, and ∆109-123 did not bind to Vn, whereas the active mutants M8, ∆109-112, A114-115, A114-118 and P73A did.

In the present study, the binding of two newly expressed PAI-1 mutants (Q55P and Q123K) which were previously described in the literature [Lawrence et al., 1994] was tested. Q55P was not inhibitory active against uPA and did not bind to Vn. In contrast to wt-PAI-1, Q123K displayed only a slight increase in binding to Vn with increasing concentrations from 0 to 45 U/ml. Neither PAI-1 nor Q123K bound to collagen type IV-coated control wells (Fig. 15).

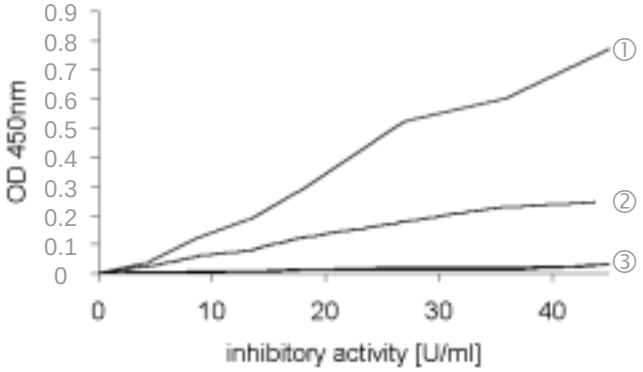

Fig. 15. Dose-dependent adhesion of PAI-1 to Vn-coated microtiter plates. The given concentrations of wt-PAI-1 (graph ①) and Q123K (graph ②) were allowed to bind to coated Vn. The proteins did not bind to wells coated with collagen type IV (graph ③).

In order to test whether this binding was specific, competition experiments were performed: PAI-1 or Q123K (in concentrations of 50 or 100 U/ml) were incubated with 10 µg/ml Vn or with 1,000 U/ml Hep prior to adding it to the wells. Wt-PAI-1 binding could be reduced by 80% by addition of soluble Vn whereas the same amount of Q123K interacted with Vn, regardless of whether soluble Vn was added or not. When incubated with Hep, about 50% of wt-PAI-1 and 20% of Q123K bound to Vn. Collagen type IV (10 µg/ml) added to wt-PAI-1 or Q123K before the solution was pipetted into the wells did not significantly change adhesion to Vn. Only very small amounts of the proteins bound to control wells which were coated with collagen type IV (Fig. 16). Similar results were obtained when the wells were washed with buffers containing higher concentrations of NaCl.

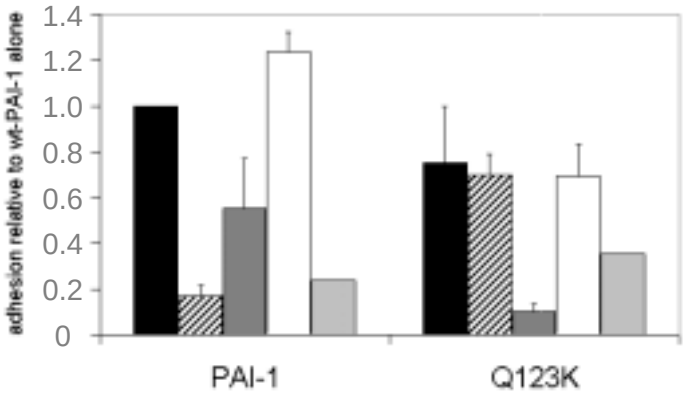

Fig. 16. Competition of binding of wt-PAI-1 and Q123K to Vn coated microtiter plates. 100 U/ml of (mutant) PAI-1 were allowed to bind to coated Vn in the presence of buffer only (black bars), 133 nM Vn (hatched bars), 1,000 U/ml Hep (dark grey bars), or collagen type IV (10µg/ml, white bars). Binding to coated collagen type IV was minimal (light grey bars). Results from two independent experiments +/- SD.

3.7.2. Surface Plasmon Resonance Spectroscopy

Wt-PAI-1, M3, M4, M8new, M9, P73A, A114-118, and Q123K were chosen for analysis by SPR. A114-115 was not tested, as it was not expected to have a lower affinity towards Vn than A114-118. Δ109-112 was not tested because of its low specific inhibitory activity. The inactive variants Δ109-123, M2, and Q55P were not

tested, because the other inactive variants M3, M4, and M9 were chosen as inactive controls.

3.7.2.1. Interaction of PAI-1 (Variants) with Immobilized Vn

Wt-PAI-1 bound with high affinity to immobilized Vn. It did not dissociate considerably during washing with buffer, but addition of uPA led to total dissociation of the bound PAI-1 from immobilized Vn (Fig. 17). The inactive mutants M3, M4, and M9 did not bind to Vn. M8new bound to Vn (data not shown), but no conclusions about its affinity towards Vn could be drawn, because bulk effects complicated the analysis of the binding profiles. With this mutant, high concentrations of total protein had to be injected due to its low specific inhibitory activity, which probably was the cause for the bulk effects. P73A behaved similarly to wt-PAI-1 (data not shown). Mutant A114-118 displayed a slower association to and a faster dissociation from Vn than wt-PAI-1. Only a low amount of mutant Q123K associated with Vn and was dissociated immediately after washing with buffer, indicating an unspecific interaction with Vn (Fig. 17).

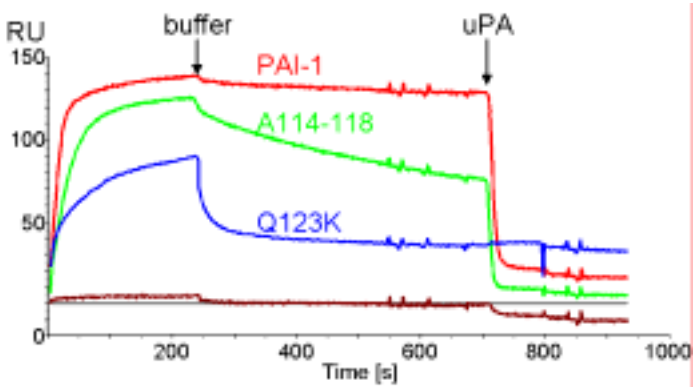

Fig. 17. SPR: Binding of (variant) PAI-1 to immobilized Vn. Wt-PAI-1 displays fast association to and nearly no dissociation from Vn. Addition of uPA leads to dissociation. A114-118 associates a little slower and dissociates faster from Vn as the wt. Q123K only shows unspecific binding (see text). The brown control curve shows that denatured wt-PAI-1 does not bind to Vn.

