FEBS Letters 352 (1994) 342–346

FEBS 14603

Determination of the vitronectin binding site on plasminogen activator inhibitor 1 (PAI-1)

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Received 15 August 1994

Abstract Vitronectin is the carrier protein of plasminogen activator inhibitor 1 (PAI-1). We used a well-characterized panel of anti-human PAI-1 monoclonal antibodies (MoAbs) to localize the vitronectin-binding site on PAI-1. By employing a direct vitronectin/PAI-1 binding assay and two vitronectin-dependent inhibition assays, we demonstrate that the anti-PAI-1 MoAbs CLB-5, CLB-10, CLB-2C8 and I1, directed against different epitopes in the region between amino acids 110 and 145, prevent the interaction of PAI-1 with vitronectin. We conclude that the region between amino acids 110 and 145 of PAI-1 harbours an important determinant for the interaction with vitronectin.

Key words: Fibrinolysis; PAI-1; Vitronectin; Thrombin

1. Introduction

Plasminogen activator inhibitor 1 (PAI-1), a member of the serine protease inhibitor ('serpin') family [1–4], is a major regulatory protein of the fibrinolytic system [5]. Serpins act as a pseudo-substrate: the P1–P1′ peptide bond of the reactive center is contained within an exposed 'stressed' loop that acts as a 'bait' for the protease [6,7]. The interaction between the reactive center and the catalytic moiety of the protease results in a tight binding, typified by the formation of an equimolar, inactive SDS-stable complex. The target specificity of a serpin is mainly determined by the nature of the reactive center P1 residue. This is illustrated, e.g., by the properties of a PAI-1 mutant protein, having a methionine instead of the genuine arginine residue at the P1 position (PAI-1 R346M) [8], having a 10,000-fold lower rate of association with tissue-type plasminogen activator (t-PA) than 'wild-type' PAI-1.

It is conceivable that the physiologically relevant configuration of PAI-1 in plasma [9,10], in the subendothelial matrix [11] and in releasates of platelets [12], is as a complex with its carrier protein vitronectin. This interaction stabilizes the inhibitory activity [10] without altering the second-order association rate (k_1) with t-PA [13]. Surprisingly, in the presence of vitronectin, the inhibitory property of the virtually inactive mutant PAI-1 R346M towards t-PA is vastly improved [8]. In addition, the interaction between PAI-1 and vitronectin endows the inhibitor with thrombin-inhibitory properties as evidenced by a 200-fold increase of the k_1 and the formation of SDS-stable thrombin-PAI-1 complexes [14,15]. The significance of the interaction between thrombin and PAI-1 in the presence of vitronectin has been demonstrated by using matrices of cultured endothelial cells as a source of vitronectin–PAI-1 complexes [16].

Apart from vitronectin, we have recently reported that the glycosaminoglycan heparin can also act as an obligatory cofactor for the inhibition of thrombin by PAI-1 [17]. The molecular interactions between thrombin, PAI-1 and heparin have been described in some detail [18,19]. In contrast, the interaction between PAI-1, thrombin and vitronectin has not been extensively studied. The present study was undertaken to better define the area on PAI-1 involved in the interaction with vitronectin. By employing several approaches, we provide evidence that the region between the amino acid residues 110 and 145 harbours an important determinant for the interaction between PAI-1 and vitronectin.

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Abbreviations: BSA, bovine serum albumin; MoAb(s), monoclonal antibody(ies); PAI-1, plasminogen activator inhibitor 1; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; S.E.M., standard error of the mean; t-PA, tissue-type plasminogen activator.

