Interaction between Plasminogen Activator Inhibitor Type 1 (PAI-1) Bound to Fibrin and Either Tissue-type Plasminogen Activator (t-PA) or Urokinase-type Plasminogen Activator (u-PA)

Binding of t-PA/PAI-1 Complexes to Fibrin Mediated by Both the Finger and the Kringle-2 Domain of t-PA

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Abstract

Plasminogen activation is catalyzed both by tissue-type- (t-PA) and by urokinase-type plasminogen activator (u-PA). This reaction is controlled by plasminogen activator inhibitor type 1 (PAI-1) that is either present in plasma or bound to fibrin, present in a thrombus. We studied the mechanism of in vitro inhibition of both t-PA and u-PA activity by PAI-1 bound to fibrin. It is shown that activation of latent PAI-1 unmasks a specific fibrin-binding site that is distinct from its reactive site. This reactive site of activated PAI-1 bound to fibrin is fully exposed to form complexes with t-PA and u-PA, that are unable to activate plasminogen. Upon complex formation with either one of the plasminogen activators, PAI-1 apparently undergoes a conformational change and loses its affinity for fibrin. Consequently, complexes of u-PA and PAI-1 dissociate from the fibrin matrix and are encountered in the fluid phase. In contrast, t-PA/PAI-1 complexes remain bound to fibrin. By employing recombinant t-PA deletion-mutant proteins, that precisely lack domains involved in fibrin binding, we demonstrate that binding of t-PA/PAI-1 complexes is mediated by both the "finger" (F) and the "kringle-2" (K2) domain of t-PA. A model is proposed that explains inhibition of the fibrinolytic process, at the level of plasminogen activation by t-PA, directed by PAI-1 bound to fibrin. An implication of the proposed model is that t-PA/PAI-1 complexes and free t-PA compete for the same binding sites on fibrin.

Introduction

Limited proteolysis of the zymogen plasminogen and of fibrin by the serine proteases tissue-type plasminogen activator (t-PA)[1] and plasmin, respectively, forms the basis of the fibrinolytic process (1). The solid-phase component fibrin plays a pivotal role in this process. First, it provides for a selective binding surface both for t-PA and its substrate plasminogen. The assembly of these three components greatly facilitates the conversion of plasminogen into plasmin (2, 3). Second, fibrin interacts with serine protease inhibitors (serpins) that restrain the action of either plasmin or t-PA. For instance, it has been shown that α2-antiplasmin (α2AP), a specific inhibitor of plasmin, is bound to fibrin and subsequently crosslinked (4). Furthermore, it has recently been reported that plasminogen activator inhibitor type 1 (PAI-1), a specific inhibitor of both t-PA and u-PA, binds to fibrin, but is apparently not crosslinked (5). In this paper, we have focussed on the interplay between fibrin, t-PA (or u-PA) and PAI-1, in the absence of plasminogen. A number of structural and biological features of t-PA and PAI-1 have been reported that are pertinent to this study.

The serine protease t-PA, the predominant plasminogen activator in blood, is synthesized and secreted by endothelial cells and circulates in a single-chain form (6–8). This form is cleaved by plasmin into the double-chain form, consisting of an amino-terminal "heavy" (H) chain and a carboxy-terminal "light" (L) chain held together by a disulfide bond (9, 10). The H chain is composed of distinct structural and functional domains that share homology with other plasma proteins (11, 12). Starting from the amino terminus, a "finger" domain (F) can be distinguished, followed by an "epidermal growth factor" domain (E), and two "kringle" structures (K1 and K2). Studies on t-PA deletion mutants revealed the presence of fibrin-binding sites on the F as well as on the K2 domain, the latter representing a lysine-binding site (13, 14). Recently, it has been indicated that another lysine-binding site, maybe involved in fibrin binding, is located on K1 (15). The L chain contains the trypsin-like serine protease moiety, that interacts with the substrate plasminogen (16). It also constitutes the major "target" for the specific plasminogen activator inhibitor PAI-1 (17).

PAI-1 belongs to the serpin family, a large group of proteins, sharing structural features and a common functional principle (18, 19). Serpins act as ideal pseudosubstrates for their target serine proteases. The serine residue of the active center of the protease reacts with the P1 residue of the reactive site of the serpin. This interaction ultimately results in a scission of the P1-P1′ peptide bond and in a tight linkage between the protease and the P1 residue. As a consequence, a 1:1 molar complex of the serpin and the target protease is formed (18). In general, serpins can interact with several serine proteases. The physiological role of a serpin and its specificity are, however, determined by the colocalization of a particular serpin and its target protease, their (local) concentration and the affinity of the serpin for its target protease (18). Taking these determi-

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1. *Abbreviations used in this paper:* α_2AP, α_2-antiplasmin; del.FEK2, deletion mutant of t-PA, lacking F, E and K2; del.F, deletion mutant of t-PA, lacking F; del.K2, deletion mutant of t-PA, lacking K2; E, epidermal growth factor-like domain; EACA, ϵ-amino caproic acid; F, finger domain; HAT, hypoxanthine, aminopterin, thymidine; H, heavy (chain of t-PA); K1, kringle-1 domain; K2, kringle-2 domain; L, light (chain of t-PA); PAI-1, plasminogen activator inhibitor type 1; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.

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nants together, two serpins appear to be the primary inhibitors of fibrinolysis, α_2AP and PAI-1.

