The Interaction of Plasminogen Activator Inhibitor 1 With Plasminogen Activators (Tissue-Type and Urokinase-Type) and Fibrin: Localization of Interaction Sites and Physiologic Relevance

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Plasminogen activator inhibitor 1 (PAI-1), an essential regulatory protein of the fibrinolytic system, harbors interaction sites for plasminogen activators (tissue-type [t-PA] and urokinase-type [u-PA]) and for fibrin. In this study, anti-PAI-1 monoclonal antibodies (MoAbs) were used to identify interaction sites of PAI-1 with these components. The binding sites of 18 different MoAbs were established and are located on five distinct "linear" areas of PAI-1. MoAbs, binding to two distinct areas of PAI-1, are able to prevent the inhibition of t-PA by PAI-1. In addition, two interaction sites for fibrin were identified on PAI-1. The area located between amino acids 110 and 145 of PAI-1 contains a binding site for both components and its significance is discussed in the context of the t-PA inhibition by fibrin-bound PAI-1. Subsequently, the MoAbs were used to assess the role of platelet–PAI-1 in clot-lysis. An in vitro clot-lysis system was used to demonstrate that clot-lysis resistance is dependent on the presence of activated platelets and that PAI-1 is a major determinant for lysis-resistance. We propose that, upon activation of platelets, PAI-1 is fixed within the clot by binding to fibrin and retains its full capacity to inhibit t-PA and u-PA.

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Plasminogen activator inhibitor 1 (PAI-1) is the fast-acting inhibitor of tissue-type plasminogen activator (t-PA) and of urokinase-type plasminogen activator (u-PA). These plasminogen activators are key components of the fibrinolytic system. Molecular cloning of full-length PAI-1 cDNA and the establishment of its nucleotide sequence have shown that the mature protein consists of 379 amino acids and, significantly, is devoid of cysteine residues. The latter feature allows expression of biologically active material in extracts of Escherichia coli cells transformed with PAI-1 cDNA. PAI-1 belongs to the superfamily of serine protease inhibitors ("serpins") and as such is thought to act as a pseudo-substrate for its target protease(s). Essential to this view is the presentation of the peptide bond of the PAI-1 P1-P1' (arg346-met347) reactive center to the catalytic site of t-PA (or u-PA) to form a tight bond.

In addition to its interaction with t-PA and u-PA, we and others have recently demonstrated that PAI-1 specifically binds to various other components involved in modulation of the fibrinolytic system, such as fibrin, vitronectin, and heparin. Moreover, in the presence of either vitronectin or heparin efficient inhibition of thrombin is achieved. The elucidation of the various binding sites on PAI-1 would lead to a better understanding of the structure and the function of the molecule. So far, only the reactive site P1 residue (arg346) is shown to be of importance for the specific interaction of PAI-1 with t-PA and u-PA. Because many serpins harbor an arginine-residue at their respective P1 position, other distinct sequences have to provide the elements for the specific, extremely fast and tight interaction. The identity of such residues has not been elucidated for any of the serpins. Similarly, no information is available on the identity of amino acid residues of PAI-1 that are involved in the binding to fibrin.

In this study we have used a panel of monoclonal antibodies (MoAbs), directed against human PAI-1, to characterize the interaction of PAI-1 with t-PA (u-PA) and fibrin. By using deletion mutants of PAI-1, produced either in vitro or in transformed E coli cells, we have determined the binding sites on PAI-1.

Subsequently, the ability of the anti-PAI-1 MoAbs to prevent either the interaction with t-PA, u-PA, or fibrin was assessed. The combination of the epitope localization and the functional properties of the MoAbs allowed us to assign binding sites to specific areas on PAI-1 for interaction with either t-PA (u-PA) or fibrin. In a more physiologic setting, the relief of platelet-induced inhibition of t-PA mediated clot-lysis by different anti-PAI-1 MoAbs is demonstrated. In this context, both the t-PA–inhibitory function of PAI-1 and its fibrin-binding capacity are shown to modulate clot lysis. The physiologic relevance of the latter observations and a possible mechanism for the interaction of t-PA with PAI-1 bound to fibrin is discussed.

MATERIALS AND METHODS

Reagents. Iscove's modified Dulbecco's medium (IMDM) and fetal calf serum (FCS) were obtained from GIBCO (Paisley, UK). Radioactive materials were from the Radiobiological Centre (Amersham, UK). Two-chain Bowes melanoma t-PA (850,000 IU/mg) was from Biopool (Umea, Sweden). High molecular weight urokinase was purchased from Calbiochem (La Jolla, CA). Chromogenic substrate S2288 (H-D-isoleucyl-prolyl-arginyl-p-nitroanilide) was obtained from KabiVitrum (Stockholm, Sweden). Human fibrinogen (grade L; KabiVitrum) was treated with 1 mmol/L diisopropylfluorophosphate and with lysine-Sepharose to inactivate potentially contaminating proteases and to remove traces of plasminogen. [3H]labeled fibrinogen was prepared using the chloramine-T method. Glu-plasminogen was purified from fresh human plasma exactly as described before. Lysine-Sepharose and plasmin psP69 DNA were from Pharmacia (Uppsala, Sweden). Nucleic acid modifying enzymes were from New England Biolabs (Beverly, MA) or BRL (Gaithersburg, MD) and were used as indicated by the supplier. For DNA sequencing, Sequenase (USB, Cleveland, OH) was used as described by the manufacturer.

