Vitronectin Governs the Interaction between Plasminogen Activator Inhibitor 1 and Tissue-type Plasminogen Activator*

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The "serpin" plasminogen activator inhibitor 1 (PAI-1) is the fast acting inhibitor of plasminogen activators (tissue-type (t-PA) and urokinase type-PA) and is an essential regulatory protein of the fibrinolytic system. Its P1-P1' reactive center (R346 M347) acts as a "bait" for tight binding to t-PA/urokinase-type PA. In vivo, PAI-1 is encountered in complex with vitronectin, an interaction known to stabilize its activity but not to affect the second-order association rate constant (kₐ) between PAI-1 and t-PA. Nevertheless, by using PAI-1 reactive site variants (R346M, M347S, and R346M M347S), we show that the binding of vitronectin to the PAI-1 mutant proteins improves plasminogen activator inhibition. In the absence of vitronectin the PAI-1 R346M mutants are virtually inactive toward t-PA (kₐ <1 x 10⁵ M⁻¹ s⁻¹). In contrast, in the presence of vitronectin the rate of association increases about 1,000-fold (kₐ of 6-8 x 10⁷ M⁻¹ s⁻¹). This inhibition coincides with the formation of serpin-typical, sodium dodecyl sulfide-stable t-PA-PAI-1 R346M (R346M M347S) complexes. As evidenced by amino acid sequence analysis, the newly created M346-M347 peptide bond is susceptible to attack by t-PA, similar to the wild-type R346M-M347 peptide bond, indicating that in the presence of vitronectin M346 functions as an efficient P1 residue. In addition, we show that the inhibition of t-PA and urokinase-type PA by PAI-1 mutant proteins is accelerated by the presence of the non-protease A chains of the plasminogen activators.

Plasminogen activator inhibitor 1 (PAI-1) is a major regulatory protein of the fibrinolytic system (for reviews see Refs. 1 and 2). It is the fast acting inhibitor of both tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (urokinase-type PA). Molecular cloning and DNA sequencing of full-length PAI-1 cDNA have revealed that the mature protein consists of 379 amino acid residues and that the protein belongs to the family of serine protease inhibitors ("serpins") (3-6). The general principle of the mode of action of the serpins is largely understood (7-9). The key reaction constitutes the formation of a tight bond between the serine residue of the catalytic triad of the protease and the P1 residue of the inhibitors' reactive center, located close to its carboxyl terminus. This reaction generates an equimolar, SDS-stable, inactive complex. Like the other inhibitory serpins, PAI-1 actually functions as a pseudosubstrate since the P1-P1' peptide bond, being R346-M347 (5), acts as a "bait" and mimics the R560-V561 peptide bond of plasminogen (10), the substrate for the plasminogen activators t-PA and urokinase-type PA. Although the reactive center P1-P1' residues of a serpin clearly are an important determinant for its target specificity, obviously other distinct amino acids are required for the highly selective interaction between a target serine protease and its serpin. This is best illustrated by comparing the distinct target specificity of PAI-1 with α2-antiplasmin (11, 12), both harboring identical P1 (R) and P1' (M) residues (5, 13). As yet, the identity of other specificity-determining amino acid residues has not been elucidated. In contrast, data are available on the areas on t-PA, apart from the serine residue (S478) of the catalytic triad, which contribute to the interaction with PAI-1. Notably, it has been shown that three basic amino acids (K296, R298, and R299) in the carboxyl-terminal B chain of t-PA are of crucial importance for the inhibition by PAI-1 (14). Furthermore, competition has been described between intact t-PA and an isolated domain (i.e. "kringle 2" domain) of the amino-terminal A chain of t-PA for the interaction with PAI-1, a finding that is indicative for a role of the kringle 2 domain in the inhibition reaction (15).