Recently, full-length cDNAs of α_2AP and PAI-1 have been constructed and the nucleotide sequences have been established (20–25). The predicted amino-acid sequences clearly pointed out that these proteins are both members of the serpin family. α_2AP is synthesized by hepatocytes, whereas the site of synthesis of PAI-1 is not clear (26). At least part of PAI-1 in blood is contained within platelets (27–29). PAI-1 is also synthesized and secreted by cultured endothelial cells and hepatocytes (30, 31). Remarkably, in such conditioned media PAI-1 is encountered predominantly as an inactive (latent) component. Upon treatment with denaturants [e.g., SDS, guanidine hydrochloride (32)] or with more physiological agents [negatively charged phospholipids (33)], PAI-1 is activated and, consequently, will inhibit its target proteases t-PA and u-PA. Undoubtedly, PAI-1 and α_2AP share many important structural and functional features. Nevertheless, in the presence of fibrin their mechanism of action may, at least in part, be rather distinct. Upon formation of a fibrin clot, α_2AP can be cross-linked to fibrin by Factor XIII and in this way protects the clot from "premature" lysis by plasmin (4, 34). Concerning the interaction of PAI-1, t-PA (u-PA) and fibrin several observations are relevant in this respect. As mentioned before, it has been shown that PAI-1 is bound to fibrin, but is not cross-linked (5). Furthermore, it has been demonstrated that platelets (in this context present in a clot) activated by thrombin release substantial amounts of PAI-1 (27). It has been proposed that this event may yield local, high concentrations of PAI-1, inhibiting premature lysis of the clot. In this case, inhibition of lysis would then conceivably occur at the level of plasminogen activation. In this paper, we report on the interaction between fibrin, t-PA (u-PA) and PAI-1 and present a model that differs from equivalent interactions, involving α_2AP.

Methods

Materials. Iscove's modified Dulbecco's medium and Ultroser G were from Gibco (Paisley, U.K.). Hypoxanthine, aminopterin and thymidine were purchased from Sigma Chemical Co. (St. Louis, MO). Human plasminogen-free fibrinogen was from IMCO (Stockholm, Sweden) and bovine thrombin was from Hoffmann-La Roche (Basel, Switzerland). [^{35}S]Methionine was purchased from The Radiochemical Centre (Amersham, U.K.). The murine monoclonal antibody anti-t-PA L-chain IgG ESP2 was purchased from Bioscot (Edinburgh, UK). The murine monoclonal antibody anti-t-PA L-chain IgM MPWIv-PA and (high-molecular-weight) u-PA were gifts from Dr. B. R. Binder (University of Vienna, Vienna, Austria) and Dr. G. Cassani (Lepetit, Milano, Italy), respectively. The chromogenic substrate H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide-dihydrochloride (S2288) to determine the amidolytic activity of t-PA was from Kabi-Vitrum (Stockholm, Sweden). Plasmid pAGO, containing the Herpes simplex virus thymidine kinase gene has been described before (35). Restriction endonucleases and DNA modifying enzymes were either from New England Biolabs (Beverly, MA) or from Bethesda Research Laboratories (Rockville, MD). Synthetic oligonucleotides were made on a DNA synthesizer (type 381A; Applied Biosystems, Foster City, CA).

Construction of rt-PA deletion mutants. The plasmid pSV2/t-PA, containing full-length human t-PA cDNA (13), was used to construct the t-PA deletion-mutant cDNAs. To construct the mutant cDNAs a 1417 base pair (bp) fragment of the pSV2/t-PA full-length cDNA, extending from the Hind III site (position 1) to the Sac I site (position 1417) was inserted into the Hind III/Sac I digested polylinker of the double-stranded replicative form of M13mp18am4 bacteriophage DNA (36). Subsequently, the "M13 gapped-duplex outlooping" mutagenesis procedure (37) was employed using both recombinant M13mp18am4 (single-stranded) and M13mp18 (double-stranded, linearized) and different synthetic oligonucleotides (36-mers) to delete the desired parts of the t-PA cDNA (Table I). It should be noted that none of the constructions would ultimately encode a protein with novel free cysteine residues. DNA sequence determinations were performed to verify correct fusion sequences (38). Finally, different mutated fragments were exchanged in the pSV2/t-PA full-length cDNA to obtain the set of t-PA deletion cDNAs under the control of the SV40 early promotor.

Expression of rt-PA deletion mutants. Mouse Ltk$^-$ cells were maintained in Iscove's modified medium, containing penicillin, streptomycin and 10% (vol/vol) FCS. Semiconfluent monolayers of Ltk$^-$ cells were exposed for 4 h to a calcium-phosphate co-precipitate (39) of both pSV2/t-PA (or variants thereof) and pAGO plasmid DNAs (20 and 1 μg, per 80 cm^2 flask, respectively). The cells were "shocked" for 2 min with 15% (vol/vol) glycerol. After recovery of the cells for 24–48 h, in medium containing serum, the cells were trypsinized and replated in selective medium containing hypoxanthine (12 μg/ml), aminopterin (1 μg/ml), and thymidine (8 μg/ml) (HAT). After 7 to 14 d, colonies resistant to this selection medium were taken. Separate colonies were grown and incubated for 2–3 d in medium without serum, before samples were collected to test them for rt-PA (variant) production. The clones secreting the highest amount of rt-PA activity were chosen and grown in Iscove's, penicillin, streptomycin, HAT, supplemented with 2% (vol/vol) Ultroser G. Confluent cells were washed with PBS and incubated with "harvest" medium (Iscove's, penicillin, streptomycin, HAT, Trasylol [60 KIE/ml]). Conditioned media were collected after 3

Table I. Details on the Construction and Composition of the t-PA Deletion Mutants

Mutant	Primer mutagenesis	Fusion	Point mutation	Mutated fragment exchanged
del.F	GAGCCAGATCTTACCAAG*GTTGCAGCGAGCCAAGGT	bp199–bp338	Val4-Gly4	HindIII(61)-NarI(517): 319bp
del.K2	CCCCTGCCTGCTCTGAGG*CCACCTGCGGCCTGAGAC	bp715–bp974	Gly176-Ala176	NarI(517)-SacI(1417): 643bp
del.FEK2	GAGCCAGATCTTACCAAG*ATACCAGGGCCACGTGCT	bp199–bp449	Val4-Asp4	HindIII(61)-NarI(517): 208bp
	CCCCTGCCTGCTCTGAGG*CCACCTGCGGCCTGAGAC	bp715–bp974	Gly176-Ala176	NarI(517)-SacI(1417): 643bp

	Plasmid	Protein
del.F	pSV2/rt-PA gly4 del (ile5-ser50)	rt-PA Gly4 del (Ile5-Ser50)
del.K2	pSV2/rt-PA ala176 del (asn177-ser262)	rt-PA Ala176 del (Asn177-Ser262)
del.FEK2	pSV2/rt-PA asp4, ala176	rt-PA Asp4, Ala176
	del (ile5-asp87)	del (Ile5-Asp87)
	del (asn177-ser262)	del (Asn177-Ser262)

d, centrifuged to remove cell debris, and stored at −20°C until further use.