Mutant Q123K did not show any change in binding to solid phase Vn in the presence or absence of soluble Vn, whereas the other tested active mutants did not bind to immobilized Vn when incubated with soluble Vn prior to injection (Fig. 18 and data not shown). All Vn-binding mutants, similar to wt-PAI-1, dissociated immediately from Vn after uPA injection. Control experiments showed no binding of denatured or latent PAI-1 to immobilized Vn (Fig. 17). Thus, these results clearly demonstrate that active (mutant) PAI-1, but not Q123K, specifically bound to Vn immobilized to the dextran matrix of the CM5 chip, and then still was able to form complexes with uPA.

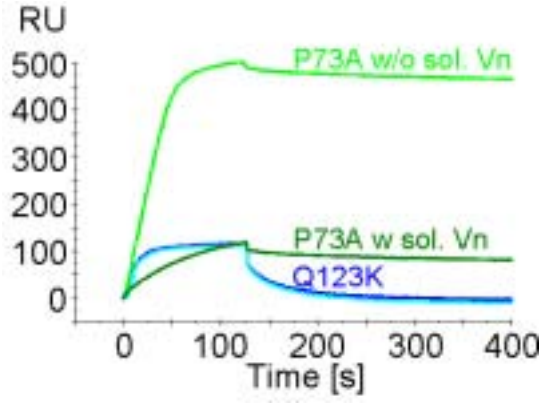

Fig. 18. SPR: Competition of variant PAI-1 binding to immobilized Vn with soluble Vn. P73A only bound to Vn in the absence of soluble Vn (light green) but not in its presence (dark green). Q123K did not show any differences in Vn binding in the presence (dark blue) or absence (light blue) of soluble Vn.

3.7.2.2. Interaction of wt-PAI-1 and Q123K with Native and Denatured Vn

A similar approach was used to test for differences in affinities of wt-PAI-1 and Q123K towards monomeric (plasma) Vn and multimeric (denatured, 3 min. at 95 °C) Vn. The monoclonal antibody to vitronectin VN7 was immobilized to the dextran surface of the CM5 sensor chip and then either native or denatured Vn were bound. Similar amounts of both types of Vn bound to this mab. Then different concentrations of wt-PAI-1 or Q123K were injected, followed by injection of uPA. The binding of both wt-PAI-1 and Q123K to denatured Vn completely resembled the binding of those two proteins to immobilized Vn (compare 3.7.2.1 with Fig. 19). The binding of Q123K to

denatured Vn was shown to be unspecific, as a control experiment in which Q123K was injected onto a surface exhibiting the antibody VN7 only, resulted in a similar binding profile. Surprisingly, no significant differences in affinities of wt-PAI-1 and Q123K towards native Vn could be detected. A significant amount of both proteins specifically bound to Vn. In contrast to wt-PAI-1, Q123K bound to native Vn did not dissociate from it upon uPA injection, neither did uPA bind to the Vn-bound Q123K (Fig. 19). However, addition of 150 nM native or denatured Vn to Q123K did not impair its inhibitory activity towards uPA, as tested in chromogenic assays. In control experiments, no binding of latent (incubation for 24h at 37 °C and pH 7.4 prior to injection) or denatured (3 min. at 95 °C) wt-PAI-1 or Q123K to native or denatured Vn could be detected. Thus, Q123K specifically binds to native Vn in a similar manner as wt-PAI-1, whereas it is not able to specifically bind to denatured Vn.

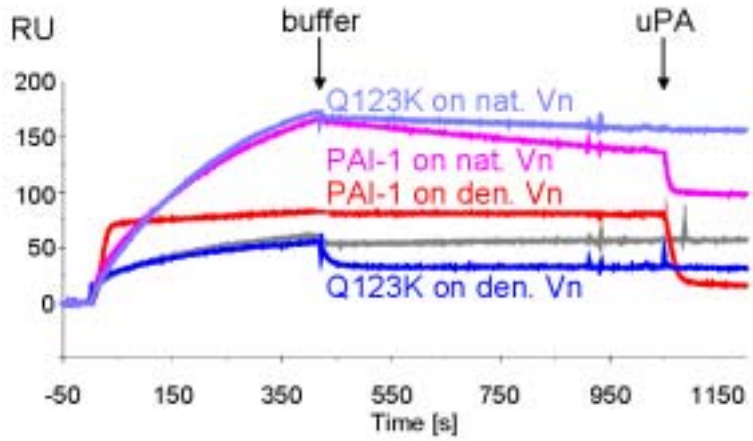

Fig. 19. SPR: Binding of wt-PAI-1 and Q123K to native and denatured Vn. Native or denatured Vn were allowed to bind to mab VN7 followed by injection of (variant) PAI-1. The binding of wt-PAI-1 to denatured Vn (red curve) as well as the binding of Q123K to denatured Vn (blue curve) were similar to the binding of these proteins to immobilized Vn. Association to native Vn did not differ between the wt (magenta curve) and Q123K (light blue curve). The grey curve shows a control experiment in which the (unspecific) binding of Q123K to the mab VN7 without bound Vn was measured.

In order to test whether native Vn becomes multimeric upon interaction with wt-PAI-1 or Q123K, the following experiment was performed. Vn was incubated with a threefold molar excess of (mutant) PAI-1 for 15 min at RT. Then the potential PAI-1/Vn complexes were allowed to bind to the antibody VN7. A high amount of the

wt-PAI-1/Vn complex bound to the mab. This complex contained active PAI-1, as subsequent injection of uPA led to dissociation of PAI-1 from the surface. After the dissociation of wt-PAI-1, mab 13H1 (which predominantly detects multimeric Vn [Stockmann et al., 1993]) was injected leading to a significant increase in RU (curve ① in Fig. 20). When the potential Q123K/Vn complex was injected, only a low amount of protein bound to the chip (curve ② in Fig. 20). This binding curve resembled the binding curve of Vn only to mab VN7 (curve ③ in Fig. 20). Upon injection of uPA no change in the signal of bound Q123K could be detected. Injection of mab 13H1 also led to an increase in RU, although this was not as high as with wt-PAI-1 (curve ② in Fig. 20).

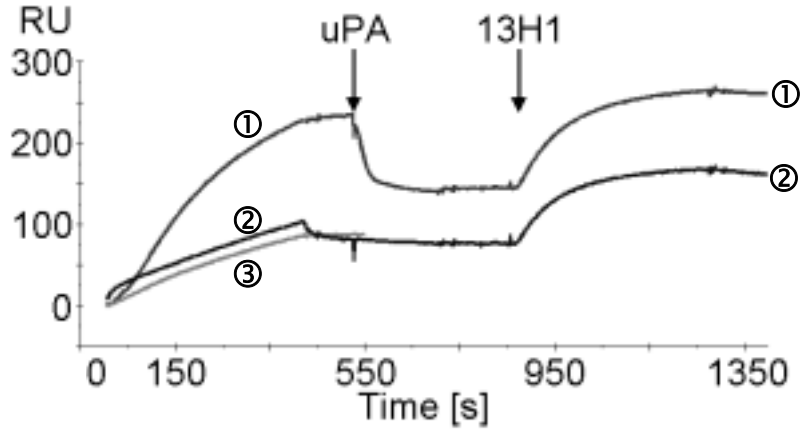

Fig. 20. SPR: Binding of wt-PAI-1/Vn complexes and Q123K preincubated with native Vn to mab VN7. Wt-PAI-1 or Q123K were preincubated with native Vn and subsequently injected onto the mab VN7 surface. Wt-PAI-1/Vn complexes bound to the surface (curve ①). Upon uPA injection the active PAI-1 dissociated from the complex (curve ①). Binding of Q123K preincubated with native Vn (curve ②) was similar to the binding of native Vn alone to the mab VN7 (curve ③). In this case no change in RU was detected upon uPA-injection (curve ②). Then 13H1 was injected. A higher amount of this mab bound to the Vn previously in complex with PAI-1 (curve ①) than to the Vn previously incubated with Q123K (curve ②).