Recently it has become evident that a detailed study of the interaction between PAI-1 and t-PA (or urokinase-type PA) requires the participation of the multifunctional protein vitronectin (16). Vitronectin has been identified as the PAI-1 binding protein both in plasma (17, 18) and in the subendothelial matrix (19). Moreover, both PAI-1 and vitronectin are found in the α-granules of platelets, and complexes of these components are released upon platelet activation (20). These findings may indicate that the complex between PAI-1 and vitronectin represents the physiologically relevant form of the inhibitor. Indeed, in the presence of vitronectin the activity of PAI-1 is substantially preserved (18). Moreover, we have recently presented additional evidence for the functional interaction between vitronectin and PAI-1 (21). Specifically, vitronectin endows PAI-1 with thrombin-inhibitory properties, enhancing in a dose-dependent manner the formation of SDS-stable complexes between the inhibitor and thrombin.

To study the mechanism of action of the three participants, PAI-1, vitronectin, and plasminogen activators, in more detail...
we have chosen the following approach. First, we have elimi-
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corresponding fragment of the expression plasmids pMBL11/Ecp-PAI-1 to yield the plasmids pEc-PAI-R346M, pEc-PAI-M347S, and pEc-PAI-R346M M347S.

Expression and Purification of PAI-1 and PAI-1 Mutants—Transformed cells were grown in 2 × YT (35), containing ampicillin (150 mg/l) and tetracycline (25 mg/ml) for 30 h (0.8 to 1.0 mg protein/ml) and were then harvested by centrifugation at 405 nm in a Titertek Twinreader (Flow Laboratories). Under these conditions, conversion of the substrate by t-PA as well as by urokinase-inhibitor complexes was analyzed by SDS-PAGE followed by autoradiography (37). A more detailed analysis of the activity of PAI-1 was done as follows. PAI-1 was activated by dialysis for 1.5 h at room temperature against 6 M guanidinium chloride (pH 7.0), 100 mM NaCl, 10 mM EDTA, and 0.1% (w/v) Tween 80. After sonication, three times for 45 s, debris was pelleted by centrifugation for 20 min at 40 000 × g and discarded. The supernatant was dialyzed against 20 mM Tris-HCl (pH 7.0), 100 mM NaCl, and finally resuspended in 25 μl of 20 mM Tris-HCl (pH 7.0), 100 mM NaCl, 10 mM EDTA, and 0.1% (w/v) Tween 80. After sonication, three times for 45 s, debris was pelleted by centrifugation for 20 min at 40 000 × g and discarded. The supernatant was dialyzed against 20 mM Tris-HCl (pH 7.0), 25 mM NaCl, and 0.1% (v/v) Tween 80 (68% binding buffer) and subsequently added to the monoclonal murine antibody CAg69 coupled to Sepharose beads and incubated overnight at 4 °C by end-over-end rotation. After washing the Sepharose three times with 69-binding buffer and twice with 69-binding buffer supplemented with 100 mM NaCl, PAI-1 was eluted from the monoclonal CAg69-Sepharose beads by end-over-end incubation for 1 h in 69-binding buffer supplemented with 1 M NaCl. Subsequently, the CAg69 epitope and the collagenase cleavage sequence could be removed by adding CaCl2 to a final concentration of 3 mM to the eluate and incubating for 1 h at 37 °C with collagenase-Sephase. Finally, the PAI-1-containing solution was dialyzed against 20 mM Tris-HCl (pH 7.2), 200 mM NaCl, 0.1% (v/v) Tween 80. Then, Q-Sepharose Fast Flow beads, washed with the same buffer, were added. After a 30-min incubation, the supernatant was taken and dialyzed against 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.1% (v/v) Tween 80, resulting in an approximately 100-fold increase in PAI-1 preparation as determined by SDS-PAGE. Purified endothelial cell-derived PAI-1 (ECCM PAI-1) was obtained as described previously (36). ECCM PAI-1 and the nonmutated rPA-1 proteins from E. coli extracts exhibited identical second-order association rate constants for the reaction toward t-PA (data not shown).