Endothelial cell culture and labeling. Endothelial cells were isolated from human umbilical cord veins according to the procedure described by Jaffe et al. (40) and cultured as described (40, 41). Conditioned medium was prepared by incubating the cells for 24 h with serum-free medium supplemented with 0.03% (wt/vol) human serum albumin. Metabolic labeling with [^{35}S]methionine was performed essentially as described before (42).

Purification of PAI-1. PAI-1 and [^{35}S]methionine metabolically labeled PAI-1 ([^{35}S]PAI-1) were purified from the respective conditioned media using a one-step immunoaffinity-purification procedure. The media were chromatographed at 4°C on the murine monoclonal anti-PAI-1 IgG (1C3), immobilized on Sepharose beads. The Sepharose beads were washed with PBS, containing 1 M NaCl, and PAI-1 was eluted using 0.1 M glycine (pH 3), supplemented with 0.01% Tween-80. The eluted fractions were collected in 1 M Tris-HCl buffer, pH 7.5 (1/10 of the eluted volume). The homogeneity of the PAI-1 preparation was assessed by SDS-polyacrylamide gelelectrophoresis (SDS-PAGE) (43), followed by Coomassie brilliant-blue staining. A single band was visualized of ~ 52,000 M_r. The identity of the purified protein was confirmed by SDS-PAGE, followed by reverse-fibrin autography (44). Reverse-fibrin autography revealed an opaque, lysis-resistant zone in an otherwise clear fibrin film, that comigrated with the single Coomassie brilliant-blue-stained protein band. Autoradiography of purified [^{35}S]PAI-1 (20,000 cpm) revealed a band that comigrated with nonlabeled PAI-1. After this purification procedure, PAI-1 and [^{35}S]PAI-1 are encountered essentially as a latent preparation, containing < 0.1% of active PAI-1. The half-maximal inhibition of the amidolytic activity of t-PA by PAI-1 preparations was determined, using the chromogenic substrate S2288. Activation was achieved by dialysis for 1 h at room temperature against 6 M guanidine hydrochloride, followed by dialysis against PBS, containing 0.1% (vol/vol) Tween-80. Activated PAI-1 preparations contained ~ 20% active PAI-1. The protein concentration of purified PAI-1 was determined by amino-acid compositional analysis as described previously (45).

Preparation of fibrin matrices. Fibrin matrices were prepared in 24-well tissue-culture plates, essentially as described previously (46). 200 μl assay buffer (PBS, 0.01% (vol/vol) Tween-80), containing 1 mg/ml BSA and 1 mg/ml plasminogen-free fibrinogen (except if otherwise stated) were added to each well, and mixed with 2.5-μl bovine thrombin (100 U/ml) in assay buffer. The plates were left at room temperature for 1 h, subsequently air-dried overnight at 37°C, and stored at 4°C until use. Control plates without fibrinogen were prepared as described above.

Binding of t-PA and u-PA, respectively, to fibrin matrices. 200 μl assay buffer, containing t-PA (0.15 pmol) or u-PA (0.1 pmol) were incubated for 1 h at 37°C with fibrin matrices. Subsequently, the supernatants were collected and the wells were washed five times with 2 ml assay buffer, containing 10 mg/ml BSA. The wells were then incubated for 1 h at room temperature with 200 μl assay buffer, containing 1 mg/ml BSA and 1% (wt/vol) SDS. As a consequence, complete lysis of the fibrin matrices occurs. For comparison, the supernatants (in the following referred to as "non-fibrin-binding" fractions) were brought to a final concentration of 1% (wt/vol) SDS as well and incubated for 1 h at room temperature before further use. The SDS-treated lysates of the fibrin matrices were diluted in an identical manner as the nonbinding fraction with assay buffer and denoted as "fibrin-binding" fractions. Aliquots of the nonfibrin binding and fibrin-binding fractions were analyzed by SDS-PAGE, followed by fibrin autography (47). Clear zones in the fibrin overlay indicate plasminogen activator activity and their position allows an estimation of the apparent molecular weights. Experiments in control wells were performed as described above.

Binding of PAI-1 to fibrin matrices. 200 μl of assay buffer, containing latent or activated [^{35}S]PAI-1 (10,000 cpm) were applied to the fibrin-binding assay as described above. Nonfibrin binding fractions and fibrin-binding fractions were characterized by SDS-PAGE, followed by reverse fibrin autography. The zones of the SDS-PAGE gels corresponding to the lysis-resistant zones in the fibrin indicator films were quantitatively recovered and counted. Subsequently, the relative distribution of [^{35}S]PAI-1 in the nonfibrin-binding and fibrin-binding fractions was calculated. Additionally, fibrin matrices were prepared following the protocol described above, using fibrinogen at different concentrations (0.04–2.5 mg/ml). Latent and activated [^{35}S]PAI-1 (10,000 cpm each), were applied to the fibrin matrices, respectively, and the resulting fibrin-binding fractions were analyzed. Aliquots of the fibrin-binding fractions were counted. The relative amounts of each PAI-1 entity (latent or activated), contained in the fibrin-binding fractions were calculated and plotted against the respective fibrin concentrations. Binding to control wells was performed to determine the specificity of the assay for binding of PAI-1 to fibrin.

Complex formation of PAI-1 bound to fibrin with u-PA, t-PA, or t-PA deletion mutants. The fibrin-binding assay described above was modified as follows: 200 μl assay buffer containing ~ 2 pmol activated PAI-1 was incubated for 1 h at 37°C with fibrin matrices. Then, the supernatants were removed and the fibrin matrices washed as outlined before. Subsequently, the fibrin matrices were further incubated for 1 h at 37°C with assay buffer containing 1 mg/ml BSA and either u-PA (0.19 pmol), t-PA (0.15 pmol) or t-PA deletion mutants (del.F, del.K2, and del.FEK2, each 0.15 pmol). Subsequently, non-fibrin-binding and fibrin-binding fractions were obtained as outlined above. Aliquots of each fraction were analyzed by SDS-PAGE followed by fibrin autography.

