The Distal Hinge of the Reactive Site Loop and Its Proximity

A TARGET TO MODULATE PLASMINOGEN ACTIVATOR INHIBITOR-1 ACTIVITY*

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Ann-Pascale Bijnen‡, Ann Gils§, Jan M. Stassen§, Andrey A. Komissarov§, Isabelle Knockaert‡, Els Brouwers‡, Joseph D. Shore¶, and Paul J. Declerck¶‡

From the ‡Laboratory for Pharmaceutical Biology and Phytopharmacology, Faculty of Pharmaceutical Sciences, Katholieke Universiteit Leuven, Van Eeomstraat 4, B-3000 Leuven, Belgium, the §Laboratory for Pharmaceutical Biology and Phytopharmacology, Boehringer Ingelheim Pharma KG, D-88397 Biberach an der Riss, Germany, and the ¶Division of Biochemical Research, Henry Ford Health Center, Detroit, Michigan 48202

The serpin plasminogen activator inhibitor type 1 (PAI-1) plays a regulatory role in various physiological processes (e.g. fibrinolysis and pericellular proteolysis) and forms a potential target for therapeutic interventions. In this study we identified the epitopes of three PAI-1 inhibitory monoclonal antibodies (MA-44E4, MA-42A2F6, and MA-56A7C10). Differential cross-reactivities of these monoclonals with PAI-1 from different species and sequence alignments between these PAI-1s, combined with the three-dimensional structure, revealed several charged residues as possible candidates to contribute to the respective epitopes. The production, characterization, and subsequent evaluation of a variety of alanine mutants using surface plasmon resonance revealed that the residues His185, Arg186, and Arg187 formed the major sites of interaction for MA-44E4. In contrast, the epitopes of MA-42A2F6 and MA-56A7C10 were found to be conformational. The epitope of MA-42A2F6 comprises residues Lys243 and Glu350, whereas the epitope of MA-56A7C10 comprises residues Glu242, Lys244, Glu350, Asp355, and Arg356. The participation of Glu350, Asp355, and Arg356 provides a molecular explanation for the differential exposure of this epitope in the different conformations of PAI-1 and for the effect of these antibodies on the kinetics of the formation of the initial PAI-1-proteinase complexes. The localization of the epitopes of MA-44E4, MA-42A2F6, and MA-56A7C10 elucidates two previously unidentified molecular mechanisms to modulate PAI-1 activity and opens new perspectives for the rational development of PAI-1 neutralizing compounds.

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§ On whom correspondence should be addressed. Tel.: 32-16-323431; Fax: 32-16-323460; E-mail: paul.declerc@farm.kuleuven.ac.be.

¶ The abbreviations used are: PAI-1, plasminogen activator inhibitor type 1; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; PCR, polymerase chain reaction; wt, wild type; stab, stable variant; PAB, p-aminobenzamidine.

Plasminogen activator inhibitor type 1 (PAI-1), a member of the serpin (serine proteinase inhibitor) superfamily (1–4), controls the plasminogen system at the level of tissue-type and urokinase-type plasminogen activator (t-PA and u-PA, respectively). Because PAI-1 is the main physiological inhibitor of t-PA in plasma (5), increased levels of PAI-1 result in a hypofibrinolytic state and are correlated with various vascular disorders such as venous thrombo-embolism, coronary artery disease, myocardial infarction, and atherosclerosis (6–9). The u-PA-inhibiting effect of PAI-1 has its main physiological implications in processes outside of the circulation (10, 11).

PAI-1 is a unique serpin because of its functional and conformational flexibility (12). PAI-1, synthesized as an active molecule, converts spontaneously into a nonreactive, latent form, which can be partially reactivated by denaturing reagents (13). Additionally, a third distinct, noninhibitory form (substrate) is identified that is reactive toward its target proteinases without the formation of a stable complex (14–16).

Elucidation of the three-dimensional structure of active PAI-1 (17, 18) reveals that the N-terminal side of the reactive site loop (extended from P16 to P3’ and including the bait peptide bond Arg345–Met346 (P1-P1’)) is exposed and accessible for the target proteinase. The C-terminal side of the reactive site loop (P4’-P13’) forms strand s1C in β-sheet C.