Moreover, the binding profiles of 13H1 to native or denatured Vn were compared to the binding profiles of this mab to Vn which was previously incubated with wt-PAI-1 or Q123K (Fig. 21). The binding profile of 13H1 binding to native Vn was exactly the same as that of 13H1 binding to Vn previously incubated with Q123K (curve ④ and ③). The response of 13H1 binding to Vn which was incubated with wt-PAI-1 (curve

②) was in between the response of 13H1 binding to native Vn (curve ④) and of 13H1 binding to denatured Vn (curve ①). This indicates that only wt-PAI-1, but not Q123K, is able to induce multimerization of Vn.

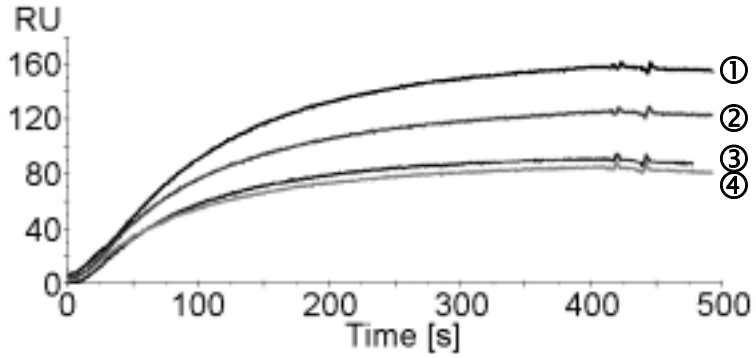

Fig. 21. SPR: Comparison of the binding profiles of 13H1 to Vn previously incubated or not incubated with (variant) PAI-1. Binding of 13H1 to native Vn (curve ④) was similar to 13H1 binding to Vn previously incubated with Q123K (curve ③). The binding profile of 13H1 to Vn previously incubated with wt-PAI-1 (curve ②) was in between the binding profiles of 13H1 binding to native Vn (curve ④) and to denatured Vn (curve ①).

3.7.2.3. Calculation of Binding Constants

The K_D values could be calculated from the data obtained with SPR analysis (Tab. 4).

	immobilized Vn	denatured Vn	native Vn
wt-PAI-1	0.18 nM	0.16 nM	14.7 nM
Q123K	no specific bind.	no specific bind.	11.4 nM
A114-118	1.87 nM	n. t.	n. t.
P73A	0.33 nM	n. t.	n. t.

Table 4. K_D values determined with SPR; n. t. not tested.

The observed K_D values for wt-PAI-1 binding to immobilized Vn and for wt-PAI-1 binding to denatured Vn were similar. P73A bound with a similar affinity to immobilized Vn as wt-PAI-1, whereas mutant A114-118 displayed a lower affinity to immobilized Vn as wt-PAI-1. For the binding of Q123K to immobilized as well as to

denatured Vn K_D values could not be calculated as the binding profiles could not be fit with a 1:1 Langmuir binding profile.

The affinities of both wt-PAI-1 and Q123K towards native Vn were approximately hundred times lower than the affinity of wt-PAI-1 towards denatured or immobilized Vn.

3.7.2.4. Interaction with Hep

All tested mutants (P73A, A114-118, and Q123K) as well as wt-PAI-1 bound to biotinylated Hep immobilized on a SA sensor chip. P73A and Q123K behaved similar to wt-PAI-1. Fig. 22 shows the binding profiles of wt-PAI-1 and A114-118 to immobilized Hep. Obviously, there are no major differences in affinity of these two proteins towards Hep.

For qualitative measurements, this analysis worked well, but in order to measure kinetic data it would have needed several adjustments of buffer and/or protein concentrations. As I could not detect big differences in heparin affinity of the PAI-1 mutants in the experiments described here, I was satisfied with the qualitative results and refrained from further studies of the measurement of PAI-1 binding to heparin.

Interestingly, after uPA injection an increase in RU could only be detected on surfaces with Hep bound wt-PAI-1 and not on surfaces with Hep bound A114-118 (Fig. 22).

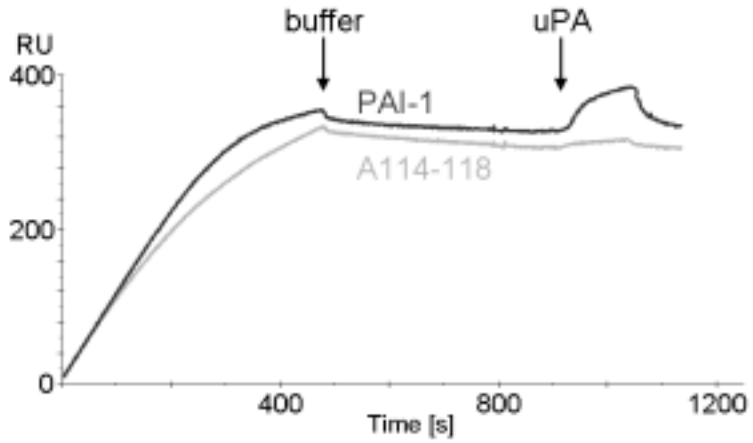

Fig. 22. SPR: Binding of wt-PAI-1 and A114-118 to surface bound Hep. A114-118's association to Hep was similar to that of the wt. The effects of washing with buffer were also similar. uPA was able to bind to heparin bound wt-PAI-1 but not to heparin bound A114-118.

Thus (as also shown in the thrombin inhibition assays, see 3.3.2), it is likely that inhibitory activity of A114-118 is reduced in the presence of Hep. This assumption was tested in chromogenic assays measuring uPA activity (Fig. 23). Inhibitory activity of A114-118 was reduced by approximately one third in the presence of 1,000 U/ml Hep, whereas wt-PAI-1 as well as Q123K displayed the same inhibitory activity in the presence or absence of 1,000 U/ml Hep.