2. Materials and methods

2.1. Materials

Wild-type PAI-1 and PAI-1 R346M were purified as described [8]. Active PAI-1 (kindly provided by Dr. T.M. Reilly, Dupont de Nemours, Wilmington, DE) was labeled with ^{125}I using the Bolton-Hunter method as described before [20], resulting in a specific radioactivity of 0.2 μCi/μg protein. Two-chain Bowes melanoma t-PA (910,000 IU/mg) was obtained from Biopool (# 1268403; Umea, Sweden). Purified human thrombin was provided by Dr. K. Mertens (Central Laboratory of the Blood Transfusion Service, The Netherlands). Active-site titration with *p*-nitrophenyl *p*′-guanidinobenzoate [21] yielded a concentration of 5.0 mg/ml. Thrombin was radiolabeled with I^{125} using the Iodogen method, resulting in a specific radioactivity of 1.6 μCi/μg protein. Vitronectin was purified from human plasma to apparent homogeneity [22]. The protein concentration of the purified preparation was determined, assuming an extinction coefficient at 280 nm of $E_{1\%}$ = 13.0 and a molecular weight of 75,000 [23]. Unfractionated heparin from porcine intestinal mucosa (# H-3125, Col 29F-0314; specific activity 178 units/mg, average molecular weight 15,000–18,000), was purchased from Sigma (St. Louis, MO). Murine anti-human PAI-1 MoAbs were obtained from different sources: hybridoma cells producing the MoAbs CLB-5, CLB-10, CLB-16, CLB-8H2 and CLB-2C8 were isolated and purified as described [24]. The MoAb MAI-12 was obtained as purified IgG from Biopool (Umea, Sweden). The MoAbs M3 and I1 were a gift of Dr. B. Holte (Monozyme, Charlottenlund, Denmark). The final concentration of all antibodies was determined by measuring the optical density at 280 nm (1 O.D. unit is 0.75 mg/ml [25]). The murine MoAb VN7, directed against vitronectin was produced and characterized as described [26]. The synthetic substrates H-D-isoleucyl-prolyl-arginine-*p*-nitroanilide (S2288) and H-D-phenylalanyl-pipecolyl-arginyl-*p*-nitroanilide (S2238) were obtained from Chromogenix (Gothenburg, Sweden).

2.2. Activation of PAI-1 (variant) and determination of the activity

Wild-type PAI-1 or PAI-1 R346M was activated by an incubation for 2 h at room temperature in 6 M guanidinium-HCl [27]. The denaturant was removed by overnight dialysis at 4°C against TST-buffer

SSDI 0014-5793(94)00990-2

(20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% Tween-80). Increasing amounts of activated wild-type PAI-1 or PAI-1 R346M were incubated for 1 h at 37°C in a total volume of 50 μl with 2.5 nM two-chain t-PA in TST-buffer, in the presence or absence of 40 nM vitronectin. Then, 200 μl 0.5 mM S2288 in TST-buffer was added and the residual t-PA activity was determined from a linear plot of the increase of the optical density at 405 nm versus time, using a Titertek Twinreader (Flow Laboratories, Irvine, UK).

2.3. Dose-dependent effect of various MoAbs on the ^{125}I-labeled-PAI-1/vitronectin interaction

The anti-vitronectin MoAb VN7 (1 μg/ 50 μl/well in 0.1 M $NaHCO_3$, pH 8.6) was coated overnight at 4°C in wells of a microtiter plate (Nunc Maxisorp, Gibco BRL, Paisley, UK). Wells coated with 2% (w/v) bovine serum albumin (BSA)/PBS served as a negative control. The wells were washed with distilled water and blocked for 1 h at 37°C with 3% (w/v) BSA/PBS. After washing with TBST-buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween-20), the wells were preincubated with [^{125}I]PAI-1 (40,000 counts per min/well) and increasing concentrations of different MoAbs in TBSB-buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (w/v) BSA) for 15 min at room temperature. Subsequently, after addition of 100 ng vitronectin/well the plate was incubated for 90 min at 37°C. The mixtures were removed and the plate was extensively washed with TBST-buffer. Bound [^{125}I]PAI-1/vitronectin complexes were eluted after addition of 200 μl 2% (w/v) SDS/TBST-buffer and incubation for 10 min at room temperature. The radioactivity was determined in a gamma-counter, and expressed as the percentage of maximal [^{125}I]PAI-1 binding. Data were corrected for binding to BSA-coated wells. The experiments were performed at least four times.