Determination of PAI-1 Activity—Initially the activity of purified PAI-1 and PAI-1 mutants was determined by SDS-PAGE followed by reverse-fibrin autography (37). A more detailed analysis of the activity of PAI-1 was done as follows. PAI-1 was activated by dialysis for 1.5 h at room temperature against 6 M guanidinium chloride containing 0.1% (w/v) Tween 80. The denaturant was then removed by overnight dialysis at 4 °C against 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.1% (v/v) Tween 80 (TBST buffer). The activity of the resulting PAI-1 preparations was determined by titration against two-chain Bowes melanoma t-PA. To that end, 2 nM t-PA was incubated in the presence or absence of 12 mg/ml vitronectin at 37 °C in a final volume of 25 μl of TBST buffer with increasing concentrations of PAI-1 or PAI-1 mutants. After a 1-h incubation, the reaction was stopped by the addition of 150 μl of 100 mM Tris-HCl (pH 8.4), 0.1% (v/v) Tween 80, and 50 μl of 5 mM S2288. The residual t-PA activity was determined from the initial increase in absorbance at 405 nm, measured in a Cary 219 spectrophotometer. The second-order association rate constant (kₐ) was calculated using a standard equation for a second-order reaction under conditions of a slight excess of inhibitor over substrate by

\[
\frac{1}{k_i} = \frac{1}{k_i(E)} + \frac{1}{k_i(I)}
\]

where \(k_i\) is the order association rate constant, \(k_i(E)\) the order association rate constant of PAI-1, and \(k_i(I)\) the order association rate constant of inhibitor. For PAI-1, the dissociation constant between PAI-1 and t-PA was determined by calculating the initial velocity as a function of PAI-1 concentration at time zero. To prepare specific binding, wells were coated for at least 16 h at 4 °C with 2% (w/v) bovine serum albumin in 50 mM sodium carbonate (pH 9.4). After coating, the wells were washed twice with phosphate-buffered saline containing 0.1% (v/v) Tween 80 and 0.1% (v/v) bovine serum albumin (PTB buffer). To each well 200 μl of fibrinogen (0-1 mg/ml), completely devoid of plasminogen and vitronectin, was added in PTB buffer to 25 μl of human thrombin (10 units/ml). The fibrin matrix was then air dried at 37 °C. Prior to use, the matrix was incubated for 30 min at 37 °C with 300 μl of PTB buffer and subsequently washed three times with PTB buffer. Labeled PAI-1 was added to 200 μl of PTB buffer in a fibrin-coated well and incubated for 2 h at 37 °C. The supernatant was then quantitatively removed, and after the addition of 200 μl of 2% (w/v) SDS, the radioactivity was determined by liquid scintillation counting. The wells were dried twice with 1.5 ml of PTB buffer. The bound PAI-1 was eluted at room temperature by gently shaking the plates for 30 s. After trypsinization, the proteins were separated by SDS-PAGE, and the radioactivity was determined by liquid scintillation counting. The level of PAI-1 was determined by comparing the amount of radioactive PAI-1.

RESULTS

Declerck et al. (18) have reported that vitronectin stabilizes the active conformation of PAI-1. Our interpretation of this finding is that the binding of PAI-1 to vitronectin allows a more stable exposure of distinct amino acids of PAI-1 leading toward a tight interaction with t-PA or urokinase-type PA. We hypothesize that these amino acid residues are not necessarily those that constitute the reactive center of PAI-1. We have previously shown that the P1′′-P1′-P1-amino acid sequence in PAI-1 is necessary but not sufficient for complex formation. Consequently, we used various PAI-1 mutants to study the role of vitronectin in the interaction between PAI-1 and t-PA, the PAI-1 reactive site residues were replaced. The choice for those novel residues is based on the corresponding amino acids of 1-antitrypsin, a serine protease inhibitor (serpin) that does not inhibit t-PA or urokinase-type PA. The P1 arginine (R346) of wild-type PAI-1 was replaced by a methionine (M346) and/or the P′′ arginine (M347) by a serine (S347). The constructed PAI-1 mutants PAI-1 R346M, PAI-1 M346M, and PAI-1 M347S were expressed in E. coli, purified to homogeneity, and analyzed by reversed-fibrin autography (Fig. 1). In accord with the consensus on the dominating role of the P1 residue for target protease specificity (7-9), we observe