Binding of preformed enzyme/inhibitor complexes to fibrin matrices. Enzyme/inhibitor complexes were formed by incubating t-PA (0.15 pmol), t-PA deletion mutants (del.F, del.K2, del.FEK2, each 0.15 pmol), or u-PA (0.19 pmol), respectively, for 1 h at room temperature with activated PAI-1 (2 pmol) in assay buffer. Incubation mixtures of t-PA or u-PA, respectively, with PAI-1 were then applied to the same fibrin binding assay as described for free t-PA or free u-PA. Non-fibrin-binding and fibrin-binding fractions obtained as above were subjected to SDS-PAGE, followed by fibrin autography. Incubation mixtures of t-PA, del.K2 and del.F, respectively, with PAI-1, were applied to the fibrin-binding assay in the absence or presence of ε-amino caproic acid (EACA) (1 mM, 10 mM or 50 mM) during the incubation and the washing steps. Fibrin-binding fractions were obtained and tested by fibrin autography after SDS-PAGE.

Immunoradiometric assay for t-PA L-chain antigen. Monoclonal anti-human L-chain IgM (MPWIv-PA) coupled to Sepharose beads was incubated with cell culture supernatants (containing del.F, del.K2, or del.FEK2), and monoclonal anti-human t-PA L-chain ^{125}I-labeled IgG ESP2 (15,000 cpm) in PBS, containing 10 mg/ml BSA, and 0.1% (vol/vol) Tween-20. Head-over-head rotation of the incubation mixture was performed overnight at room temperature in polystyrene tubes. Then, the Sepharose beads were washed five times with 1.5 ml of 0.15 M NaCl, 0.1% (vol/vol) Tween-20, 10 mM EDTA, and the bound radioactivity was determined. Serial dilutions of Bowes melanoma t-PA were used as standard.

Miscellaneous. SDS-PAGE, fibrin autography and reversed-fibrin autography were performed essentially according to the original protocols (43, 44, 47). Mapping of the t-PA L-chain-specific monoclonal antibodies anti-t-PA ESP2 and MPWIv-PA, directed against different epitopes, was performed as described previously (48). Radiolabeling of monoclonal ESP2 was done as described before (48). The murine monoclonal anti-PAI-1 IgG was prepared in the Department of Blood Coagulation of this Institute and has been described previously (49).

Results

Fibrin-binding assay. A specific fibrin-binding assay has previously been described for Glu-plasminogen (46). It is based on fibrin generated by thrombin digestion of intact fibrinogen that has subsequently been dehydrated before use. This assay has been employed throughout this study as well and is shown

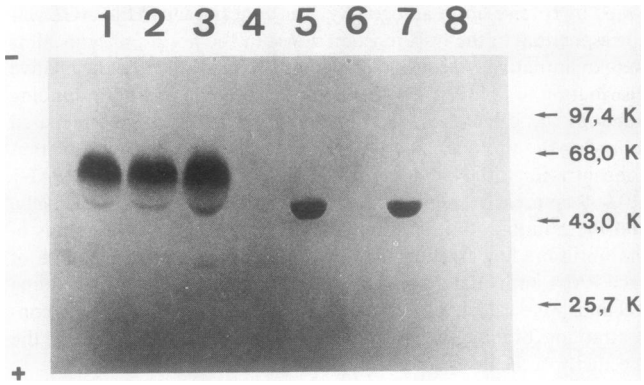

Figure 1. Binding of t-PA and u-PA to fibrin matrices. Fibrin matrices were prepared in 24-well tissue culture wells using a mixture of BSA, plasminogen-free fibrinogen and thrombin, as described in Methods. Control wells were prepared as outlined, omitting fibrinogen. T-PA (0.15 pmol) and u-PA (0.19 pmol), respectively, were incubated for 1 h at 37°C either with fibrin matrices or with control wells. Subsequently, supernatants were collected and adjusted to a final concentration of 1% (wt/vol) SDS. After extensive washing, fibrin-binding fractions were obtained by incubating the wells with 1% (wt/vol) SDS. Nonbinding and binding fractions were subjected to SDS-PAGE, followed by fibrin autography. Lane *1:* nonbinding fraction of t-PA with fibrin. Lane *2:* binding fraction of t-PA with fibrin. Lane *3:* nonbinding fraction of t-PA with control well. Lane *4:* binding fraction of t-PA with control well. Lane *5:* nonbinding fraction of u-PA with fibrin. Lane *6:* binding fraction of u-PA with fibrin. Lane *7:* nonbinding fraction of u-PA with control well. Lane *8:* binding fraction of u-PA with control well.

here to be convenient and specific for an analysis of the binding of different fibrinolytic components to fibrin. A semiquantitative determination of a fibrin-binding and a non-fibrin-binding fraction is performed by the fibrin-autography overlay technique, after SDS-PAGE. In accord with previous observations, we show that t-PA specifically binds to fibrin (Fig. 1), but not to an irrelevant protein (BSA). In this experiment, ~ 50% of the t-PA applied was found to be associated with fibrin. In contrast, u-PA binds neither to fibrin nor to BSA. We conclude that intact fibrin exposes specific binding sites for t-PA, known to bind to fibrin, whereas no binding sites are present for u-PA.