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M13mp9am4 DNA was obtained from Anglian Biotechnology Ltd (Colchester, UK). Oligonucleotides were made using an automated DNA synthesizer (Applied Biosystems model 318A, Foster City, CA). Polyclonal antihuman factor X was from this institute and was purified using diethyl aminoethyl (DEAE)-Sepharose chromatography. Asparagine, prostatic lipase and, human thrombin were purchased from Sigma (St Louis, MO).

**MoAbs.** MoAbs directed against human PAI-1 were obtained from different sources. Hybridoma cells producing the MoAbs CLB-1C3, CLB-1B10, and CLB-9H7 were a gift from Drs J.A. van Mourik and H. Lambers (Department of Blood Coagulation, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). Human PAI-1, purified from umbilical cord vein endothelial cell (HUVEC)-conditioned medium, was used to raise these MoAbs. Hybridoma cells producing the MoAbs CLB-1C5, CLB-2, CLB-5, CLB-7, CLB-10, CLB-14, CLB-15, CLB-16, CLB-17, CLB-22, CLB-26, CLB-33, CLB-2C8, CLB-8H2, and CLB-103 were isolated in this institute. In this case, PAI-1 purified from conditioned medium of human HepG2 cells was used as the immunogen. Purified antihuman PAI-1 MoAbs M2, M3, M5, M7, M1, and M2 were a gift of Dr B. Hoke (Monozyme, Charlottenlund, Denmark). The MoAbs MAI-111 and MAI-121 were obtained as purified IgG from Biopool. The MoAbs TCI and TC3 (purified IgG) were purchased from Technoclone (Vienna, Austria).

The CLB-type MoAbs were purified from hybridoma cell-conditioned medium as follows. Hybridoma cells were grown in IMDM, supplemented with 5% (vol/vol) FCS, 50 μmol/L 2-mercaptoethanol, penicillin (100 IU/ml), streptomycin (100 μg/mL), and 80 U/mL interleukin-6 (IL-6; a gift of Dr L. Aarden, this institute) ("hybridoma growth medium"). To produce MoAbs, 40 x 10^6 cells were transferred to a roller bottle, containing 1 L of the hybridoma growth medium supplemented with 2.5% (vol/vol) FCS. After 3 weeks of cell growth at 37°C, the medium was collected and centrifuged to remove residual cells. The MoAb preparation was concentrated by ammoniumsulphate precipitation and subsequently purified using protein A-Sepharose (Pharmacia) according to the instructions of the supplier. The purification resulted in homogeneous preparations as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing, as well as nonreducing conditions. The final concentration of all antibodies was determined by measuring the optical density (OD) at 280 nm (1 OD unit is 0.75 mg/mL). The MoAbs were coupled to cyanogen-bromide-activated Sepharose (Pharmacia) (1 mg MoAb/mL of Sepharose), as indicated by the supplier.

**Construction and expression of PAI-1 and PAI-1 variants.** The construction of plasmids encoding human PAI-1 that are suitable for the expression of biologically active recombinant PAI-1 in E.coli has been described in detail. Essentially, the full-length PAI-1 coding sequence is inserted into the expression plasmid pMBL11, downstream of an epitope (E) and a cleavage sequence for collagenase (c), respectively, resulting in plasmid pMBL11/ EcPAI-1. Expression of the fusion product is directed by the E.coli tryptophan (trp) promotor. The anti-factor VIII MoAb CAG69 binds to the epitope (E), derived from the amino acid sequence of human factor VIII, and was used for immuno-affinity purification. The epitope can be removed from the fusion protein, to liberate mature PAI-1, upon incubation with collagenase coupled to Sepharose. The PAI-1 deletion mutants PAI-1-de17-28 (lacking amino acids tyr7 to lys28), PAI-1-de56-83 (lacking amino acids lys55 to met83), and PAI-1-de17-145 (lacking amino acids tyr7 to lys145) were constructed as follows. An EcoRI-Bgl II restriction fragment, containing the entire PAI-1 coding region (from nucleotide 52 till 1476), was inserted into the multiple cloning site of M13mp9am4 RF, using standard recombinant DNA techniques.