2.4. Inhibition of t-PA by PAI-1 R346M and vitronectin in the presence of different anti-PAI-1 MoAbs

5 nM active PAI-1 R346M was incubated for 30 min at room temperature in 30 μl TST-buffer, containing 40 nM vitronectin and 0.5 μg of either one of the anti-PAI-1 MoAbs. Subsequently, t-PA was added to a final concentration of 5 nM followed by an additional incubation of 1 h at 37°C. Finally, 200 μl of 0.5 mM of S2288 was added and the residual t-PA activity was determined as described above.

2.5. Inhibition of thrombin by wild-type PAI-1 and vitronectin or heparin in the presence of different anti-PAI-1 MoAbs

5 nM active wild-type PAI-1 was incubated either with 40 nM vitronectin or with 1 U/ml heparin and with 0.5 μg (or a range of 0–1 μg in case of the dose–response assay) of one of the anti-PAI-1 MoAbs as described above. After complex formation of PAI-1 with either one of the cofactors, 5 μl of 0.7 nM thrombin was added. After an incubation of 1 h at 37°C, the reaction was arrested by the addition of 200 μl of 0.5 mM S2238 and the residual thrombin activity was determined as described above.

Results and discussion

3.1. Effect of different anti-PAI-1 MoAbs on [^{125}I]PAI-1 binding to vitronectin

Previously, we described the properties of a panel of the anti-human PAI-1 MoAbs and determined the corresponding epitopes on the protein [24]. A summary of relevant data on the anti-PAI-1 MoAbs employed is outlined in Fig. 1. It should be noted that the corresponding epitopes, involved in the inhibitory activity of PAI-1, are located in two distinct areas namely, encompassing the reactive center P1–P1′(R346-M347) and the region between residues 110 and 145 [24]. We devised a binding assay and avoided immobilization of either vitronectin or PAI-1, since such manipulations are known to induce irreversible conformational changes and inactivation [15]. To that end, [^{125}I]PAI-1 was preincubated with increasing concentrations of different anti-PAI-1 MoAbs followed by the addition of vitronectin. Formed [^{125}I]PAI-1/vitronectin complexes were then bound to coated anti-vitronectin MoAb VN7. [^{125}I]PAI-1 bound to vitronectin is fully competed with a 100-fold excess of unlabeled PAI-1 (data not shown). The generation of [^{125}I]PAI-1/vitronectin complexes is partially prevented by the presence of the anti-PAI-1 MoAbs CLB-10, CLB-2C8, CLB-5 and I1 (53–66% competition), whereas none of the other MoAbs affects the interaction between [^{125}I]PAI-1 and vitronectin (EC_{50} values are given in Table 1). Combinations of the inhibitory MoAbs did not further reduce the binding of PAI-1 to vitronectin. Significantly, the corresponding epitope for each of the inhibitory MoAbs is exclusively located in the area between amino-acid residues 110 and 145 of PAI-1, suggesting that PAI-1 contains a single binding site for vitronectin. However, the observation that the indicated antibodies do not fully prevent complex formation does not exclude the presence of additional binding sites. Taken together, these data supports our conclusion that region 110 to 145 of PAI-1 harbors an essential determinant for the binary interaction with vitronectin. It should be noted that this area is also involved in the interaction of PAI-1 with serine proteases and with fibrin [24]. The importance of this site in ternary interactions, i.e. in the presence of serine proteases, will be subsequently assessed.