Binding of PAI-1 to fibrin. The specificity and the extent of binding of PAI-1 to immobilized fibrin matrices was subsequently investigated. For that purpose, we used a homogeneous preparation of PAI-1, purified from cultured, human vascular endothelial cells that were metabolically labeled with [^{35}S]methionine. Samples of the purified preparation were either activated with guanidine hydrochloride or not activated (i.e., latent PAI-1) and binding to fibrin was analyzed by the reversed-fibrin overlay technique after SDS-PAGE. Lysis-resistant zones, indicative for the activity and the apparent relative molecular mass of PAI-1, were quantitatively removed and the radioactivity was determined (Fig. 2). Clearly, activated PAI-1 binds to fibrin to a larger extent than nonactivated (latent) PAI-1. Approximately 23% of the activated PAI-1 preparation was found to bind to fibrin, whereas only ~ 6% of latent PAI-1 binds to the matrices. Apparently, activation of latent PAI-1 with guanidine hydrochloride better exposes the fibrin-binding capacity of this protein. It should be noted that the extent of binding, exhibited by latent PAI-1, is significantly

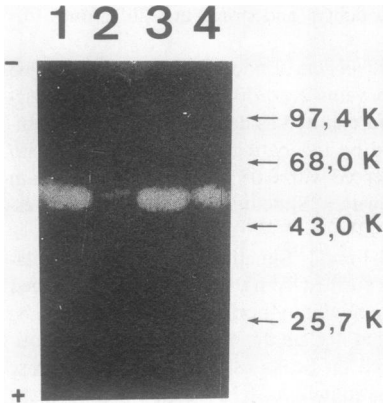

Figure 2. Binding of PAI-1 to fibrin. Purified [^{35}S]PAI-1, obtained in an inactive, latent conformation, was activated with guanidine hydrochloride, as described in Methods. Binding of latent and activated [^{35}S]PAI-1 (10,000 cpm each) to fibrin was assayed using the assay as described in the legend to Fig. 1. Non-fibrin-binding and fibrin-binding fractions were obtained, and aliquots were subjected to SDS-PAGE, followed by reversed fibrin autography. [^{35}S]PAI-1 was quantitatively removed from the zones of the gel, corresponding to the lysis-resistant areas in the fibrin-indicator films, and was subsequently counted. The relative amount of PAI-1 in the non-fibrin-binding and fibrin-binding fractions was determined and is given below. Lane *1:* latent [^{35}S]PAI-1 (non-fibrin-binding fraction, 93.7%). Lane *2:* latent [^{35}S]PAI-1 (fibrin-binding fraction, 6.3%). Lane *3:* activated [^{35}S]PAI-1 (non-fibrin-binding fraction, 76.6%). Lane *4:* activated [^{35}S]PAI-1 (fibrin-binding fraction, 23.4%).

higher than the fraction of active PAI-1 in this preparation (< 0.1%). This observation indicates that the percentage of fibrin binding is not related to the percentage of active PAI-1.

Furthermore, we established the optimal conditions for the binding of PAI-1 to fibrin. To that end, a fixed amount of either form of PAI-1 was incubated with fibrin matrices, prepared from various fibrinogen concentrations. Subsequently, the amount of radioactive material bound to the immobilized fibrin matrices was determined (Fig. 3). Our results indicate that a fibrin (monomer) concentration of ~ 3.7 μM (1.25 mg/ml) provides for maximal binding of 0.2 nM of PAI-1, either activated or nonactivated. Moreover, the optimal binding and the relative efficiency of both PAI-1 species corresponds with the data presented in Fig. 2.

Complex formation of PAI-1 bound to fibrin with t-PA and u-PA. Several reports have shown that t-PA- (or u-PA) activity can be recovered in situ from 1:1 molar complexes with PAI-1 after treatment with SDS and Triton X-100 (50, 51). This observation forms the basis of the following experiments. Activated PAI-1 was bound to fibrin matrices that were subse-

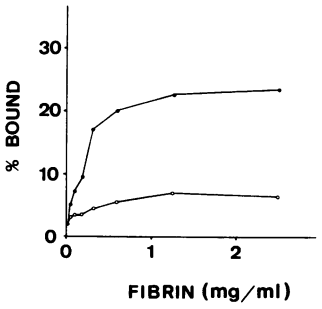

Figure 3. Binding of PAI-1 to fibrin at different concentrations. Inactive (latent) and activated [^{35}S]PAI-1 (10,000 cpm each), respectively, were allowed to bind to fibrin matrices prepared from fibrinogen at different concentrations (0.04–2.5 mg/ml). Fibrin-binding fractions were obtained and counted. The percentage of the radioactive material, binding to fibrin, is plotted against the respective fibrin concentrations. ●, activated [^{35}S]PAI-1; ○, latent [^{35}S]PAI-1.

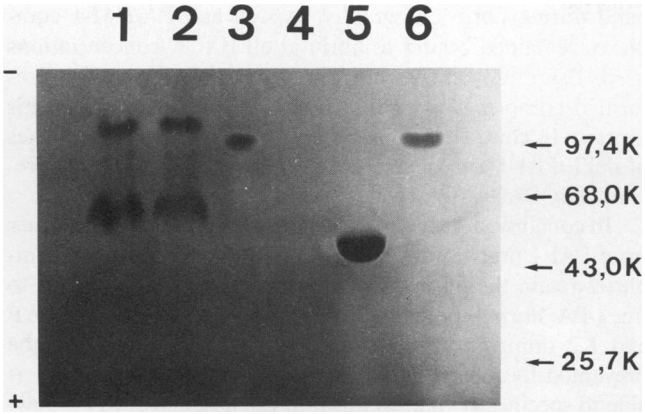

Figure 4. Complex formation of PAI-1 bound to fibrin with t-PA and u-PA, respectively. Activated PAI-1 (2 pmol) was bound to fibrin matrices as described in the legend to Fig. 2. After extensive washing, the fibrin matrices were incubated for 1 h at 37°C with t-PA (0.15 pmol) or u-PA (0.19 pmol), respectively. After the incubation, non-fibrin-binding and fibrin-binding fractions were obtained and assayed by SDS-PAGE followed by fibrin autography. Lane *1:* fraction of t-PA not bound to the fibrin/PAI-1 matrix. Lane *2:* fraction of t-PA bound to the fibrin/PAI-1 matrix. Lane *3:* fraction of u-PA not bound to the fibrin/PAI-1 matrix. Lane *4:* fraction of u-PA bound to the fibrin/PAI-1 matrix. As a control, fibrin/PAI-1 matrices were incubated for 1 h at 37°C without u-PA. Then, the supernatant was removed and u-PA (0.19 pmol) was added either to the supernatant (analyzed in lane *5*) or to the fibrin/PAI-1 matrix (analyzed in lane *6*) and both were incubated for another 1 h at 37°C.