"Out-looping mutagenesis" was performed as described, using the following oligonucleotides: 5' GTGCACATCCCCCATC- CGACCAAGCTTGGTATCTT3', 5' CAAGAGCTATGGGAT- TCGGGCCATGGACAGGAC 3', and 5' GTGCACATC- CCCCATTGATATGACGACTT3' for PAI-1-de7-28, PAI-1-de65-83, and PAI-1-de17-145, respectively. After out-looping, the mutated fragments that were isolated to substitute the nonmutated corresponding fragments of the expression plasmid pMBL11/EcPAI-1 were entirely sequenced. For PAI-1-de7-28, the ApaLI-Sac I (nucleotide positions 196-436, respectively) fragment was used. Similarly, for PAI-1-de65-83 the ApaLI (position 196) -Nco I (position 446) fragment was used. For PAI-1-de7-145, the ApaLI-Bam I fragment (positions 196 and 693, respectively) was used. Expression of the PAI-1 proteins in E.coli, in some cases metabolically labeled with [35S]-methionine, and the subsequent immunopurification was performed as described. Human endothelial PAI-1, in some cases metabolically labeled with [35S]-methionine, was purified from the conditioned medium of HUVEC. The anti-PAI-1 MoAb CLB-IC3 was used for the one-step immuno-affinity procedure.

**In vitro transcription and translation of PAI-1 deletion mutants.** The metabolically labeled [35S]-PAI-1 and [35S]-PAI-1 variants, which were used to determine the localization of the corresponding epitopes of the MoAbs, were produced in extracts of transformed E.coli cells as described above. Alternatively, we used in vitro "run-off" transcription, using the SP6 RNA polymerase system, followed by in vitro translation in a rabbit reticulocyte lysate. All materials for the transcription and the translation reactions were from Promega (Madison, WI) and were used according to protocols of the supplier. The plasmids used for transcription were constructed as follows. The cDNA of PAI-1, including the complete coding region, the 3' untranslated region, and the polyadenylation site (EcoRI [52]-HindIII [from the multiple cloning site] fragment), was cloned into the multiple cloning site of transcription plasmid pSP69, yielding plasmid pSP/PAI-1. Run-off transcription from the SP6 promoter of pSP/PAI-1 DNA, truncated by cleavage with Bgl II (position 1476) and followed by translation of the resulting RNA, yields full-length human PAI-1 protein. Carboxyl-terminal deletion mutants were made by using plasmids in the transcription reaction, which were truncated by cleavage with various restriction endonucleases. The PAI-1 translation product, after cleavage of pSP/PAI-1 DNA with Nco I (position 448) before transcription, contains amino acids 1-108. Similarly, using Bam I (693) to cleave the DNA, the protein contains amino acids 1-166; using Sal I (1045) it contains amino acids 1-283; using Hae II (1152) it contains amino acids 1-319; and using Ava I (1240) the protein contains amino acids 1-346. Amino terminal deletion mutants were made by cleaving pSP/PAI-1 DNA with EcoRI (position 52) and an appropriate restriction endonuclease. After purification and blunting of the resulting termini, the plasmid DNA was ligated to position the promoter upstream of the PAI-1 "open reading frame." This was confirmed by sequence analysis. Before transcription, the plasmids were cleaved with Bgl II (1476). In all cases estimation of the molecular weight of the in vitro translation products indicated strong initiation of translation from the utmost amino terminal methionine, except for the deletion plasmid resulting from cleavage with Bam I (693). Here, the methionine at amino acid position 234 was used as initiator for translation instead of either one of the methionines at positions 201 or 201, as determined by an estimation of the size of the translation product on SDS-PAGE. The PAI-1 translation product, resulting from plasmid pSP/PAI-1 (position 436), lacks amino acids 1-82. Similarly, the pSP/PAlde1Nco (448)-derived product lacks amino acids 1-109, the pSP/PAIdeBamI-derived product lacks amino acids 1-234 and the pSP/PAIdeSal (1045)-derived product lacks amino acids...
acids 1-294. The proteins used for the epitope mapping are schematically presented in Fig 1.