3.2. Prevention of t-PA inhibition by PAI-1 R346M in the presence of vitronectin by anti-PAI-1 MoAbs

The inhibition of t-PA by PAI-1 is independent of the presence of vitronectin [10]. Remarkably, however, vitronectin converts a virtually inactive PAI-1 mutant (PAI-1 R346M) into a rather efficient inhibitor of t-PA [8]. This observation was exploited to test the influence of selected anti-PAI-1 MoAbs in a ternary interaction, including t-PA, vitronectin and PAI-1 R346M, to gain insight into the cofactor-dependent inhibitory mechanism. We are particularly interested in the effect of MoAb CLB-10, since we showed before that MoAbs CLB-2C8, CLB-5 and I1 also directly prevented the interaction between

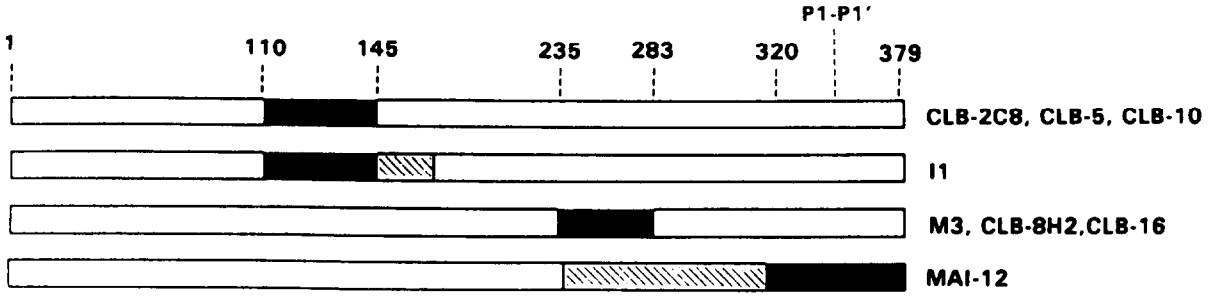

Fig. 1. Position of epitopes of anti-PAI-1 MoAbs on PAI-1. The binding site on PAI-1 for the MoAbs has been determined as described [24] and are indicated by black bars. The positions of the amino acids, bordering the areas in which the binding sites are located, are given. The location of the epitope of the MoAbs I1 and MAI-12 may be slightly extended in one direction as indicated by the shaded areas.

PAI-1 and t-PA in the absence of vitronectin, in contrast to CLB-10 [24]. The effect of different anti-PAI-1 MoAbs on vitronectin-dependent t-PA inhibition was measured using an endpoint assay with the chromogenic substrate S2288. As a control, t-PA inhibition by wild-type PAI-1 in the absence of vitronectin was determined in the presence of different anti-PAI-1 MoAbs (data not shown; see [24]). As expected, inhibitory MoAbs that map either around the reactive center (MAI-12) or within the 110–145 region (CLB-2C8,CLB-5, I1), also prevent inhibition of t-PA by PAI-1 R346M in the presence of vitronectin (Fig. 2, white bar). The presence of the antibody M3 (or CLB-16, CLB-8H2: data not shown) does not affect the inhibition of t-PA by wild-type PAI-1 nor of t-PA by PAI-1 R346M in the presence of vitronectin. Clearly, the anti-PAI-1 MoAb CLB-10 completely prevented the inhibition of t-PA by PAI-1 R346M in the presence of vitronectin (Fig. 2, white bar), but had no effect on the inhibition of t-PA by wild-type PAI-1 (data not shown; see [24]). These observations were confirmed by SDS-PAGE analysis of the formation of 'serpin specific', SDS-stable complexes between [^{125}I]labeled t-PA and PAI-1 R346M and prevention of complex formation by CLB-10 (data not shown). It is noteworthy that the virtually complete blockade of the vitronectin-dependent inhibitory activity by CLB-10 contrasts with the partial prevention of PAI-1/vitronectin complex formation (see previous section). Apparently, the ternary interaction (and ultimately inhibition of t-PA) fully relies on the availability of the 110–145 area of PAI-1 R346M to vitronectin, whereas other regions may contribute to the binary interaction, next to the 110–145 region. Consequently, we conclude that binary complexes of PAI-1 R346M and vitronectin are a prerequisite for the ternary inhibition reaction with t-PA.