quently washed and then incubated with either t-PA or u-PA. The reaction mixture was analyzed by fibrin autography with respect to free plasminogen activator (PA) activity and to PA activity complexed with PAI-1, either bound to fibrin or nonbound. The data, presented in Fig. 4, demonstrate that both plasminogen activators indeed form complexes with PAI-1 initially bound to fibrin. Complexes of t-PA or u-PA with PAI-1 migrated at apparent M_r positions of ~ 120,000 and 100,000, respectively, as expected from the sum of the apparent relative molecular masses of t-PA (69,000) or free u-PA (54,000) and PAI-1 (52,000). The extent of complex formation between t-PA and u-PA differs: part of t-PA remains uncomplexed, whereas u-PA is entirely contained within complexes. This observation has also been made in the absence of fibrin (results not shown) and may reflect partial dissociation of the t-PA/PAI-1 complex during electrophoresis, in contrast to the u-PA/PAI-1 complex. However, we found a significant difference between the interaction of t-PA with PAI-1 bound to fibrin and that of u-PA. Upon addition of t-PA to PAI-1 bound to fibrin, t-PA/PAI-1 complexes are encountered that are bound to fibrin, whereas after addition of u-PA the u-PA/PAI-1 complexes are detected exclusively in the nonbound fraction.

As a control, we verified that the plasminogen activators indeed interact with PAI-1 bound to fibrin and not merely with PAI-1 dissociating from the matrix during the 1-h incubation with the enzymes. Accordingly, PAI-1 bound to fibrin was exposed for the same period to buffer without u-PA. Subsequently, the supernatant was incubated with u-PA and analyzed by fibrin autography. As anticipated, only free u-PA was detected and no PA/PAI-1 complexes (Fig. 4, lane *5*). In contrast, addition of u-PA to PAI-1 bound to fibrin after the incubation with buffer resulted in the formation of complexes (Fig. 4, lane *6*) that are again encountered in the nonbound fraction. This observation clearly shows that no detectable amounts of PAI-1 dissociate from the matrix during the incubation with the plasminogen activators. Moreover, both t-PA and u-PA interact with PAI-1 that is specifically bound to fibrin. As a result of these interactions t-PA/PAI-1 complexes remain bound to fibrin, whereas u-PA/PAI-1 complexes dissociate from the fibrin matrix.

Essentially identical results were obtained for the interaction of the fibrin matrices with preformed complexes of the plasminogen activators with PAI-1. The data (Fig. 5) demonstrate that t-PA/PAI-1 complexes bind to fibrin, while no binding is detected with u-PA/PAI-1 complexes. The extent of binding of preformed t-PA/PAI-1 complexes is comparable to that of free t-PA, visualized by the lower band (~ 69,000 M_r) after fibrin autography.

Two explanations can be advanced for the different mechanism of interaction between t-PA and u-PA with PAI-1 bound to fibrin. First, PAI-1 might retain its affinity for fibrin upon complex formation with t-PA, whereas its affinity would be lost upon complex formation with u-PA. Second, PAI-1 might lose its affinity for fibrin upon interaction with either plasminogen activator. However, t-PA/PAI-1 complexes would then remain bound to fibrin, mediated by t-PA, whereas u-PA/PAI-1 complexes would not harbor fibrin-binding sites. To address this question and to obtain insight into the mechanism of interaction of t-PA/PAI-1 complexes with fibrin, we performed similar experiments with recombinant deletion mutants of t-PA.

Domains on t-PA that enable t-PA/PAI-1 complexes to interact with fibrin. Previously, we have demonstrated that binding of t-PA to forming fibrin is mediated by both the finger (F) domain and the kringle-K2 domain (13). Recently, it has been indicated that the kringle-K1 domain would also be implicated in fibrin binding (15). With respect to the involvement of these three different domains of free t-PA on the interaction with fibrin, we employed the following mutants: del.F, del.K2 and del.FEK2 (i.e., containing K1). The effect of these mutants on complex formation with PAI-1, prebound to fibrin, was analyzed essentially as described in the previous paragraphs (Fig. 6).

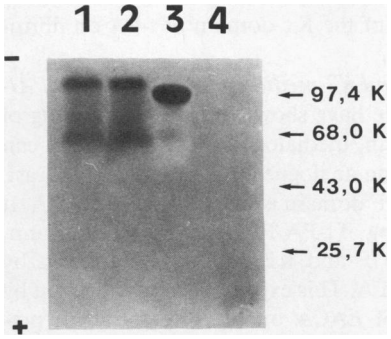

Figure 5. Binding of preformed complexes to fibrin. Activated PAI-1 (2 pmol) was incubated for 1 h at 37°C with either t-PA (0.15 pmol) or u-PA (0.19 pmol) before addition to the fibrin-binding assay. After 1 h of incubation at 37°C with the fibrin matrices, non-fibrin-binding and fibrin-binding fractions were obtained and tested by fibrin autography after SDS-PAGE. Lane *1:* non-fibrin-binding fraction of preformed t-PA/PAI-1 complexes. Lane *2:* fibrin-binding fraction of preformed t-PA/PAI-1 complexes. Lane *3:* non-fibrin-binding fraction of preformed u-PA/PAI-1 complexes. Lane *4:* fibrin-binding fraction of u-PA/PAI-1 complexes.