methanol) onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). After an incubation of 2 min at room temperature in 1 M NH₄OH to dissociate the complex, the filter was quickly washed three times with distilled water and air dried. After staining the filter with Coomassie Brilliant Blue R-250, the band representing the complex between the mutant PAI-1 R346M M347S protein and t-PA was excised and directly sequenced by automated Edman degradation (Applied Biosystems model 477A) (38). The liberated phenylthiohydantoins were identified by means of on-line high performance liquid chromatography (Applied Biosystems model 120A).
that both PAI-1 mutants containing a methionine (R346M) in the P1 position are inactive. In contrast, PAI-1 M347S exhibits a specific activity comparable to that of wild-type PAI-1.

It is well documented that PAI-1 from conditioned medium of cultured vascular endothelial cells or from extracts of E. coli transformed with PAI-1 cDNA is encountered as a mixture of active and latent PAI-1 (4, 11, 40). The latent form can be activated by treatment with e.g. guanidinium chloride (41-43). In addition, we recently reported that the active form of PAI-1 specifically binds to fibrin whereas a much lower binding to fibrin was observed with the latent form (44). Thus, monitoring the capacity of PAI-1 and its variants to bind to fibrin can be taken as a parameter for the ability of the different PAI-1 preparations to be activated. Indeed, upon activation the binding of the inactive mutants PAI-1 R346M and PAI-1 R346M M347S to fibrin is similar to that of wild-type PAI-1 whereas there is hardly any binding of the latent forms (Fig. 2). From these data we conclude that the various PAI-1 derivatives differ in inhibitory activity although their abilities to be activated are identical.

To study the potential effect of vitronectin on the interaction between PAI-1 (variants) and t-PA or urokinase-type PA, we employed an end point assay with the direct chromogenic substrate S2288 and subsequently performed a more detailed analysis of the inhibition kinetics. First, the residual amidolytic activity of t-PA was determined in the absence of vitronectin after a 1-h incubation of t-PA with the different PAI-1 derivatives. In accordance with the analysis by reversed-fibrin autography (Fig. 1C), we observed efficient inhibition both with wild-type PAI-1 and with PAI-1 M347S whereas no measurable inhibition of t-PA activity was seen with either PAI-1 R346M or PAI-1 R346M M347S (Fig. 3). A further prolongation of the reaction time of t-PA with PAI-1 R346M or PAI-1 R346M M347S to 4 h did not result in significant t-PA inhibition (data not shown). Second, we performed the end point assay in the presence of a 4-fold molar excess of vitronectin over active PAI-1. Clearly, the
mutants PAI-1 R346M and PAI-1 R346M M347S, which are inactive in the absence of vitronectin, are converted into active inhibitors in the presence of vitronectin. The observed influence of vitronectin on the activity of these PAI-1 mutants is dose dependent, and a half-maximal effect is found at approximately equimolar concentrations of vitronectin and active PAI-1 (data not shown). We have reported before that heparin, like vitronectin, can also endow PAI-1 with thrombin-inhibitory properties (45). Furthermore, we presented evidence indicating that the mechanism of these cofactors in promoting the association between PAI-1 and thrombin is quite different (21, 45). Here, we show that heparin, in contrast to vitronectin, does not promote the activity of the “inactive” mutants PAI-1 R346M and PAI-1 R346M M347S (Fig. 3). This observation provides additional evidence for a different mechanism of interaction between PAI-1 and either heparin or vitronectin.