Conversion to the latent state implies the insertion of the N-terminal side of the reactive site loop into β-sheet A, the loss of strand s1C from β-sheet C, and the formation of an unusual extended loop by the C-terminal side of the reactive site loop, resulting in the distortion of the PAI-1’ “bait” peptide bond (19). It is hypothesized that the active-to-latent conversion involves the movement of the C-terminal side of the reactive site loop through a gap formed by two surface-exposed loops, i.e. loop 1 (residues 185–200, connecting strand s4C with s3C) and loop 2 (residues 242–246, connecting strand s3B with helix hG) (19–21). In previous studies, the unique flexibility of loop 1, in combination with the presence of charged residues (20, 22), has been assumed to contribute to the mobility of the reactive site loop in PAI-1.

Previously, we have characterized a panel of monoclonal antibodies with PAI-1 inhibitory properties (23, 24). For MA-44E4 the major interaction sites were suggested to be located within a segment from residue 81 to 187 comprising helices hD, hE, and hF, β-strands s1A, s2A, s3A, and s4C and the loops connecting these elements. The major determinants for the binding of MA-42A2F6 and MA-56A7C10 were suggested to be located C-terminal from residue 327 in a region containing the reactive site loop.

In the present study, the evaluation of the differential reactivity of these PAI-1 inhibitory monoclonal antibodies with PAI-1 from different species and in various conformations, in combination with sequence alignments and the analysis of the three-dimensional structure of PAI-1, is used to identify the major residues involved in the binding of these inhibitory an-
tibodies. In addition, alanine-scanning mutagenesis is used to confirm the contribution of surface-exposed charged residues located in the vicinity of or comprising residues in the distal hinge of the reactive site loop. Even though the possible contribution of other noncharged residues cannot be excluded when using this approach, it is generally accepted that charged residues form the major interaction sites governing the specificity of the interaction between an antigen and an antibody (25).

The localization of two previously unknown epitopes reveals new target regions to modulate PAI-1 activity. Furthermore, the localization of the epitopes of MA-42A2F6 and MA-56A7C10 forms a molecular explanation for the differential reactivity of these antibodies with the different conformations of PAI-1.

**EXPERIMENTAL PROCEDURES**

**Materials—PfuTurbo** DNA polymerase was purchased from Stratagene (La Jolla, CA). Synthetic oligonucleotides (for PCR and DNA sequencing) were synthesized by Amersham Pharmacia Biotech (Uppsala, Sweden). The vectors pIGE20-humanPAI-1 and pIGE20-PAI-1-stab were constructed as described previously (26, 57). pGE20 containing a heat-inducible promoter, pAel, encoding a thermolabile replicator and the Escherichia coli strains DH11 and MC1061 used for cloning and expression, respectively, were kindly provided by Innogenetics (Ghent, Belgium). Luria Bertani broth (LB) growth medium was purchased from Life Technologies, Inc. Tissue-type plasminogen activator (Actilase) from Life Technologies, Inc. Tissue-type plasminogen activator (Actilase) was produced and purified as described previously (26–29). Most chemical reagents including diithiothreitol and the protease inhibitors leupeptin, phenylmethanesulfonyl fluoride, pepstatin, benzamidine hydrochloride, and antipain were from Sigma. SP-Sepharose® Fast Flow and heparin-Sepharose® CL-4B were purchased from Pharmacia Biotech. The recombinant monomeric antibodies MA-42A2F6, MA-14E4, and MA-56A7C10, directed against human PAI-1 (humPAI-1), were produced and purified as described previously (23).

**General DNA Techniques—DNA manipulation techniques were carried out according to standard procedures and following the instructions of the manufacturers. Plasmid DNA was isolated using Nucleobond cartriges ( Machery-Nagel, Düren, Germany). DNA fragments were purified using the QIAquick® gel extraction kit (Qiagen GmbH, Hilden, Germany), and PCR was performed using the GeneAmp® 2400 (PerkinElmer Life Sciences). DNA was sequenced with the Autoread Sequencing kit and the Automated Laser Fluorescent ALF® apparatus (both from Amersham Pharmacia Biotech).