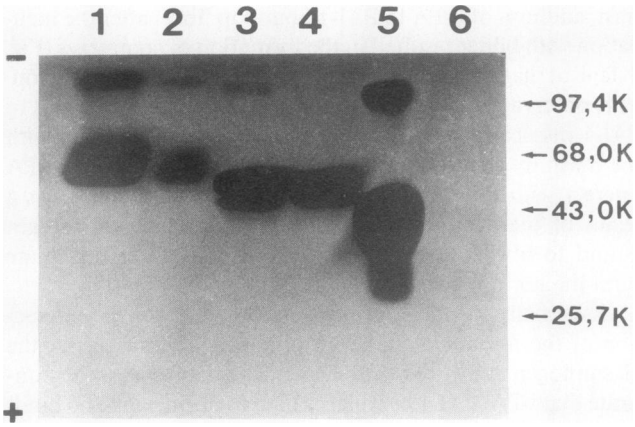

Figure 6. Complex formation of PAI-1 bound to fibrin with t-PA deletion mutants. The mutants del.F, del.K2, and del.FEK2 (each 0.15 pmol) were incubated with fibrin matrices carrying activated prebound PAI-1 as described in the legend to Fig. 5. Non-fibrin-binding and fibrin-binding fractions were analyzed by fibrin autography after SDS-PAGE. Lane *1:* fraction of del.F not bound to fibrin/PAI-1 matrix. Lane *2:* fraction of del.F bound to fibrin/PAI-1 matrix. Lane *3:* fraction of del.K2 not bound to fibrin/PAI-1 matrix. Lane *4:* fraction of del.K2 bound to fibrin/PAI-1 matrix. Lane *5:* fraction of del.FEK2 not bound to fibrin/PAI-1 matrix. Lane *6:* fraction of del.FEK2 bound to fibrin/PAI-1 matrix.

Clearly, all three t-PA mutant proteins formed complexes with PAI-1 bound to fibrin. Complexes between del.F and del.K2 with PAI-1 were found to be associated with fibrin, in contrast to complexes with del.FEK2 that were detected exclusively in the nonbound fraction. Hence, the effect of mutant del.FEK2, lacking both F and K2 but retaining K1, on PAI-1 bound to fibrin is similar to that of u-PA. The same conclusion can be drawn for the absence of binding of del.FEK2 and u-PA to fibrin that had not been exposed to PAI-1 (results not shown). On the other hand, the effect of the mutants del.F and del.K2, lacking either only F or only K2, on PAI-1 bound to fibrin is comparable to that of the complete t-PA protein. These results indicate that PAI-1 loses its affinity for fibrin not only when complexed with u-PA, but also upon interaction with t-PA. Subsequently, t-PA/PAI-1 complexes released from the matrix are rebound to fibrin, mediated by both the F and the K2 domain in accord with binding of free t-PA to fibrin (13). At least in this assay system, and employing the indicated mutants, a contribution of the K1 domain of t-PA on fibrin-binding is not detectable.

On the nature of F- and K2-mediated binding of t-PA/PAI-1 complexes to fibrin. We have shown before that binding of free t-PA to forming fibrin, mediated by the K2 domain, can be prevented by the lysine analogue EACA (14). In contrast, binding mediated by the F domain is not affected by EACA. It is conceivable that binding of t-PA/PAI-1 complexes to fibrin, mediated as well by the F and K2 domains, is affected by EACA similarly to free t-PA. This expectation was born out by determining the effect of EACA on the interaction of preformed t-PA (mutant)/PAI-1 complexes with fibrin. For that purpose, we employed t-PA, del.K2 and del.F, respectively, incubated preformed complexes with fibrin in the presence of increasing concentrations of EACA and analyzed the material that was bound to fibrin (Fig. 7). Both free t-PA, present due to incomplete complex formation and visualized by the lower band during fibrin autography, as well as t-PA/PAI-1 complexes remained bound to fibrin at all EACA concentrations used. Essentially the same results were obtained with preformed complexes of del.K2 and with the free form of this mutant. In contrast, in this system binding of both complexes of del.F/PAI-1 and of free del.F to fibrin was effectively prevented by EACA (final concentration ~ 10 mM).

In conclusion, complexes of t-PA with PAI-1 formed either from PAI-1 prebound to fibrin or employing preformed complexes retain the fibrin-binding properties of t-PA. Similar to free t-PA, fibrin binding of the complexes is mediated by the F and K2 domains, the latter exhibiting binding that can be prevented by the lysine analogue EACA. Although PAI-1 is able to specifically bind to fibrin in the absence of t-PA, however, as part of a complex with t-PA it apparently does not contribute to the interaction with fibrin.

Discussion

Recent reports have indicated that in blood two pools of PAI-1 exist that may originate from different cell types. The first one is the free plasma pool, the source of which has not been unambiguously established. It is possible that this pool is synthesized by endothelial cells and/or by hepatocytes, since in vitro cultures of these cell types synthesize and secrete PAI-1 (30, 31). The second pool of PAI-1 in blood is contained within the α-granules of platelets (27) and, most probably, is synthesized in megakaryocytes. These two separate pools may serve different functions. The plasma pool, having a concentration usually in excess over that of t-PA, inhibits t-PA in the fluid phase by the formation of inactive t-PA/PAI-1 complexes. By this mechanism the generation of plasmin in the circulation is efficiently prevented. The pool of platelet PAI-1 may serve a different function, related to the presence of platelets within a thrombus. Upon activation with specific stimuli (e.g. thrombin), platelets can release PAI-1 (27). Since platelets are able to aggregate at the site of a vascular lesion, release may result in very high, local concentrations of PAI-1. The local concentration of a serpin has been established as one of the crucial determinants for its specificity and its physiological

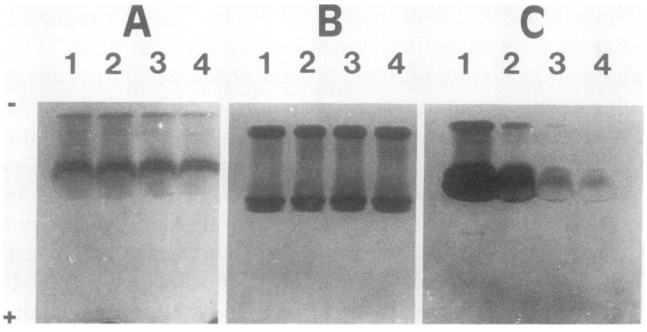

Figure 7. Influence of EACA on the binding of preformed complexes of t-PA or mutants thereof and PAI-1 to fibrin matrices. Activated PAI-1 (2 pmol) was incubated for 1 h at 37°C with t-PA (*A*), del.K2 (*B*), or del.F (*C*), before addition to the fibrin matrices. The incubation with fibrin as well as the subsequent washing step was performed in the absence or presence of EACA. The respective fibrin-binding fractions were collected and subjected to fibrin autography after SDS-PAGE. Lane *1:* no EACA. Lane *2:* 1 mM EACA. Lane *3:* 10 mM EACA. Lane *4:* 50 mM EACA.