3.3. *Effect of anti-PAI-1 MoAb CLB-10 on thrombin-inhibition by PAI-1 in the presence of either vitronectin or heparin*

We reported before that vitronectin, but also heparin, provides PAI-1 with thrombin-inhibitory properties and increases k_i at least two orders of magnitude [14,17]. Consequently, we incubated wild-type PAI-1, vitronectin or heparin, thrombin and various anti-PAI-1 MoAbs and measured residual amidolytic thrombin activity by using the chromogenic substrate S2238 (Fig. 2, black and hatched bars, respectively). Obviously, the inhibitory MoAbs CLB-2C8, CLB-5, I1 and MAI-12 prevent thrombin inhibition by PAI-1 both in the presence of vitronectin or heparin (data are shown for CLB-2C8 with vitronectin (Fig. 2, black bar) and with heparin (Fig. 2, hatched bar)). The antibody M3 (and similarly CLB-16, CLB-8H2; data not shown), did not affect the inhibition of thrombin by PAI-1 in the presence of either cofactor, in accordance with their lack of effect in the t-PA-based assays. MoAb CLB-10 fully prevented thrombin-inhibition in the presence of vitronectin, similarly as the effect of CLB-10 in the t-PA/PAI R346M assay in the presence of this cofactor (Fig. 2, black bar). The anti-PAI-1 MoAb CLB-10 did not alter the inhibition of thrombin by PAI-1 in the presence of heparin, in agreement with the finding that the heparin-binding site does not coincide with the 110–145 area [18] (Fig. 2, hatched bar). In addition, we assessed the formation of SDS-stable complexes between [^{125}I]thrombin and PAI-1 in the presence of vitronectin or heparin and, in particular, the effect of different anti-PAI-1 MoAbs on complex formation. Again, complex formation with vitronectin as cofactor is prevented in the presence of CLB-10 (and CLB-2C8 or MAI-12), but not in the presence of, e.g., M3 and CLB-16, whereas with heparin no effect is observed for CLB-10 (data not shown). From these results, we conclude inhibition of thrombin requires the full association of PAI-1 and vitronectin, mediated by the 110–145 area of PAI-1. This conclusion is in accordance with our recent observations that thrombin, PAI-1 and vitronectin form ternary complexes (M. van den Nieuwenhuizen and H. Pannekoek, manuscript in preparation).

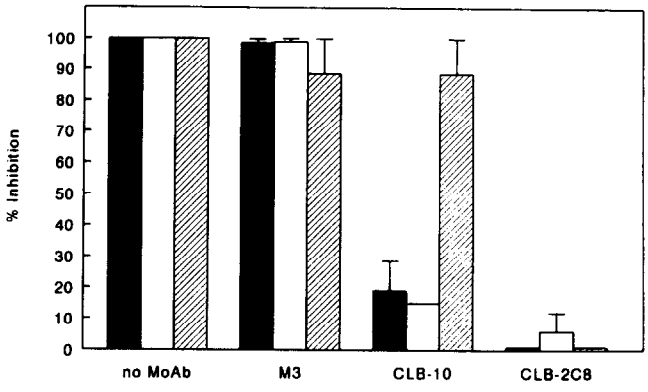

Fig. 2. Prevention of t-PA or thrombin inhibition by PAI-1 (variant) and vitronectin in the presence of different anti-PAI-1 MoAbs. The residual inhibition of t-PA or thrombin after incubation with PAI-1 (variant) and vitronectin in the presence of different anti-PAI-1 MoAbs is presented. Inhibition of t-PA by PAI-1 R346M or inhibition of thrombin by wild-type PAI-1 and heparin or vitronectin in the absence of MoAbs is indicated by 100%. Results of the representative MoAbs M3, CLB-10 and CLB-2C8 are presented. Data are presented as the mean of at least two experiments, while error bars indicated the standard deviation. Symbols: black bars, thrombin inhibition by wild- type PAI-1 and vitronectin; white bars, t-PA inhibition by PAI-1 R346M and vitronectin; hatched bars, thrombin inhibition by wild- type PAI-1 and heparin.