**Immunoprecipitation.** Immunoprecipitation of PAI-1 and PAI-1 variant proteins was performed as follows. The Sepharose-coupled MoAbs were washed twice with 10 mmol/L Tris-HCl (pH 8.0), 500 mmol/L NaCl, 2% (wt/vol) bovine serum albumin (BSA), 0.1% (vol/vol) Tween-80, and 10 mmol/L benzamidine ("precipitation buffer"). [3S]-labeled PAI-1 (mutants) were added to 10 μL MoAb-Sepharose beads in a volume of 500 μL precipitation buffer. After rotating overnight at 4°C, the beads were washed twice with 1 mL precipitation buffer, once with 1 mL precipitation buffer without BSA, and, finally, once with 20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 0.1% (vol/vol) Tween-80. Subsequently, the beads were resuspended in 35 μL 5% (wt/vol) SDS, 2% (vol/vol) 2-mercaptoethanol, 3.5% (vol/vol) glycerol, 25 mmol/L Tris-HCl (pH 6.8), and 0.002% (wt/vol) bromophenol blue and heated for 5 minutes at 100°C. After centrifugation, the supernatant was subjected to SDS-PAGE. Finally, the precipitated product was visualized by fluorography. Under these conditions, a purified antihuman factor X antiseraum coupled to Sepharose, that is used as a negative control, did not give any significant signal in immunoprecipitation of [35S]PAI-1 (variants), which readily precipitated [35S]factor X. Immunodetection of PAI-1, immobilized on nitrocellulose after SDS-PAGE, by means of alkaline phosphatase-conjugated goat antimouse IgG (Promega) is performed as indicated by the supplier, except that 0.1% (vol/vol) Tween 80 was used in all buffers, instead of the indicated 0.05% (vol/vol) Tween 80. Under these conditions, the antihuman factor VIII MoAb CAg69 that was used as a negative control, did not give any significant signal.

**Competition assay.** [35S]-labeled endothelial PAI-1 (2,500 cpm per incubation) was incubated overnight at 4°C with an increasing amount of an anti-PAI-1 MoAb in a total volume of 500 μL precipitation buffer. Subsequently, 10 μL of another anti-PAI-1 MoAb, coupled to Sepharose, was added and the suspension was incubated for 3 hours at room temperature. After centrifugation, the supernatant was collected and the radioactivity was determined. The beads were washed twice with precipitation buffer and the bound radioactivity was measured.

**t-PA inhibition assay.** The influence of the anti-PAI-1 MoAbs on the inhibition of t-PA or u-PA by PAI-1 was tested as follows. Recombinant PAI-1 was activated by dialysis for 1.5 hours at room temperature against 6 mol/L guanidinium chloride, containing 0.1% (vol/vol) Tween-80. The denaturant was removed by dialysis for 2 hours at 4°C against 150 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.8), and 0.1% (vol/vol) Tween-80 ("TBST-buffer"), followed by overnight dialysis at 4°C against the same buffer. The activity of the resulting PAI-1 preparation was determined by titration against two-chain Bowes melanoma t-PA, t-PA (1.76 nmol/L) or u-PA (1.8 IU) was incubated at 37°C with increasing concentrations of PAI-1 in a total volume of 40 μL in TBST-buffer. After 15 minutes the inhibition reaction was arrested by adding 150 μL 100 mmol/L Tris-HCl (pH 8.4), 0.1% (vol/vol) Tween-80, and 50 μL 5 mmol/L S2288. The residual t-PA activity was determined by continuously measuring the conversion of the chromogenic substrate at 405 nm in a TiterTek twinreader (Flow Laboratories, Irvine, UK). Conversion of the chromogenic substrate was linear for at least 6 hours. To determine whether anti-PAI-1 MoAbs prevent the inhibition of t-PA or u-PA by PAI-1, this assay was adapted as follows. Active PAI-1 (1.60 nmol/L) was incubated for 1 hour at room temperature with increasing concentrations of the MoAb (0.01, 0.1, 1, and 10 μg) in a total volume of 40 μL TBST-buffer. Subsequently, 1.76 nmol/L t-PA was added and the mixture was incubated for 15 minutes at 37°C. Finally, the reaction was arrested and the residual t-PA activity was determined as described above. To block the u-PA inhibition with anti-PAI-1 MoAbs, 1.8 U of u-PA was used and an adequate amount of active PAI-1 to inhibit 80% to 90% of the u-PA activity. As negative controls, polyclonal anti-factor X and the anti-factor VIII MoAb CAg69 were used.

**Fibrin binding assay.** The antihuman PAI-1 MoAbs were assayed for potential inhibition of PAI-1 binding to fibrin matrices as follows. Fibrin matrices in 24-well tissue culture plates were prepared essentially according to Tran-Thang et al. To prevent aspecific binding, wells were coated for at least 16 hours at 4°C with 2% (wt/vol) BSA in 50 mmol/L sodium carbonate (pH 9.4). After coating, the wells were washed twice with phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween-80, and 0.1% (wt/vol) BSA ("PTB buffer"). In each well 250 μL fibrinogen (250 mg/mL) fully depleted of plasminogen and vitronectin, was added in PTB buffer to 25 μL human thrombin (10 U/mL). The formed fibrin matrix was air dried at 37°C. Before use, the matrix was incubated for 30 minutes with 300 μL PTB buffer and, subsequently, washed three times with PTB buffer. Simultaneously, [35S]-labeled PAI-1 (2,500 cpm/well) was incubated at room temperature for 30 minutes with increasing amounts of a particular MoAb (0.01, 0.1, and 10 μg in a total volume of 100 μL). Subsequently, the PAI-1/MoAb mixtures were added to 100 μL PTB buffer present in a fibrin coated well or to 100 μL PTB buffer in a control well without fibrin and incubated for 1.5 hours at 37°C. The supernatant was then quantitatively removed and, after the addition of 200 μL 2% (wt/vol) SDS, the radioactivity was determined by liquid-scintillation counting. The wells were rapidly washed twice with 1.5 mL PTB buffer. The bound PAI-1 was eluted at room temperature by gently shaking with 200 μL 1% (wt/vol) SDS. After transfer of each eluate to a scintillation vial, the well was washed with 100 μL 1% (wt/vol) SDS, which was subsequently transferred to the corresponding vial for determination of the radioactivity. The antihuman factor VIII MoAb CAg69 was used as a negative control.