relevance (18). Thus, the platelet pool may function as the primary local source of PAI-1 in the initial stage of hemostasis and would contribute to stabilization of a (provisional) hemostatic plug by preventing the local action of fibrin-bound t-PA. This view is supported by the recent finding that PAI-1 binds to fibrin, apparently independent of the crosslinking enzyme Factor XIII, and that the fibrin-bound conformation of PAI-1 is able to inhibit t-PA activity (5). Furthermore, it has been reported that platelets contain and release both active and latent PAI-1 (29). Interestingly, negatively charged phospholipids, that are also released from activated platelets and play a key role in the activation of coagulation factors, can activate latent PAI-1 as well (33). Hence, these phospholipids might be particularly involved in the activation of the latent fraction of platelet PAI-1 after release into the hemostatic plug. In conclusion, a number of observations have indicated that PAI-1 may inhibit the fibrinolytic system by different pathways, involving either free PAI-1 or PAI-1 immobilized on fibrin.

In this paper, we have investigated the mechanism of inhibition of t-PA activity by fibrin-bound PAI-1 in the absence of additional fibrinolytic components. To that end, a specific binding assay for PAI-1 to fibrin was developed. Using this assay, it is revealed that activated PAI-1 binds to a larger extent than latent PAI-1. Activation of latent PAI-1 obviously not only results in exposure of the reactive site for the interaction with its target serine proteases, but also substantially unmasks its fibrin-binding site. We subsequently have compared the effect of t-PA on activated PAI-1 bound to fibrin with that of u-PA and of t-PA deletion mutant proteins, lacking specific structural and functional domains located on the amino-terminal H chain. All plasminogen activators contain an intact catalytic center, located on their respective carboxy-terminal chains, that would be available for an interaction with the reactive site of PAI-1. From our data, it is evident that the reactive site of PAI-1 is not directly involved in binding to the fibrin matrix, since after binding it is fully available for the formation of complexes with any of the plasminogen activators employed.

Taking our results together, a model can be formulated for the interaction of t-PA with activated PAI-1 bound to fibrin that is composed of three sequential steps: (*a*) The catalytic center of t-PA interacts with the reactive center of PAI-1, bound to fibrin, resulting in a stable complex. The preferential binding of t-PA to PAI-1, instead of binding to fibrin, is in accord with the kinetic parameters reported for these interactions with the separate components (52, 53). (*b*) Upon formation of a stable complex with t-PA, PAI-1 apparently undergoes a conformational change and loses its affinity for fibrin. (*c*) The 1:1 molar complex of t-PA and PAI-1 dissociates from the fibrin matrix and subsequently rebinds to fibrin. This fibrin binding is dependent on the presence of the finger (F) and/or the kringle-2 (K2) domain of t-PA and is independent of the PAI-1 moiety of the complex.

Actually, at this point we cannot distinguish whether the t-PA/PAI-1 complex dissociates and rebinds to fibrin or that dissociation and binding, mediated by the F and/or K2 domain of t-PA, are simultaneous events. Furthermore, it should be noted that our observations divert from other reports on two aspects. First, Okada et al. (54) could demonstrate only involvement of the K2 domain in binding of t-PA/PAI-1 complexes to fibrin. These authors suggested that, due to complex formation with PAI-1, the fibrin-binding feature of the F domain is apparently masked. Second, our experiments do not reveal involvement of the K1 domain on fibrin binding of either free t-PA (as suggested by others [15]) or of t-PA/PAI-1 complexes. It is conceivable, as indicated before (55), that different observations may be due to the use of different assay systems, including other fibrin preparations, and/or differently composed mutant proteins.

Apart from inhibition of fibrinolysis at the level of plasminogen activation by PAI-1, this process is also controlled at the level of plasmin activity by α2AP (4, 34). As outlined before, both inhibitors (serpins) form a 1:1 molar complex with their respective target proteases and exhibit a significant structural homology. Similar to the action of PAI-1, α2AP also displays two modes of inhibition, i.e., in the fluid phase and crosslinked to fibrin. However, in the latter case, the mechanism of inhibition is clearly distinct from that of PAI-1 bound to fibrin. First, complexes of α2AP/plasmin are associated with fibrin by the α2AP moiety, crosslinked to the matrix. In contrast, we have shown here that PAI-1/t-PA complexes are bound, mediated by the t-PA part. Second, the binding sites for the α2AP/plasmin complex are distinct from those of plasmin, whereas identical binding sites are proposed in this report for free t-PA and for the PAI-1/t-PA complex, based on the F and the K2 domain of t-PA. At present, the physiological significance of the apparently distinct mechanisms of these fibrin-associated inhibitors is not resolved.

The mechanism proposed above offers an explanation for the inhibition of the fibrinolytic system, catalyzed by t-PA, due to PAI-1 bound to fibrin. Essential for this explanation is our finding that the fibrinolytically inactive complex of t-PA and PAI-1 still displays fibrin-binding properties. The structural entities of the complex that enable binding, i.e. the F and K2 domains of t-PA, are identical to those of free t-PA. Hence, it is likely that complexes of t-PA/PAI-1 and free t-PA compete for the same binding sites on fibrin and, consequently, complexes will inhibit fibrinolysis by preventing t-PA binding to fibrin, an event that would greatly augment t-PA activity. In conclusion, our results demonstrate that, besides inhibiting t-PA activity in the fluid phase, PAI-1 bound to fibrin can also effectively inhibit t-PA: a mode of action is proposed that ultimately results in the formation of t-PA/PAI-1 complexes that are able to occupy the fibrin-binding sites of free t-PA.

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