Table 1
Maximal inhibition and EC_{50} values of various anti-PAI-1 MoAbs to prevent PAI-1/vitronectin binding

MoAb	Epitope (area on PAL-1)	Reduction binding (% ± S.E.M.)	EC_{50} (nM ± S.E.M.)
CLB-10	110–145	66 ± 6.0	92.6 ± 14.2
CLB-2C8	110–145	55 ± 2.4	5.5 ± 0.8
CLB-5	110–145	53 ± 6.4	10.4 ± 1.9
I1	110–145	54 ± 6.7	17.4 ± 8.2
M3	235–283	≈0	>667
CLB16	235–283	≈0	>667
CLB-8H2	235–283	≈0	>667
MAI-12	320–379	≈0	>2600

3.4. *Quantitative analysis on the prevention of vitronectin-mediated inhibition of t-PA and thrombin by anti-PAI-1 CLB-10*

We compared the amount of anti-PAI-1 MoAb CLB-10 required to prevent PAI-1/vitronectin binding in either one of the assays employed. For that purpose, different concentrations of CLB-10 were applied in both vitronectin-dependent assays, measuring either t-PA-inhibition by PAI-1 R346M or thrombin-inhibition by wild-type PAI-1. The anti-PAI-1 MoAb M3 of the same subclass as CLB-10 (IgG_1) [24] was used as a control (Fig. 3). In accord with our previous results, no effect was detected with M3 in either one of the available assays. A

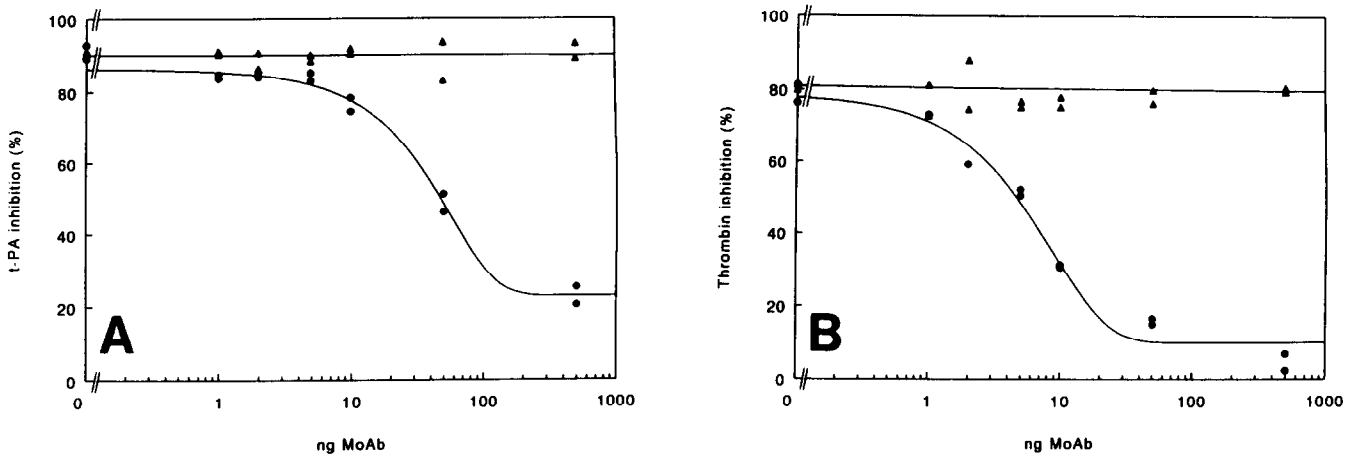

Fig. 3. Quantitative analysis on the prevention of vitronectin-mediated inhibition of t-PA and thrombin by anti-PAI-1 CLB-10. The prevention of vitronectin-dependent t-PA inhibition by PAI R346M (panel A) or thrombin inhibition by PAI-1 using increasing amounts of the MoAb CLB-10 is presented. MoAb M3 serves as a control. Symbols: ●=MoAb CLB-10; ▲=MoAb M3.