To evaluate the extent of the vitronectin-induced inhibition of t-PA by the various PAI-1 mutants, we determined the second-order association rate constants ($k_1$) in the presence and in the absence of vitronectin. As shown in Table 1, the $k_1$ values of wild-type PAI-1 and PAI-1 M347S are virtually identical ($k_1 = 3 \times 10^7 \text{M}^{-1} \text{s}^{-1}$) and are not affected by the presence of vitronectin. In the absence of vitronectin, both PAI-1 R346M and PAI-1 R346M M347S are virtually inactive toward t-PA, as evidenced by $k_1$ values below $1 \times 10^6 \text{M}^{-1} \text{s}^{-1}$. In the presence of vitronectin, the association rate of PAI-1 R346M and PAI-1 R346M M347S increases almost 1,000-fold, reaching $k_1$ values of $8 \times 10^8$ and $6 \times 10^8 \text{M}^{-1} \text{s}^{-1}$, respectively. Consequently, the addition of vitronectin to the PAI-1 mutants, which are virtually inactive in the absence of vitronectin, results in a rate of t-PA inhibition which is only 40-50-fold slower than t-PA inhibition by wild-type PAI-1 either with or without vitronectin. The mechanism of vitronectin-promoted inhibition of t-PA by the “P1” mutants of PAI-1 was investigated further. For that purpose we monitored the formation of SDS-stable complexes, a characteristic feature of a serpin-target protease interaction, either in the presence or in the absence of vitronectin. Analysis was performed using SDS-PAGE (Fig. 4). Clearly, in the presence of vitronectin, t-PA inhibition by PAI-1 R346M coincides with the formation of equimolar, SDS-stable complexes of t-PA and PAI-1 R346M. Identical results were obtained with the mutant PAI-1 R346M M347S. In contrast, no complex formation is observed in the absence of vitronectin.

To establish whether the M346-M/S347 peptide bond of the PAI-1 variants is attacked by the catalytic center of t-PA, analogous to the R346-M347 peptide bond of wild-type PAI-1, we determined the amino-terminal amino acid sequence of the postcomplex peptide of one of the variants. For that purpose, a complex was formed between PAI-1 R346M M347S and t-PA in the presence of vitronectin. The complex was isolated, subsequently dissociated on a filter by treatment with NH$_2$OH, and the filter was directly used for automated amino acid sequence analysis. Each cycle of the Edman degradation resulted in the identification of four amino acid derivatives in equimolar amounts. These residues represent the known amino termini of PAI-1 (5), of the A chain of t-PA, and of the B chain of t-PA (46) and an additional fourth sequence. The latter amino acid sequence is recorded as: S41-A2-P3-E4-E5-I6-X7-M8. This sequence is present in PAI-1 R346M M347S (S474-A348-P349-E350-E351-I352-I353-M354) and precisely coincides with that of the postcomplex peptide of wild-type PAI-1 (5). Omission of the NH$_2$OH treatment of the complexes resulted in a recovery of only 10% of the amount of the other three amino-terminal sequences that are present in equimolar amounts (data not shown). In agreement with observations on complex formation between wild-type PAI-1 and t-PA (47), the dissociation of the complex and concomitant release of the postcomplex peptide were effectively promoted by treatment of the complex with NH$_2$OH and resulted in equimolar amounts of all four amino-terminal sequences. It should be noted that the amino-terminal sequence of the postcomplex peptide with or without treatment of the complex with NH$_2$OH is identical.