**In vitro clot-lysis assay.** The antihuman PAI-1 MoAbs were tested in vitro to determine their influence on t-PA-mediated lysis of platelet-rich clots. Platelets were isolated essentially as described. For the isolation of platelets only plastic materials were used. Blood (42.5 mL) from healthy donors was collected in a tube, containing 7.5 mL of 100 mmol/L glucose, 85 mmol/L trisodiumcitrate, and 66 mmol/L citric acid. After centrifugation for
20 minutes at 225g the platelet-rich plasma was carefully collected. To prevent platelet activation, apyrase and prostaglandine E, were added to final concentrations of 25 µg/mL and 100 nmol/L, respectively. The suspension was centrifuged for 10 minutes at 1,500g and the pelleted platelets were resuspended in a buffer containing 105 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 5 mmol/L glucose, 36 mmol/L citric acid, and 0.35% (wt/vol) BSA at pH 6.5. Again, apyrase (25 µg/mL) and prostaglandine E, (100 nmol/L) were added. This step was repeated twice and the platelets were finally resuspended at a concentration of 2 x 109 platelets/mL in Tyrode’s buffer (137 mmol/L NaCl, 2.68 mmol/L KCl, 2 mmol/L CaCl2, 1 mmol/L MgCl2, 0.36 mmol/L NaH2PO4, 11.9 mmol/L NaHCO3, 5 mmol/L HEPES, 0.1% [wt/vol] glucose, and 0.35% [wt/vol] BSA at pH 7.35). To assay for clot-lysis, fibrinogen (1 mg), [125I]-fibrinogen (20,000 cpm), glu-plasminogen (20 µg), two-chain t-PA (1.5 ng), and, facultatively, 2.5 x 106 platelets and increasing amounts of anti-PAI-1 MoAbs (0, 0.1, 1, 10, and 100 µg) were mixed in a volume of 1 mL in Tyrode’s buffer. Subsequently, 100 µL of the mixture was added to 10 µL thrombin (10 Ul/mL) and incubated for increasing periods at 37°C, after which the clot was centrifuged for 4 minutes at 40,000g. The radioactivity in 10 µL of the supernatant was determined. This value was related to 10 µL of an incubation without thrombin (input). Clot-lysis was dependent on the presence of t-PA, because in its absence no lysis was observed for at least 4 hours. Upon addition of 2.5 x 109 platelets, the lysis by t-PA was inhibited for 80% to 95% after incubation for 2 hours at 37°C.

RESULTS

General properties of the anti-PAI-1 MoAbs. The 28 different MoAbs that have been used in this study are listed in Table 1. These MoAbs were raised against human PAI-1 purified from conditioned medium of vascular endothelial cells or HEP-G2 cells. The determination of the Ig subclass was performed as described and we found most of them to belong to the IgG1 subclass (Table 1). Furthermore, we established that in immunoprecipitation experiments all of the antibodies are able to recognize PAI-1 in solution (Table 1). Significantly, none of the MoAbs showed any difference in forming immune complexes either with PAI-1 isolated from endothelial cell-conditioned medium or recombinant PAI-1 produced in E.coli. In accord with our previous data, we conclude that the conformation of these two PAI-1 species is similar. In addition, we conclude that none of the antihuman PAI-1 MoAbs is directed against the carbohydrate moiety of the original immunogen, i.e., human endothelial PAI-1 or HEP-G2 PAI-1. Binding of antibodies to native proteins in solution may differ from the binding to immobilized, partially denatured proteins. Indeed, four of the anti-PAI-1 MoAbs (CLB1B10, CLB1H7, CLB26, and CLB103) did not recognize PAI-1 after SDS-PAGE28 followed by immunoblotting25 on nitrocellulose (Table 1), indicating a conformation-dependent recognition of PAI-1 by the respective MoAbs.