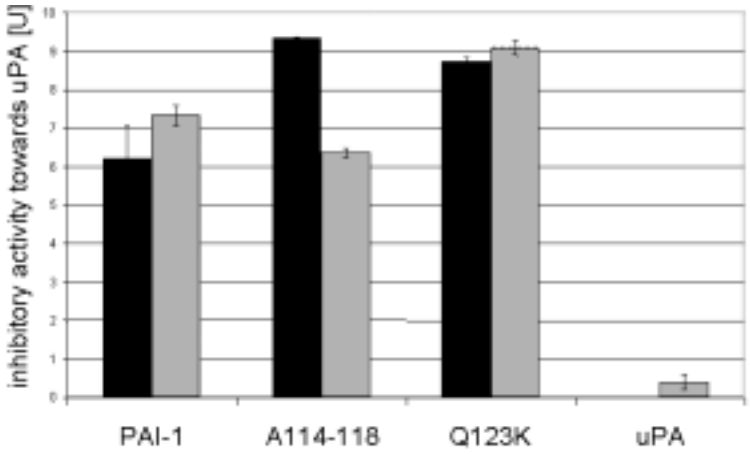

Fig. 23. Inhibitory activity of A114-118 is reduced by Hep. Inhibitory activity of (mutant) PAI-1 against uPA was measured with (grey bars) and without (black bars) 1,000 U/ml of Hep. in a chromogenic assay. Q123K and wt-PAI-1 had the same activity regardless of whether Hep was present or not, whereas the inhibitory activity of A114-118 was significantly reduced in the presence of 1,000 U/ml Hep. Heparin alone did not inhibit uPA (right grey bar). Data are from 3 independent experiments, each measured in duplicate +/- SD.

3.8. Cell Invasion Assays

The cell lines OV-MZ-6 and OV-MZ-19 were chosen, because both express similar amounts of uPA, tPA, and uPAR, but differ in expression of PAI-1 (Tab. 5). OV-MZ-19 does not express detectable levels of PAI-1, whereas OV-MZ-6 expresses a high amount of PAI-1 [Will et al., 1994]. Thus, with the OV-MZ-19 cell line all the PAI-1 present during invasion was assumed to be exogenous.

Cell line	uPA		t-PA		PAI-1		uPAR
	Cell extract	Culture medium	Cell extract	Culture medium	Cell extract	Culture medium	Cell extract
OV-MZ-6	10.5	13.7	<1.0	3.7	8.6	53.8	4.0
OV-MZ-19	18.4	24.2	<1.0	<1.0	<0.05	<0.05	1.9

Table 5. Antigen levels in the cell lines OV-MZ-6 and OV-MZ-19 as determined by ELISA. The antigen content in cell extracts is expressed in ng/mg protein and in the cell culture medium as ng/ml. From [Will et al, 1994].

Invasion of the tumor cell lines through Matrigel with or without Vn was compared: OV-MZ-6 was significantly more invasive than OV-MZ-19. This is in line with results from previous invasion assays [Will et al., 1994]. In contrast to the invasion of OV-MZ-6 cells, the invasion of OV-MZ-19 cells depended on the presence of Vn in the Matrigel. OV-MZ-19 cells were significantly more invasive in the presence of Vn (hatched bars) than in its absence (black bars in Fig. 24).

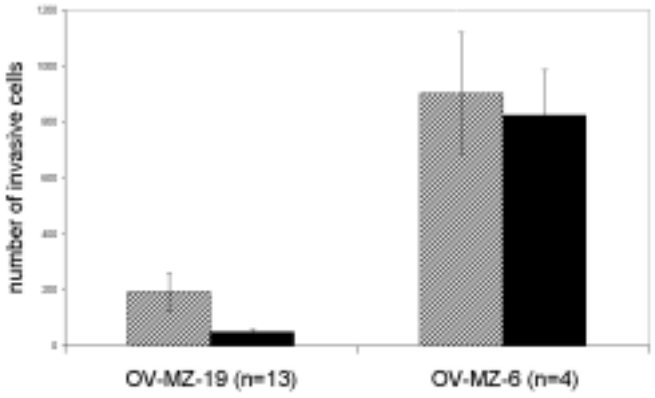

Fig. 24. Vn-dependent invasion of OV-MZ-19 but not OV-MZ-6. The ovarian tumor cell lines OV-MZ-19 and OV-MZ-6 were allowed to invade through Matrigel-covered membranes for 72 h. Then the cells on the lower side of the filter were counted under a microscope. Assays were performed with matrigel supplemented with 5% Vn (hatched bars) or without Vn (black bars). n indicates the number of experiments performed. Data are shown +/-SD.

Recently, studies have shown a dose-dependent effect of PAI-1 on angiogenesis [McMahon et al., 2001] [Devy et al., 2002]. Therefore, it was analyzed whether there is a dose-dependent effect of PAI-1 on tumor cell invasion in these *in vitro* assays of

Vn-dependent cell invasion. Different amounts (0.03 to 100 U/ml) of active wt-PAI-1 were added every 24 hours during the incubation. No differences in invasion of OV-MZ-19 cells could be detected at concentrations between 0.03 and 1 U/ml of wt-PAI-1 due to the high intra- and inter-assay variations and the probably rather small effects of the added PAI-1. Nevertheless, it was possible to significantly inhibit invasion of OV-MZ-19 cells at PAI-1 concentrations of 100 U/ml (approximately 2.6 µg/ml; Fig. 25).

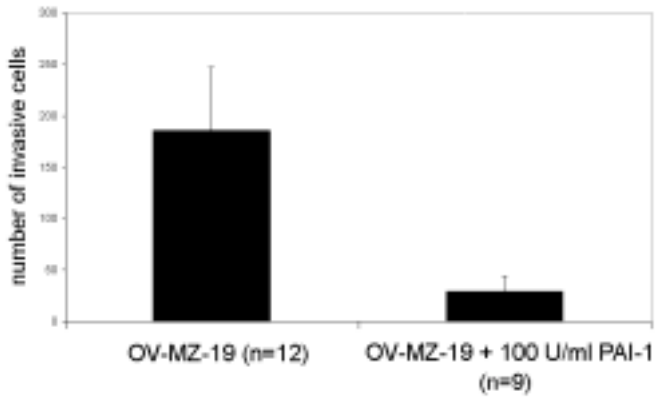

Fig. 25. 100 U/ml of wt-PAI-1 inhibit cell invasion. OV-MZ-19 cells were allowed to invade through the Vn-containing Matrigel for 72 h. Addition of 100 U/ml of wt-PAI-1 every 24 h decreased the number of invasive cells significantly. n indicates the number of experiments performed. Data are shown +/- SD.