### Table 1

| Second-order association rate constants ($k_1$) for the inhibition of t-PA by wild-type PAI-1 (wt) and PAI-1 active site variants in the absence and the presence of vitronectin |
|-----------------|-----------------|-----------------|
|                  | t-PA            | t-PA + vitronectin |
| P1-P1'           |                 |                 |
| RM (wt)          | $2.6 \pm 0.5 \times 10^7$ | $3.1 \pm 0.6 \times 10^7$ |
| MM               | $<1 \times 10^7$ | $8.1 \pm 0.3 \times 10^7$ |
| MS               | $<1 \times 10^7$ | $5.5 \pm 0.4 \times 10^6$ |
| RS               | $2.6 \pm 0.6 \times 10^7$ | $3.0 \pm 0.5 \times 10^7$ |

A serine residue (S) was identified at position 1 (X1) but could not be assigned to this sequence since it is also the amino-terminal t-PA residue. In position 7 (X7) an isoleucine residue (I) was identified, but it appeared at half the level of the other residues.
We conclude that the novel M346-M/S347 peptide bond of the PAI-1 variants functions as the P1-P1' bond for t-PA, in a manner similar to the R346-M347 peptide bond of wild-type PAI-1.

The vitronectin-dependent inhibition with the inactive mutants PAI-1 R346M and PAI-1 R346M M347S was analyzed further by using another target protease of PAI-1, i.e. high molecular weight urokinase-type PA. Results similar to those for the inhibition of t-PA were obtained, namely that inhibition of urokinase-type PA by the PAI-1 mutants is only observed in the presence of vitronectin. Surprisingly, however, low molecular weight urokinase-type PA, containing essentially only the protease domain, is inhibited to a significantly lower extent than high molecular weight urokinase-type PA (data not shown). This unexpected finding may indicate an involvement of the non-protease part of urokinase-type PA, and possibly of t-PA, in the vitronectin-dependent inhibition.

To investigate this option in more detail, we compared the inhibition of either full-length recombinant t-PA and a recombinant t-PA mutant (t-PA del.FEK1K2) which contains only the protease domain (i.e. “B” or “light” chain) by the mutant proteins PAI-1 R346M and PAI-1 R346M M347S. As a control, it is shown that wild-type PAI-1 inhibits t-PA and t-PA del.FEK1K2 to the same extent both in the presence or in the absence of vitronectin (Fig. 5). Interestingly, both in the end point assay and in the determination of the second-order association rate constants (k1), the presence of vitronectin causes a significant difference between the k1 of the PAI-1 mutants with either t-PA or t-PA del.FEK1K2. For the inhibition by PAI-1 R346M, the full-length protein displays a k1 value of 8 \times 10^7 M^{-1} s^{-1} whereas t-PA del.FEK1K2, lacking the amino-terminal A (“heavy”) chain, displays a 6-fold lower k1 (1.3 \times 10^6 M^{-1} s^{-1}). For the double mutant PAI-1 R346M M347S, these values are 6 \times 10^5 M^{-1} s^{-1} and 9.8 \times 10^6 M^{-1} s^{-1}, respectively. These observations indicate that the non-protease part of t-PA (and of urokinase-type PA) contributes to the interactions between t-PA, PAI-1, and vitronectin.

**FIG. 5.** Inhibition of full-length t-PA and of a t-PA variant (t-PA del.FEK1K2), lacking the A chain, by PAI-1 variants in the absence and presence of vitronectin (VN). The residual t-PA activity after a 1 h inhibition is shown for both mutant PAI-1 R346M and PAI-1 R346M M347S. In addition, the second-order association rate constants (k1, expressed in M^{-1} s^{-1}) for the reactions in the presence of vitronectin are given. Experiments are performed as outlined under “Experimental Procedures.” The 100% value is the activity of the t-PA in the absence of inhibitor. A+B, full-length t-PA; A+ t-PA del.FEK1K2.

**DISCUSSION**

The nature of the P1’ amino acid residue undoubtedly is of crucial importance for the specificity of a particular serine protease inhibitor (8). This is best illustrated by the pathological manifestations of a patient who presented a bleeding diathesis caused by the substitution for the P1 residue (M) of \(\alpha_1\)-antitrypsin (\(\alpha_1\)-AT) by an arginine (R) residue (\(\alpha_1\)-AT “Pittsburgh”), being the P1 residue of e.g. antithrombin III (48). As a result, the patient had acquired increased antithrombin activity and a simultaneous lack of elastase-inhibitory activity. Our data on the properties of PAI-1 mutants having an altered P1 residue (53). In agreement with the view that this residue is a prime determinant for the target specificity of a serpin. Replacement of the P1 arginine residue (R346) of PAI-1 by a methionine virtually inactivates the inhibitor. However, as will be discussed in a following paragraph, apart from the P1 residue, clearly other amino acid residues also perform an essential function in the specific interaction with the target serine proteases t-PA and urokinase-type PA.