dose-dependent prevention of the inhibition by CLB-10 was observed both for the interaction between t-PA, PAI-1 R346M and vitronectin and between thrombin, wild-type PAI-1 and vitronectin. When heparin was used as a cofactor for the inhibition of thrombin by PAI-1, no influence of both of the MoAbs was observed (data not shown). Half-maximal inhibition in the t-PA-based assay is obtained with approximately 39.8 ± 5 ng of MoAb CLB-10 (panel A), whereas about 6.2 ± 1.3 ng of the antibody is required for half-maximal inhibition of the thrombin-based assay (panel B). The requirement of MoAb CLB-10 to achieve half-maximal inhibition in each of the vitronectin-dependent reactions matches with the established k_i values, i.e. 8.1×10^5 and 1.6×10^5 $M^{-1} \cdot s^{-1}$ for t-PA inhibition by PAI-1 R346M [8] and thrombin inhibition by PAI-1 [14], respectively. We conclude that the inhibition of both serine proteases heavily relies on the availability of the 110–145 area on PAI-1 for vitronectin binding.

3.5. Concluding remarks

Our conclusion that the region between amino acids 110 and 145 on PAI-1 harbors an essential determinant for vitronectin binding and, consequently, for vitronectin-dependent inhibition of thrombin and t-PA inhibition by PAI-1 (R346M), is supported by preliminary data reported by Sane and Padmanabhan [28]. These authors showed by ligand blotting that vitronectin binds to two overlapping, proteolytic fragments of PAI-1, sharing the region between amino acids 91 and 128. In addition, by constructing chimeric proteins of PAI-1 and the serpin plasminogen activator inhibitor 2, it was indicated that the region between amino acids 115 and 166 binds to vitronectin. Furthermore, during the preparation of this paper, Lawrence and collaborators [29] showed by using random-directed mutagenesis that the amino acid residues 55, 109, 110, 116 and 123 of PAI-1 are involved in binding to (immobilized) vitronectin. Although our data and those of others [28,29] are indicative of a single vitronectin-binding site on PAI-1, we do not exclude the presence of other sites. First, although MoAb CLB-10 fully prevents thrombin- and t-PA inhibition, as outlined here, it should be emphasized that the antibody only partially inhibits the direct binding of vitronectin to PAI-1. Second, a Scatchard analysis of the binding of vitronectin to PAI-1 suggested the presence of two binding sites [30]. A future localization of the epitopes of other MoAbs that prevent the binding of vitronectin to PAI-1 may resolve this enigma [31].

In previous studies, we have shown that the region between amino acid residues 110 and 145 of PAI-1 is involved in binding to several other components, namely to t-PA, urokinase and fibrin [24]. Examination of the three-dimensional structure of PAI-1 reveals that the region spanning amino acid residues 110 and 145 is composed of the subdomains helix E, sheet A1 and helix F [7,32]. It can be speculated that each of these juxtaposed subdomains might be responsible for the various interactions, although further experiments are required to substantiate this option.

Acknowledgements: We thank Ing. H. Stringer for ^{125}I-labeled PAI-1 and Dr. M. Otter for helpful discussions. A. Smilde and B. Richter are acknowledged for technical assistance. This study was supported by tTe Netherlands Organization for Scientific Research (Grant 900-526-132), The Netherlands Thrombosis Foundation (Grant 92.003) and the Deutsche Forschungsgemeinschaft (Grant Pr 327/1-2).

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