**Construction of PAI-1 Mutants—**All mutants were created using a method based on the QuickChange™ site-directed mutagenesis kit from Stratagene. Therefore, pIGE20-PAI-1-wt (26) and pIGE20-PAI-1-stab (27) were used as template to introduce mutations in PAI-1-wt and in PAI-1-stab, respectively. PCR was performed using 2.5 units of PfuTurbo** DNA polymerase, 50 ng of template, 125 ng of each primer, and 0.2 mmol of each dNTP in 50 μl of buffer containing 10 mm KC1, 10 mm (NH4)2SO4, 20 μM Tris-HCl (pH 8.8), 2 mM MgSO4, 0.1% Triton X-100, and 100 μg/ml nuclelease-free bovine serum albumin. After an initial DNA denaturation step (95 °C, 30 s), 16 PCR cycles were performed (95 °C, 30 s; 55 °C, 60 s; 68 °C, 12 min). Subsequently, DNA was subjected to a DpnI digestion prior to transformation of DH11 E. coli. For all mutants, large scale DNA preparations were made, and the PAI-1 encoding region was sequenced entirely.

**Expression and Purification of PAI-1-wt and PAI-1 Mutants—**PAI-1-wt and PAI-1 mutants were expressed in MC1061 E. coli cells using a method described previously (26). In brief, competent MC1061 cells were cotransformed with pAc1 and either pIGE20-PAI-1-wt or one of the pIGE20-PAI-1 mutant constructs. Clonal isolates were grown and the PAI-1 containing supernatant was collected and immediately subjected to purification as described previously (30).

After evaluation of PAI-1 content and purity, PAI-1-containing elution fractions were examined for their inhibitory activity toward t-PA by SDS-polyacrylamide gel electrophoresis. Fractions containing the highest proportion of functionally active PAI-1 were pooled and used for further experiments.

**Determination of the Functional Distribution of PAI-1-wt and PAI-1 Mutants—**Samples of PAI-1-wt and PAI-1 mutants were diluted on ice to a final PAI-1 protein concentration between 60 and 360 μg/ml with 0.02 M Tris-acetate buffer with diluent solution to 0.02 mol NaHPO4, 70 mM NaCl, pH 7.2–7.4. To determine the functional distribution, aliquots were incubated immediately with a 2-fold molar excess of t-PA at 37 °C for 30 min. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis using 10–15% gels followed by Coomassie Brilliant Blue staining. Quantitation of the formed reaction products (complexed, nonreactive, and cleaved, corresponding to the presence of active, latent, and substrate conformation, respectively) was done by densitometric scanning of the gels using the Imagemaster™ system (Amersham Pharmacia Biotech) (26).

**PAI-1 Neutralization Assay—**Residual PAI-1 activity was quantitated (31) after preincubation of human PAI-1 with monoclonal antibody. Briefly, 100 μl of PAI-1 (50 ng/ml with an activity of 80% of the theoretical maximal value) was incubated with 100 μl of serial 2-fold dilutions of the antibodies (MA-44E4, MA-42A2F6, and MA-56A7C10) ranging from 0.125- to 16-fold molar excess over total PAI-1. The reaction mixture was allowed to react for 2 h at room temperature. 50-μl samples of this reaction mixture were incubated with 50 μl of t-PA (20 IU/ml) at 37 °C for 15 min in the wells of a microtiter plate. Then, 100 μl of fluorogenic substrate (0.04% NBD-labeled fibrinogen, 1 μM) and 100 μl of substrate (KH2PO4-NaHPO4, 70 mM NaCl, pH 7.4) were added. The residual plasminogen activator activity was measured by recording the absorbance change at 405 nm. 100% PAI-1 activity was defined as the PAI-1 activity observed in the absence of monoclonal antibody. The percentage inhibition by the monoclonal antibody was then calculated from the residual PAI-1 activity measured in the presence of the monoclonal antibody.