The importance of the nature of the P1’ residue of a serpin for the target specificity is less obvious than that of the P1 residue. In some cases the alteration of the P1’ residue, notably the replacement of the P1’ serine (S) residue of \(\alpha_1\)-antitrypsin by alanine (A) (49) and the conversion of the antithrombin III P1’ serine (S) residue to leucine (L) (50), results in mutant inhibitors with decreased efficiency toward their respective target proteases elastase and thrombin. In general, however, the nature of the P1’ residue seems to be less critical than that of the P1 residue although limitations have been proposed with regard to the size and the hydrophobicity of the side chains (51). This view is supported further by the properties of the P1’ mutant protein PAI-1 M347S described here. Clearly, neither the specificity nor the rate of association with t-PA is affected by this alteration. Similarly, another mutant, PAI-1 M347V, displays a k1 similar to that of wild-type PAI-1.1 From these observations we conclude that the nature of the P1’ residue of PAI-1 is relatively insignificant for the efficacy and specificity of this inhibitor.

The difference in the rate of association between full-length t-PA and t-PA lacking the A chain (and high and low molecular weight urokinase-type PA) toward the PAI-1 variants R346M and R346M M347S suggests a role for the respective non-protease chain in the inhibition reaction. Recently, similar observations have been made on the involvement of in particular kringle 2 on the A chain of t-PA in the interaction with PAI-1 (15, 52). First, it was demonstrated that PAI-1 binds with low affinity to isolated kringle 2 and that the isolated kringle 2 partly abolishes the inhibition of intact t-PA by PAI-1 (15). Second, using reaction conditions different from those employed here it was reported that the presence of kringle 2 within the t-PA molecule slightly decreases the rate of association between t-PA and PAI-1 (52). It should be noted, however, that in our hands in the absence of vitronectin the second-order association rate of PAI-1 with either t-PA or t-PA del.FEK1K2 is not significantly different. We assume that the apparent discrepancy with the aforementioned report is a result of the quite distinct conditions during the inhibition reaction. At face value our data and those of the others mentioned above coincide. In the notion that the non-protease A chain of t-PA and urokinase-type PA is implicated in the interaction with PAI-1. At present, the available data are insufficient to describe the interaction of the non-protease part of t-PA with PAI-1 in more detail.

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1. H. J. Ehrlich, J. Keijer, H Pannekoek, unpublished data.
In this paper we report that the presence of vitronectin results in the conversion of virtually inactive P1 mutants of PAI-1 (PAI-1 R346M and PAI-1 R346M M347S) into potent inhibitors of plasminogen activators. Furthermore, we demonstrate that in the presence of vitronectin the peptide bond M346-M/S347 of the PAI-1 mutant proteins is attacked by t-PA. In the absence of vitronectin, no complex formation is detected, and attack of the M346-M/S347 peptide bond does not occur. Hence, we conclude that in the presence of vitronectin, residues M346 and M/S347 perform the function of P1 and P1' residues, similar to the genuine P1 (R346) and P1' (M347) of wild-type PAI-1 both in the presence and absence of vitronectin. In view of the concept of a serpin acting as a pseudosubstrate (7,8), our finding that the novel M346 residue of the mutant PAI-1 proteins effectively functions as the P1 reactive center residue is surprising. According to this concept and supported by the altered phenotype of a-antitrypsin “Pittsburgh” mentioned before (48), the P1 residue should be identical to the P1 residue of the substrate, being arginine (R560) of plasminogen (10) in the t-PA/PAI-1 system. However, although vitronectin increases the association rate between PAI-1 R346M (R346M M347S) and t-PA about 1,000-fold, the interaction between wild-type PAI-1 and t-PA is still 40–50-fold faster, illustrating the important contribution of the arginine at the P1 position of PAI-1.