4. Discussion

4.1. Antiproteolytic Activity of (Variant) PAI-1

For the purpose of functionally analyzing (variant) PAI-1, sufficient amounts of purified protein had to be obtained. The described expression and purification procedure, initially developed by Nuria Arroyo de Prada auf our research group [Arroyo de Prada, N., Schroeck F., et al., 2002], yielded high amounts of active PAI-1 (variants) only after denaturation and refolding of the proteins. In contrast, PAI-2 and its deletion variant PAI-2 ΔP66-P98 were active immediately after purification, which underlines the uniqueness of the metastability of PAI-1. The purification of PAI-1 proved to be very efficient, as a high proportion of the isolated protein was active (*e.g.* 95% active protein was obtained in a preparation with a specific inhibitory of 95,745 U/mg with a theoretical maximum of 100,000 U/mg [Gaffney and Heath, 1990]). The specific inhibitory activity of the PAI-1 mutants inversely correlated with the number of aa changed. Thus, the two point mutants Q123K and P73A reached a higher specific inhibitory activity than mutants in which more aa were deleted or changed (Δ109-112, A114-118, M8), whereas the other mutants with even more extensive changes were inactive. An exception was the point mutant Q55P, which was also inactive in this study, although it has been reported as inhibitorily active and non-Vn-binding previously [Lawrence et al., 1994]. In conclusion, it is likely that the modifications in the mutants did not only affect the vicinity of α-helix E, but also changed the overall structure of the whole PAI-1 molecule, leading to a lower inhibitory activity towards uPA.

The results from the assays directly measuring uPA activity with a chromogenic substrate were reproduced in plasminogen activation assays as well as in SDS-PAGE and Western blots visualizing SDS-stable complexes. This gives additional credibility to the results obtained in the routinely used assays.

4.2. Functional Interaction of PAI-1 with Vn

In order to more closely define the Vn-binding site of PAI-1, the interaction of PAI-1 variants with Vn was evaluated with four different techniques. Vn binding was tested on Vn-coated microtiter plates, with SPR, in thrombin inhibition experiments, and in Western blots visualizing SDS-stable complexes of PAI-1 with thrombin.

With SPR analysis, K_D-values for (variant) PAI-1 binding to Vn could be determined. Generally, accurate binding constants can be calculated with kinetic data from SPR. Still, in the case of PAI-1 the binding constants given in the result section cannot be absolutely accurate, because of two reasons: First, in order to calculate the binding constants the exact concentration of active PAI-1 in the preparation has to be known. For wt-PAI-1, the amount of active PAI-1 could be calculated just by measuring its inhibitory activity against uPA, as the theoretical maximum of PAI-1 activity was defined as 100,000 U/mg [Gaffney and Heath, 1990]. For the mutants however, this is not possible, because other maximal specific inhibitory activities could exist for them. Second, PAI-1 rather rapidly converts to its latent form at physiologic pH. Thus, from the time of dilution to the time of injection still another unknown part of the proteins converted to their latent form and were therefore unable to bind to Vn.

Because of these problems, the total amount of protein in the preparation was used for the calculation of the constants. Therefore, the numbers given should not be seen as absolute values, but rather used for comparison of the different affinities. This is still possible, because preparations with similar specific inhibitory activities of (mutant) PAI-1 were used in the SPR analysis. Nevertheless, the K_D-value for the PAI-1/Vn interaction in this study (K_D of 0.18 nM) was in a similar range as that of a previous study, also using SPR (K_D of 0.10 nM [Ehnebom et al., 2000])

As expected, the inactive mutants did not bind to Vn, as latent, RCL-cleaved, or misfolded PAI-1 cannot bind to Vn [Lawrence et al., 1997]. Therefore, conclusions about the Vn binding site of PAI-1 cannot be drawn from these probably misfolded mutants, but only from mutants with inhibitory activity. All inhibitorily active PAI-1 variants (with exception of Q123K) also bound to Vn-coated microtiter plates [Arroyo

de Prada, N., Schroeck, F., et al., 2002]. These results were confirmed with the use of the three other techniques, underlining that the mutants do not just bind to Vn, but are also able to functionally interact with it. Still, A114-118 had a slightly lower affinity to Vn than wt-PAI-1 in SPR analysis. In summary, neither deletion of the N-terminal part of hE (Δ109-112) nor changes in the C-terminal part of hE (A114-118) were able to completely abolish Vn binding of PAI-1. Therefore, the integrity of hE of PAI-1 is not a prerequisite for Vn binding.

Concerning the mutant Q123K, the results of Lawrence *et al.*, 1994, were reproduced. This mutant displayed only unspecific binding to Vn, as binding could not be competed by addition of soluble Vn. Moreover, this mutant dissociated very rapidly from immobilized Vn after washing with buffer, which also indicates unspecific binding. The small amount of Q123K which remained on the surface after washing with buffer did not interact with uPA, indicating the presence of only non-functional protein on the surface.

In addition to Q123K, Lawrence *et al.*, 1994, presented another PAI-1 mutant (L116P) which did not bind to Vn. In contrast to this report, two of the mutants studied in the present work (A114-118, M8), in which L116 (among other aa) was changed to alanine, still bound to Vn. This may indicate that the relatively conservative mutation of L116 to alanine (compared to the exchange of this aa with proline) was not dramatic enough to eliminate the Vn binding ability of these mutants.

Another group [Padmanabhan and Sane, 1995] localized the Vn binding site of PAI-1 to aa 115-130 employing PAI-1/PAI-2 chimeras as well as protease-digested PAI-1. This seems to be contradictory to the results presented here, but due to the compact tertiary structure of PAI-1 it seems very likely that digestion of PAI-1 by proteases leads to fragments with an altered overall structure. Furthermore, all of the PAI-1/PAI-2 chimeras that did not bind to Vn in the study of Padmanabhan and Sane, 1995, were not only altered around hE but also in the vicinity of Q123.

Van Meijer *et al.* used epitope-mapped monoclonal anti-PAI-1 antibodies that inhibited PAI-1/Vn interaction in order to map the Vn binding region of PAI-1 to aa

110-145 [van Meijer et al., 1994]. Again, this region not only comprises hE but also the area around the strand 1 edge of β-strand A, where Q123 is located [Sharp et al., 1999]. Crosslinking studies by Deng *et al.* implemented the same region in Vn binding [Deng et al., 1995].

However, Sui & Wiman could not detect any changes in Vn binding behavior of PAI-1 variants with mutations of single aa in the region F113 to D138, which is in line with the results presented here [Sui and Wiman, 1998].

Jensen *et al.* (2002) described aa M110, K122, and Q123, among others, as important for Vn-binding, applying a novel approach in which they scanned alanine mutants for protection by Vn from bis-ANS induced inhibition. The importance of K122 and Q123 is in line with our data and indicates an importance of ß-strand 1A for Vn-binding. M110 was deleted in our mutant Δ109-112, but our mutant still bound to Vn. A possible explanation could be that the deletion of these four aa in hE had another impact on the PAI-1 surface than the mutation M110A described in this paper [Jensen et al., 2002]. It is possible that one of the aa of the C-terminal region of hE could substitute the function of M110 in the mutant Δ109-112.