Mapping of the MoAbs on “linear” areas of PAI-1. The MoAbs characterized in the previous paragraph were used to localize the interaction sites on PAI-1 with t-PA, u-PA, and fibrin. To that end, it is imperative to determine the area on PAI-1 that binds to these respective MoAbs. For such “epitope mapping” experiments, we prepared defined deletion mutants of PAI-1 that were used in immunoblotting and immunoprecipitation assays. In Fig 1 these mutants are schematically presented and their respective designations indicate the positions of the amino acids that were deleted. The mutants PAI-1del17-28, PAI-1del65-83, and PAI-1del17-145 were produced in E.coli and used in blotting as well as in immunoprecipitation experiments. The other mutants, truncated either from the amino terminus or the carboxyl terminus, were made in vitro by transcription from the SP6 promoter, followed by translation in a rabbit reticulocyte lysate in the presence of [35S]-methionine, and were used exclusively in immunoprecipitation experiments. Taking this approach, only epitopes for MoAbs that bind to denatured “linear” areas of PAI-1 can be mapped, because the in vitro-produced mutants will not be properly folded. In accord with this view, the deletion mutants produced in E.coli are found to be defective in fibrin binding and plasminogen activator inhibition and are not recognized by the conformation-depen-

![Table 1. Properties of Antihuman PAI-1 MoAbs](attachment:table1.jpg)
dent antibodies CLB-1B10 and CLB-9H7 (data not shown), in contrast to the complete E coli-derived PAI-1 molecule. These findings indicate that deletions in PAI-1 result in inactive, malfolded PAI-1 proteins. The results of the mapping experiments are shown in Fig 2. From our panel of 28 different anti-PAI-1 MoAbs we were able to localize the corresponding epitope of 18 MoAbs. The epitopes are located in five distinct immunodominant areas on PAI-1. The MoAbs TC3, TC1, CLB-1C3, CLB-2C8, CLB-5, CLB-10, CLB-14, CLB-15, CLB-33, M2, and I1 bind to an epitope between amino acids 110 and 145. The binding site of antibodies M3, CLB-8H2, CLB-16, and CLB-17 is located between amino acids 235 and 283. The position on PAI-1 to which the MoAbs M5, CLB-7, and MAI-12 bind is from 284 to 294, from 295 to 320, and from amino acid 320 to the carboxyl terminus of PAI-1, respectively. In addition to the four nonblotting antibodies, the epitope for six other anti-PAI-1 MoAbs (CLB-1, CLB-2, CLB-22, M7, I2, and MAI-11) could not be assigned to a distinct, linear area on PAI-1. The properties of the remaining 18 anti-PAI-1 MoAbs, for which the corresponding epitope on PAI-1 now has been established, were further studied.

**Competition for the binding to PAI-1.** To determine whether MoAbs share part of their epitope on PAI-1, we performed competition experiments with different MoAbs. We found that all of the MoAbs that bind to an epitope localized between amino acid residues 110 and 145 could be competed by the anti-PAI-1 MoAb CLB-1C3. Consequently, this class of MoAbs indeed share at least part of their epitope. In contrast, none of the antibodies that were mapped outside this region was competed by CLB-1C3. The MoAb M3 was able to compete with CLB-16, CLB-17, and CLB-8H2, while it did not compete with M5, CLB-7, or MAI-12. The MoAbs M5, CLB-7, and MAI-12 have unique binding sites and exhibit only "self-competition." Hence, no competition is observed between pairs of each one of the MoAbs CLB-1C3, M3, CLB-7, M5, or MAI-12, being representatives of anti-PAI-1 MoAbs that bind to five different epitopes on PAI-1. This conclusion is reached irrespective of whether any of the MoAbs are coupled to Sepharose or whether it is present as competitor in solution. It should be noted that all competition experiments are performed using a 10-fold excess of competitor in solution over the amount of MoAb that yields complete self-competition. We conclude that the data of these competition experiments fully agree with the data of the epitope mapping experiments.