To explain the role of vitronectin in the inhibition of plasminogen activators by PAI-1, we attempt to fit our observations in the best current model for inhibition of serine proteases (E) by their cognate serpin (I) (7). This model distinguishes the generation of a reversible complex (EI) followed by conversion to an SDS-stable tight complex (EI') and, finally, dissociation of the protease (E) and the cleaved inhibitor (I'). The inhibition of t-PA by PAI-1 is characterized by the following pertinent observations. (a) In vivo, PAI-1 is encountered as a complex bound to its binding protein vitronectin (17–20); (b) the addition of t-PA to vitronectin-PAI-1 complexes results in the formation of t-PA/PAI-1 complexes devoid of vitronectin (18); (c) no cleavage of PAI-1 R346M or PAI-1 R346M M347S by t-PA is observed in the absence or presence of vitronectin, as is the case for wild-type PAI-1; and (d) there is no evidence for a direct interaction between t-PA and vitronectin; neither by studies using immobilized vitronectin nor by immunoprecipitation techniques could association between t-PA and vitronectin be detected (data not shown). In the absence of vitronectin, the EI complex between t-PA and the PAI-1 R348 M mutants is not formed, as illustrated by the lack of SDS-stable complexes. Apparently, the altered P1 residue of PAI-1 is not positioned appropriately, and proteolytic attack on the P1-P1' peptide bond does not occur. However, the formation of the reversible (EI) complex cannot be excluded, and arguments can be advanced which support this possibility. The formation of the EI complex may be mediated, at least in part, by the positively charged amino acid residues (R296, R298, R299) of t-PA which are distinct from the catalytic triad but are crucial for t-PA inhibition by PAI-1 (14). It is conceivable that the counterparts of these basic t-PA residues on PAI-1 do not comprise the reactive center that interacts with the catalytic triad of t-PA but are located elsewhere on the protein. From the observation that the mutant proteins bind equally well to fibrin as wild-type PAI-1 (Fig. 2) we infer that the overall structure of these proteins is comparable. Consequently, we deduce that amino acids of the PAI-1 mutants, supposed to interact with the basic region of t-PA to form the reversible EI complex, interact in a manner similar to those of wild-type PAI-1.

In the presence of vitronectin efficient generation of the SDS-stable EI' complex can be observed without a subsequent cleavage reaction. Consequently, with the use of PAI-1 reactive site mutants as a model system, we deduce the following alternatives for the functioning of vitronectin in the inhibition reaction. (a) Vitronectin may promote the formation of the reversible complex (EI) or decrease the decay of such complexes, and/or (b) vitronectin may increase the formation of the subsequent tight SDS-stable complexes (EI'). For both options we conclude that vitronectin exerts its function by interacting with PAI-1. We are currently investigating the different alternatives for the action of vitronectin on the inhibition reaction. It is anticipated that such studies may provide additional insight in the role of vitronectin in the inhibition of t-PA by wild-type PAI-1.

In general, the data reported here indicate that vitronectin substantially alleviates a suboptimal inhibitory condition for t-PA or urokinase-type PA. Although in this study such conditions are created by using PAI-1 P1 mutants, we envision that in vivo suboptimal inhibitory conditions may occur as well. Specifically, it has been reported that phosphorylated urokinase-type PA is partly refractory to inhibition by PAI-1 (50). Furthermore, it has been shown that t-PA complexes with α2-macroglobulin are unable to activate plasminogen, is inhibited by PAI-1 at an approximately 1,000-fold slower rate (30). Possibly, under those conditions, vitronectin may restore full inhibition of the respective plasminogen activators by PAI-1.

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