In summary, it is very likely that the importance of hE for Vn binding of PAI-1 was overestimated previously. None of the studies described above were able to narrow down the Vn binding region of PAI-1 to hE only. Moreover, the results presented in this study provide evidence, that the integrity of hE in PAI-1 is not an essential prerequisite for Vn binding. Still, hE does play a role in Vn binding of PAI-1, as demonstrated by the slower association to and especially the faster dissociation from immobilized Vn of A114-118 in SPR. Altogether, it is likely that there is some cooperativity of hE and the region around β-strand 1A (where Q123 is located) of PAI-1 in Vn binding.

Furthermore, the relevance of PAI-1 and Vn for tumor cell invasion could be shown with *in vitro* cell invasion assays. In these assays, OV-MZ-19 cells were able to penetrate significantly more effectively through Vn-supplemented Matrigel than

through Matrigel lacking Vn. A possible explanation for this observation could be that this cell line is primarily adhesive via αvβ3-integrins. Binding of this integrin to Vn could then lead to upregulation of components of the plasmin activation system, increased surface bound plasminogen levels, and eventually also to a more invasive phenotype as already described for a melanoma cell line [Khatib et al., 2001]. Vn-dependent invasion of OV-MZ-19 cells through Matrigel was blocked by repeated addition of 100 U/ml PAI-1 (approximately 2.6 µg/ml) to the cell medium, which is in line with previous studies showing the ability of Vn-bound PAI-1 in high concentrations to inhibit invasion of tumor cells into amnion membranes *in vitro* [Bruckner et al., 1992]. The PAI-1 concentration used in the present study is an approximately fifty fold excess of PAI-1 naturally present in the supernatant of OV-MZ-6 cells (these cells express approximately 50 ng/ml of PAI-1 [Will et al., 1994]). Because of this high PAI-1 concentration, the migration inhibiting effect is most likely due to the antiproteolytic activity of PAI-1. Still, it is possible that stabilization of the active PAI-1 conformation by Vn plays a role during this process.

Due to the fact that Q123K is now widely used as a non-Vn-binding mutant in *in vitro* and *in vivo* studies (see introduction), the established SPR analysis was used to further characterize the Vn-binding behavior of wt-PAI-1 and this mutant. For this, differences in interaction between wt-PAI-1 and Q123K in binding to immobilized, denatured or native Vn were observed. The fact, that binding of wt-PAI-1 and Q123K to denatured Vn fully resembled the binding of these proteins to immobilized Vn indicates that Vn was denatured during the immobilization process. Denaturation of even conformationally more stable proteins than Vn such as antibodies occurring during the adsorption of proteins to microtiter plates is a widely known problem [König and Skerra, 1998]. It is likely that similar conformational changes occurred in Vn during the immobilization to the dextran matrix.

Surprisingly, the two proteins did not differ in binding to native Vn, as the observed K_D-values were similar. Nevertheless, their affinity to native Vn was approximately

hundred times lower than that of wt-PAI-1 to denatured Vn, although it was still in the nM-range. This is in line with data indicating that most ligands preferentially interact with the multimeric form of Vn [Schvartz et al., 1999]. Up to now, there have not been any studies performed testing the interaction of PAI-1 with monomeric Vn: Two studies tested PAI-1 binding to coated Vn [Lawrence et al., 1994] [Sui and Wiman, 1998]. This Vn most probably is in the multimeric state due to conformational changes occurring during the adhesion to the plastic. The studies incubating PAI-1 with Vn in solution and then detecting the formed PAI-1/Vn complexes [Padmanabhan and Sane, 1995] [van Meijer et al., 1994] still do not really measure PAI-1 binding to native Vn as interaction of PAI-1 with Vn leads to multimerization of Vn [Seiffert and Loskutoff, 1996] [Minor and Peterson, 2002]. In fact, the SPR studies described here also provide evidence that presence of PAI-1 leads to multimerization of Vn. Preformed PAI-1/Vn complexes that were bound to mab VN7 displayed enhanced binding of mab 13H1 (which preferentially binds to multimeric Vn) after PAI-1 dissociation compared to the native Vn bound to VN7. Although the binding profile of 13H1 to the Vn previously in complex with PAI-1 was not totally identical with the binding profile of 13H1 to denatured Vn, the result still suggests that at least part of the Vn present in the complex was multimeric.

In contrast to wt-PAI-1, Q123K apparently was not able to multimerize Vn, as binding of 13H1 to the Vn previously incubated with Q123K was similar to 13H1 binding to native Vn. Thus, Q123K is able to bind to native Vn, but does not induce its multimerization.

Q123K (in several variations) has been and still is widely used in both *in vitro* and *in vivo* studies as a “non-Vn-binding” PAI-1 mutant (see introduction). Taking into account the results of this study, conclusions of studies ascribing a certain function of PAI-1 (*e. g.* cell migration or angiogenesis) to either its inhibitory activity or its interaction with Vn based only on observations of differences between wt-PAI-1 and Q123K have to be taken with caution. These results probably are rather due to differences in induction of multimerization of Vn or in binding to multimeric Vn and not

due to differences in overall Vn-binding capacity between wt-PAI-1 and Q123K. Moreover, several groups introduced other mutations in addition to Q123K in PAI-1, *e. g.* in order to increase the half life of this mutant [Redmond et al., 2001] [Devy et al., 2002]. It is questionable whether the still incomplete biochemical data about Q123K can easily be transferred to PAI-1 mutants harboring mutations in addition to Q123K. Therefore, further research is needed to exactly define the biochemical interaction of Q123K and its variants with Vn.

4.3. Functional Interaction of PAI-1 with Hep

Interaction of PAI-1 (variants) with Hep was evaluated in three different assays. Wt-PAI-1 was able to bind to immobilized Hep in SPR and interaction with Hep also provided PAI-1 with thrombin inhibitory properties as shown by measurement of thrombin activity in a chromogenic assay as well as in Western blots visualizing SDS stable complexes of PAI-1 with thrombin. These data are in line with previous reports concerning the ability of Hep to provide PAI-1 with thrombin inhibitory properties [Ehrlich et al., 1991]. Moreover, the ideal Hep concentration for thrombin inhibition by PAI-1 in this study was 1 U/ml which confirms the data of Ehrlich *et al.*, 1991.

The mutants Q123K and P73A did not differ from wt-PAI-1 in interaction with Hep. Both bound to immobilized Hep in SPR and also inhibited thrombin in the presence of Hep like wt-PAI-1. This was expected for mutant Q123K, but not for mutant P73A, because the heparin binding region of PAI-1 was previously located to α-helix D (hD), in which P73 is located [Ehrlich et al., 1992]. Ehrlich *et al.*, 1992, implicated the basic aa K65, K69, R76, K80 and K88 of PAI-1 in Hep binding. Three of them (K69, R76, K80) are located in hD. In P73A a distortion of hD was expected due to the mutation of the proline residue, but probably this distortion was not strong enough to impair Hep binding of the basic aa still present in hD.