**Identification of the interaction sites on PAI-1 with t-PA, u-PA, and fibrin.** To align the position of an epitope on PAI-1 with a potential functional interaction site, we further assessed the properties of the 18 anti-PAI-1 MoAbs, characterized in a previous paragraph. To that end, we assayed whether the different MoAbs could prevent inhibition of t-PA (or u-PA) by PAI-1 or interfered with binding of PAI-1 to fibrin. First, increasing concentrations of a particular MoAb were incubated for 1 hour with PAI-1 and the mixture was subsequently added to t-PA. The residual t-PA activity was then determined using the chromogenic substrate S2288. The results are presented in Fig 3. Five different MoAbs that interact with two different epitopes efficiently prevent, in a dose-dependent manner, the inhibition of plasminogen activators by PAI-1 (Fig 3A and B). The inhibition-preventing properties of MoAbs M2 and MAI-12 have been described before and are in accordance with our observations. One of the inhibitory MoAbs, ie, MAI-12, binds to an area relatively close to the reactive site (P1-P1') of PAI-1. The remaining four inhibitory MoAbs, ie, CLB-2C8, CLB-5, M2, and I1, bind to a region on PAI-1 bordered by the amino acid residues at position 110 and 145. Obviously, this area is distinct from the reactive center and, consequently, our data indicate that two different areas on PAI-1 are essential for the interaction between the inhibitor and t-PA. Furthermore, identical results were obtained for the prevention of PAI-1-mediated u-PA inhibition by the aforementioned MoAbs. It should be noted that the inhibition of plasminogen activators by PAI-1 may include intermediate interactions between protease and inhibitor as has been observed for t-PA and PAI-1. In the endpoint assay used in this study, we only detect the formation of the ultimate, inactive serpin-protease complexes, while potential intermediate interactions will not be shown. We conclude that the inhibition of plasminogen activators requires at least two distinct interaction sites on the PAI-1 molecule.

Second, increasing concentrations of either one of 18 MoAbs were included into a mixture containing [35S]-labeled PAI-1 and a fibrin matrix. Indeed, several MoAbs were found to prevent the fibrin binding of PAI-1 (Fig 3A and C). Again, the MoAbs belong to two different categories: all but one MoAb (ie, M5) bind to the area between amino acid 110 and 145, which is also recognized by some of the MoAbs that prevent the inhibitory action of PAI-1. In
addition MoAb M5, which efficiently prevents the binding of PAI-1 to fibrin, binds to a different region of PAI-1, namely between the amino acid residues spanning positions 283 and 294. Clearly, this relatively short area represents a unique binding site for fibrin, because no MoAbs were found that prevent the inhibitory function of PAI-1 and simultaneously bind to this region. An analysis of the properties of MoAbs, which bind to the region between amino acids 223 and 294, allows us to conclude that in part the same amino acid residues are responsible for both fibrin binding and t-PA inhibition, whereas in addition other residues have a unique contribution in these reactions. This conclusion can best be illustrated by comparing the properties of, eg, CLB-5 (preventing both functions), CLB-2C8 (preventing only PAI-1’s inhibitory activity), and CLB-1C3 (preventing only the fibrin binding of PAI-1).

### DISCUSSION

The first aim of this study has been to localize interaction sites on PAI-1 for plasminogen activators and fibrin by using anti-PAI-1 MoAbs. The corresponding epitopes of a panel of MoAbs, raised by different immunizations, were mapped on PAI-1. Subsequently, the ability of the MoAbs to prevent either inhibition of t-PA/u-PA by PAI-1 or fibrin binding of PAI-1 was established. Both for the interaction of PAI-1 with t-PA (or u-PA) and for the interaction with fibrin, two interaction sites were identified. Each interaction is sustained both by a unique stretch of amino acid residues, ie, typified by MoAb MAI-12 for t-PA/u-PA inhibition and by MoAb M5 for fibrin binding, and by a common amino acid sequence, namely between position 110 and 145. To fully exclude that interference of MoAbs binding to the area between residues 110 and 145 is due to steric hindrance, we are currently performing experiments using peptides derived from this area as competitors for the interaction between either t-PA or fibrin with PAI-1 as well as site-directed mutagenesis to precisely delineate the residues involved. At present, we cannot discriminate whether each of the two interactions act simultaneously or in a sequential manner to enable the formation of stable complexes. In retrospect, our finding that a common area is

<table>
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<tr>
<td>TC3</td>
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Fig 3. Prevention of t-PA inhibition and fibrin binding of PAI-1 by the anti-PAI-1 MoAbs. (A) The MoAbs and the position of their respective binding sites are listed and the results are summarized. t-PA inhibition with 10 μg MoAb: an effect of less than 5% on the inhibition is indicated by the symbol [−], while a decrease of the inhibitory activity is indicated of 65% or more is indicated by [+]. Relative fibrin binding with 10 μg MoAb: no significant effect on the binding (less than 5%) is indicated by the symbol [−], a reduction of PAI-1 binding of 40% to 80% is indicated by (+), while complete prevention (90% to 100%) of PAI-1 binding is indicated by (++). No significant effect (less than 5%) is observed with 10 μg purified antihuman factor Xa IgG. The results are illustrated in (B) and (C). (B) The dose-dependency of the prevention of t-PA inhibition is presented for a set of selected MoAbs (CLB-1C3 [X], CLB-2C8 [O], CLB-5 [+] M2 [*], I1 [V], M5 [E], and MAI-12 [L]). The residual t-PA activity after inhibition with PAI-1 in the presence of increasing amounts of MoAbs is shown. Identical results are obtained with u-PA instead of t-PA. (C) The dose-dependency of the prevention of fibrin binding is given for the same MoAbs as presented in (B). The relative fibrin binding of PAI-1 in the presence of increasing amounts of MoAbs, is shown. Background binding to wells coated with BSA (less than 10% of total binding in the absence of a MoAb) has been subtracted.