Interestingly, two other basic residues (R115 and R118) located in hE were also made responsible for Hep binding of PAI-1, as the mutants R115D and R118D were eluted earlier from a heparin-sepharose chromatography column [Sui and Wiman,

1998]. These two aa (among others) were changed to alanine in the mutant A114-118. In contrast to the results of Sui & Wiman, 1998, this mutant still bound to immobilized Hep in SPR. Nevertheless, it did differ from wt-PAI-1, as Hep-bound A114-118 did not interact with uPA as well as wt-PAI-1 in SPR. Moreover, Hep did not provide A114-118 with inhibitory properties against thrombin as efficiently as wt-PAI-1 and its inhibitory activity against uPA was reduced in the presence of Hep. A possible explanation for these results could be that the rather conservative change to alanine in A114-118 compared to the exchange of basic aa to acidic aa (R115D and R118D) was not sufficient to completely abolish Hep binding of PAI-1, but still did alter the functional interaction with it. Together with the results from Ehrlich *et al.*, 1992, and Sui & Wiman, 1998, it seems likely that the basic aa in hD as well as in hE are involved in Hep binding of PAI-1.

5. Recent Developments and Outlook

After termination of the laboratory work for this study, several new studies have been published further characterizing the PAI-1/Vn interaction. Most importantly, Zhou *et al.*, 2003, published the crystal structure of a stable PAI-1 variant [Berkenpas et al., 1995] in complex with the SMB domain of Vn. Here, the SMB domain binds across hE and hF of PAI-1 with the beta strands 1A and 2A lying in-between these helixes (Fig. 26).

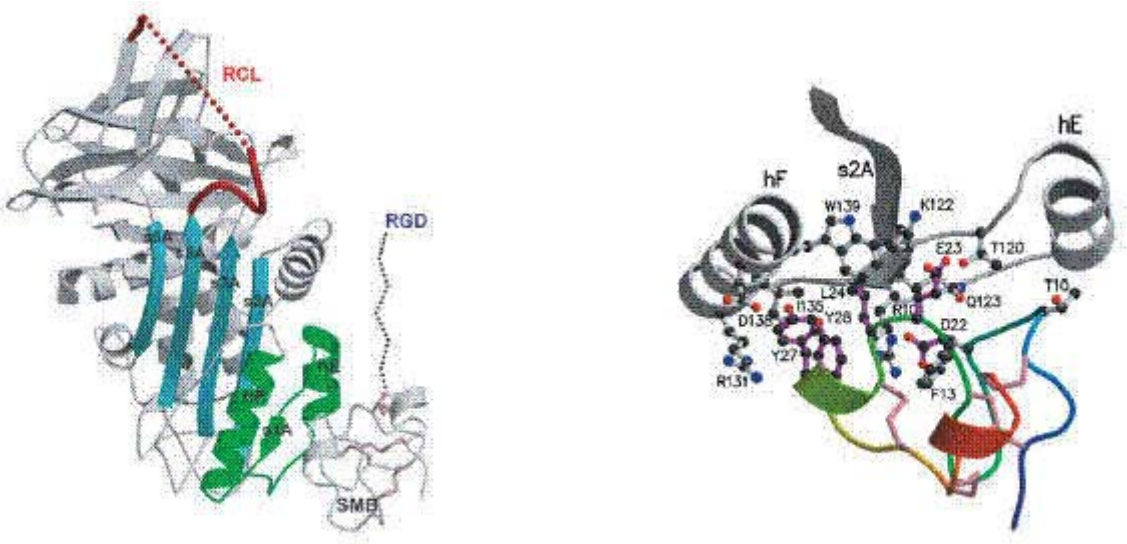

Fig. 26. Structure of the SMB/PAI-1 complex. SMB (on the right side of the left picture and in color in the right picture) binds across hE and hF of PAI-1. From [Zhou et al., 2003].

This is in line with the data from the present study as well as with data from various other studies attributing the area around hE and s1A to Vn binding (see 4.2). The authors also provide a rather simple explanation for the stabilization of the active conformation of PAI-1 by Vn: binding of the SMB domain to PAI-1 prevents the movements of s1A and s2A which are occurring during latency transition of PAI-1 [Zhou et al, 2003]. As discussed above, the widely used PAI-1 mutant Q123K cannot simply be described as non-Vn-binding. Therefore, Jensen *et al.*, 2004, developed a non-Vn-binding PAI-1 mutant (the triple mutant R101A M110A Q123A) which does not differ from wt-PAI-1 with respect to specific inhibitory activity, latency transition

rate, or binding of the uPA/PAI-1 complex to LRP, but did not functionally interact with multimeric Vn [Jensen et al., 2004]. Still, this group did not evaluate binding to native Vn so further research is needed to evaluate this interaction. Provided that this new mutant also does not interact with native Vn, it should be used in all further studies investigating the PAI-1/Vn interaction as a non-Vn-binding PAI-1 variant rather than Q123K.

Concerning the possible use of specific PAI-1 inhibitors for therapeutic purposes, a lot of work is currently being performed to characterize various inhibitors *in vitro* and *in vivo*. Most of these inhibitors are either antibodies or low molecular weight inhibitors targeting the antiproteolytic function of PAI-1 rather than the Vn-binding site. Inhibition of PAI-1 by these compounds is due to direct interference with the RCL, due to induction of substrate behavior, or due to induction of an increased rate of latency transition of PAI-1 (for a review see [Gils and Declerck, 2004]). Nevertheless, Chavakis *et al.*, 2002, described a 17mer peptide derived from domain 5 of high molecular weight kininogen which inhibits the binding of PAI-1 to multimeric Vn.

As more and more data are available about both the function and the structure of PAI-1, it is likely that in the near future more potentially therapeutically applicable PAI-1 inhibitors will be found which eventually will lead to new therapeutic strategies in cardiovascular disease and cancer.

6. Summary

The serpin plasminogen activator inhibitor type-1 (PAI-1) is of importance in physiological processes such as fibrinolysis and thrombolysis as well as in pathophysiological processes like thrombosis, tumor cell adhesion or invasion, and metastasis. The interaction of PAI-1 with the extracellular matrix protein vitronectin (Vn) was implicated to play an important role in several of these processes and is therefore a possible target for therapeutic strategies. Understanding the PAI-1/Vn interaction is a substantial prerequisite for the development of therapeutically applicable compounds. Thus, in the present study, the Vn-binding behavior of PAI-1 was analyzed by characterizing twelve different PAI-1 variants with variations in regions previously implicated in Vn-binding of PAI-1 (*i.e.* mainly around alpha-helix E (hE) of PAI-1). For this, variant PAI-1 was expressed in *E. coli* and subsequently its inhibitory activity towards uPA was determined. Interaction with Vn was measured with four different techniques, including surface plasmon resonance. Moreover, several PAI-1 mutants were also characterized concerning their functional interaction with the extracellular matrix component heparin. On the one hand evidence is provided that the integrity of hE of PAI-1 is not essential for Vn-binding, on the other hand some cooperativity of hE and beta strand 1A of PAI-1 in Vn-binding may exist, as the mutant A114-118 (change of amino acids F114-R118 into AAAAA) shows reduced affinity towards Vn and the mutant Q123K only shows unspecific binding to multimeric Vn. Interestingly, the mutant Q123K which is now widely used as a non-Vn-binding PAI-1 variant in several *in vitro* and *in vivo* studies, only differs from wt-PAI-1 in binding to multimeric Vn, but not in binding to native Vn. Nevertheless, this mutant is not able to functionally interact with native Vn like wt-PAI-1 does.

The biological relevance of PAI-1 and Vn in tumor cell invasion was studied in *in vitro* cell invasion assays. Here, the ovarian carcinoma cell line OV-MZ-19 displayed Vn-dependent invasiveness, which could be inhibited by addition of recombinant PAI-1.

7. Zusammenfassung

Plasminogen Aktivator Inhibitor Typ-1 (PAI-1), ein Serinprotease-Inhibitor, spielt eine wichtige Rolle sowohl in vielen physiologischen Vorgängen wie Fibrinolyse und Thrombolyse, als auch in vielen pathologischen Vorgängen wie Thrombosen, Tumorzelladhäsion oder -invasion und Metastasierung. Die Interaktion von PAI-1 mit dem extrazellulären Matrixprotein Vitronektin (Vn) ist bei diesen Prozessen von hoher Relevanz und ist deshalb ein möglicher Angriffspunkt für zukünftige therapeutische Strategien. Das Verständnis der Interaktion von PAI-1 mit Vn ist hierbei eine wichtige Voraussetzung für die Entwicklung neuer Substanzen. Um einen Beitrag hierzu zu leisten, wurden in der vorliegenden Arbeit zwölf PAI-1-Varianten mit Mutationen in der mutmaßlichen Vn-Bindungsstelle (um Alphahelix E (hE) herum) in *E. coli* exprimiert und auf ihr Vn-Bindungsverhalten hin untersucht. Zunächst wurde die inhibitorische Aktivität dieser Mutanten bestimmt, dann wurde ihre Interaktion mit Vn mit vier verschiedenen Methoden, darunter Oberflächenplasmonresonanz, untersucht. Außerdem wurden einige Mutanten auch im Hinblick auf ihre Interaktion mit der extrazellulären Matrixkomponente Heparin untersucht. Einerseits wird hier belegt, dass die Integrität von hE in PAI-1 nicht unbedingte Voraussetzung für die Interaktion mit Vn ist. Andererseits deuten die hier vorgelegten Daten daraufhin, dass eine Kooperation in der Vitronektinbindung zwischen hE und dem Betastrang 1A von PAI-1 besteht, da die Mutante A114-118 (Austausch der Aminosäuren F114-R118 gegen AAAAA) eine reduzierte Affinität zu Vn zeigt und die Mutante Q123K nur unspezifisch an Vn bindet. Interessanterweise unterscheidet sich Q123K, das mittlerweile viel in *in vitro* und *in vivo* Studien als nicht-vitronektinbindende PAI-1-Variante benutzt wird, von Wildtyp-PAI-1 nur im Bindungsverhalten gegenüber multimerisiertem Vn, nicht aber nativem Vn. Trotzdem interagiert Q123K mit nativem Vn funktionell anders als Wildtyp-PAI-1.

Die biologische Relevanz von PAI-1 und Vn für die Tumorzellinvasion konnte in *in vitro* Versuchen untersucht werden. Hier war die Ovarialkarzinomzelllinie OV-MZ-19 nur in der Gegenwart von Vitronektin invasiv und die Invasion der Zellen konnte durch Zugabe von rekombinantem PAI-1 inhibiert werden.

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9. Appendix

9.1. Characterized PAI-1 (Variants) and their Corresponding Amino Acid Alterations

Mutant 1 (Δ109-123)	Deletion of aa F109-Q123
Mutant 2 (M2)	F109-Q123 versus AAGAGAA
Mutant 3 (M3)	F109-Q123 versus homologue PAI-2 sequence[1] and point mutation E128G
Mutant 4 (M4)	F109-Q123 versus AAAA
Mutant 5 (Δ109-112)	Deletion of aa F109-H112
Mutant 6 (A114-115)	Point mutations F114A and R115A
Mutant 7 (A114-118)	F114-R118 versus AAAAA
Mutant 8 (M8 or M8 new[2])	F114-R118 versus AAAAA and point mutation D68G
Mutant 9 (M9)	V284-G294 versus homologue PAI-2 sequence[3]
Mutant 10 (P73A)	Point mutation P73A
Mutant 11 (Q123K)	Point mutation Q123K
Mutant 12 (Q55P)	Point mutation Q55P

Numbering of PAI-1 according to Ny *et al.*, 1986. [1] 141YIRLCQKYYSSEPQA155, [2] "M8 new" is protein from a subclone of M8-expressing *E. coli*, [3] 318YELRSILRSMG328, numbering of PAI-2 according to PIR protein sequence database: A32853.

9.2. One Letter Amino Acid Code

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartic acid	P	Proline
E	Glutamic acid	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophan
L	Leucine	Y	Tyrosine

9.3. Abbreviations

aa	amino acid(s)
Ab	antibody(ies)
bFGF	basic fibroblast growth factor, also called FGF2
BGG	bovine gamma globulin
BSA	bovine serum albumin
DMEM	Dulbecco's modified Eagles Medium
ECM	extra cellular matrix
FCS	fetal calf serum
FGF2	fibroblast growth factor 2
GPI	glycosyl-phosphatidyl-inositol
hD	alpha-helix D
hE	alpha-helix E
Hep	heparin
hF	alpha-helix F
HMW-uPA	high molecular weight uPA
IPTG	isopropyl thio-beta-D-galactoside
LDL	low density lipoprotein
LRP	LDL receptor-related protein
mab	monoclonal antibody(ies)
Min	Minutes
NTA	nitrilotriacetic acid
OD	optical density
PA	plasminogen activator
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor type 1
PAI-2	plasminogen activator inhibitor type 2
PAI-3	plasminogen activator inhibitor type 3, also known as protein C inhibitor
PBS	phosphate buffered saline
RCL	reactive center loop
RGD	arginine/glycine/aspartate
RT	room temperature
RU	reflectivity units (arbitrary units)
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulfate
Serpin	serine protease inhibitor
SMB	somatomedin B
SPR	surface plasmon resonance
TNF-α	tumor necrosis factor alpha
tPA	tissue type plasminogen activator
uPA	urokinase type plasminogen activator
uPAR	uPA receptor
Vn	vitronectin
wt	wild type

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