Inhibition of PAI-1, released from activated platelets, by anti-PAI-1 MoAbs. To assess the role of PAI-1 in a more physiologic setting, we studied the influence of anti-PAI-1 MoAbs, which prevent PAI-1 activity and fibrin binding in vitro, in a clot-lysis assay. The assay mixture consists of fibrinogen, [125I]-fibrinogen (tracer), plasminogen, and thrombin. The absence of t-PA, no clot-lysis (measured by release of soluble [125I]-fibrin degradation products) can be observed up to 4 hours at 37°C. In contrast, in the presence of t-PA, clot-lysis was completed after 30 minutes of incubation. The presence of an increasing number of freshly isolated human platelets during the clot formation resulted in an increasing clot-lysis resistance (data not shown). Inclusion of increasing amounts of the anti-PAI-1 MoAb CLB-2C8 in a platelet-rich clot resulted in a dose-dependent increase of t-PA-mediated lysis (Fig 4B). Similar results were obtained with the other inhibitory MoAbs CLB-5, M2, I1, and MAI-12 (Fig 4A). The MoAbs CLB-10, CLB-14, CLB-15, and CLB-33 also diminished lysis resistance, but to a lesser extent than the aforementioned MoAbs. Other noninhibitory MoAbs did not effect the rate of clot-lysis even at a 100-fold higher concentration than, eg, MoAb CLB-2C8. Interestingly, MoAb M5, which effectively prevents fibrin binding of PAI-1 but has no influence on the inhibitory activity of PAI-1, promoted t-PA-mediated clot-lysis as well. This observation strongly supports the notion that PAI-1, released from the α-granules of activated platelets, is retained within the clot by binding to fibrin, which is an important determinant for the efficacy of clot lysis.
involved in both t-PA/u-PA inhibition and fibrin binding is of interest to our previously reported data on the fate of fibrin-bound PAI-1 upon addition of u-PA. In that study it is shown that the reactive center of fibrin-bound PAI-1 is not involved in fibrin binding and available to form a stable complex with u-PA. That observation is in accordance with the finding reported here that the MoAb MAI-12, which only prevents the inhibiting activity of PAI-1, does not interfere with its fibrin binding. Upon inhibition, u-PA/ PAI-1 complexes dissociate from fibrin and are encountered in the fluid phase. These complexes do not rebind to fibrin, an observation that may indicate that the "common" area between amino acids 110 and 145 is now required for stable u-PA binding instead of fibrin binding. All MoAbs that prevent the inhibition of t-PA and bind either to the area between 110 and 145 or close to the reactive center equally interfere with the inhibition of u-PA by PAI-1. Therefore, we assume that the interaction between PAI-1 and t-PA is similar to the interaction of the inhibitor with u-PA. Interestingly, on the t-PA molecule, a defined region, distinct from the catalytic triad, has been identified that is crucial for the interaction with PAI-1. Substitution of the basic amino acids lys296, arg298, and arg299 by negatively charged residues renders t-PA virtually resistant to PAI-1. Furthermore, these authors have suggested that the indicated positively charged region on t-PA interacts with the negatively charged glu350 and glu351 residues on PAI-1, located just downstream of the reactive center. With regard to the data presented here, it is conceivable that the complementary negatively charged region on PAI-1 is situated within the area between positions 110 and 145. Indeed, this area harbors a negatively charged stretch, asp125-phe126-ser127-glu128-val129-glu130, which may represent an alternative or additional interaction site for the indicated positively charged region of t-PA. Site-directed mutagenesis of the region between amino acid residues 110 and 145 of PAI-1 is in progress to substantiate this point.

The second aim has been to study the role of PAI-1 released from activated platelets in the regulation of t-PA-mediated clot-lysis. First, it is shown that an increasing number of platelets within the clot results in an increased clot-lysis resistance. This observation agrees with...
the well-documented in vivo situation: platelet-rich clots are associated with thrombolysis resistance, whereas erythrocyte-rich thrombi are relatively readily dissolved. By using anti–PAI-1 MoAbs, which specifically prevent the inhibition of t-PA, we demonstrate that platelet–PAI-1 is a major determinant for clot-lysis resistance. However, it remains to be established whether these MoAbs will also be effective when administered in vivo after formation of a blood clot. Currently, we explore the application of anti-PAI-1 MoAbs as an adjunctive agent for thrombolytic therapy with t-PA.

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The interaction of plasminogen activator inhibitor 1 with plasminogen activators (tissue-type and urokinase-type) and fibrin: localization of interaction sites and physiologic relevance

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