**Effect of Monoclonal Antibodies on the Reaction Products Generated during Interaction of PAI-1 and t-PA—**PAI-1-wt was diluted in phosphate-buffered saline to a concentration of 0.2 mg/ml and incubated for 10 min at 37 °C with either phosphate-buffered saline or a 3-fold molar excess of monoclonal antibody. The samples were further incubated with a 2-fold molar excess of t-PA for 2 min at 37 °C. The reaction was terminated by adding SDS (final concentration of 1%) and heating for 30 s at 100 °C. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis using 10–15% gels followed by Coomassie Brilliant Blue staining. Quantitation of the formed reaction products (complexed, nonreactive, and cleaved, corresponding to the presence of active, nonreactive, and substrate conformation, respectively) was done by densitometric scanning of the gels using the Imagemaster™ system (Amersham Pharmacia Biotech) (26).

**Affinity of monoclonal Antibodies with PAI-1-wt and PAI-1 Mutants—**Affinity constants for the binding between monoclonal antibodies and PAI-1-wt and PAI-1 mutants were determined using the BIAcore™ 3000 analytical system equipped with the CM5 sensor chip (BIAcore AB) as described previously (32). In brief, the monoclonal antibodies were coupled covalently to 2000 resonance units (using a concentration of 10 μg/ml in 10 mM acetate buffer, pH 4.5) using the automatic Wizard mode. Subsequently, PAI-1 variants diluted in 0.01 M Hepes, 0.15 M NaCl, 3 mM EDTA, 0.005% Tween 20, pH 7.4 to a final PAI-1 antigen concentration ranging from 18 to 1200 nM were injected at a flow rate of 20 μl/min (injection volume is 40 μl). After each cycle the chip was regenerated using 10 μl of a 15 mM HCl solution. The analyses of the association and dissociation phases were made with the software of the BIAcore™ 3000 (Langmuir binding, local, or global fit). **Isolation of the Latent and Substrate Conformation of PAI-1—**Isolation of the latent and substrate conformation of PAI-1-wt was performed as described previously (14). In brief, active PAI-1-wt was converted into the latent form by incubation at 37 °C for 24 h. To separate the latent from the substrate conformation, the inactivated sample was applied to immobilized t-PA-S478A; the nonbinding fraction (i.e., the latent conformation) was collected, and bound PAI-1 (i.e., the substrate conformation) was eluted using a 0.1 M acetic acid buffer containing 2 mM glutathione and 1.5 M NaCl, pH 5.5.

**Effect of Monoclonal Antibodies on the Rate of PAI-1-Proteinase Complex Formation—**The displacement of p-aminobenzimidazolene (PAB), a fluorescent probe inhibitor of serine proteinases (33), was used to determine the effect of monoclonal antibody binding on formation of the initial PAI-1-proteinase complexes. p-PA (0.08–0.22 μM) or t-PA (0.15–1.1 μM) were preincubated with PAB (100 and 180–400 μM, respectively) for 10 min at 25 °C in 0.1 M Hepes/NaOH (pH 7.4) containing 0.1 M NaCl. PAI-1 (1.6 μM) or its complex with each monoclonal antibody, when reacted with the PAB-proteinase complex, causes a decrease in
fluorescence emission of the PAB because of its displacement from the active site of the enzyme (34). The reactions were carried out using an SX-18MV microvolume stopped-flow reaction analyzer (Applied Photophysics Ltd.) equipped with a fluorescence detector or an SLM 8000 spectrofluorimeter for slower reactions. Excitation wavelengths were 320 and 330 nm for u-PA and t-PA, respectively. Progress of the PAI-1-protease reaction was monitored by changes in the fluorescence emission intensity at 360 nm for u-PA and 375 nm for t-PA in the SLM 8000 or through 335 nm cutoff filter in the stopped-flow instrument. Data were analyzed by fitting to a single exponential process from which the values of $k_{on}$ were calculated. The second order rate constants ($k_{on}$) for binding of u-PA and t-PA to PAI-1 were determined from the slope of the dependence of $k_{on}$ on PAI-1 concentration, which equals $k_{on}/(1 + ([PA]/K_{PAB}))$ (34), where $K_{PAB}$ is a dissociation constant of the PAB-protease complex. The values of $K_{PAB}$ have been determined by fluorescence titration of proteinase with PAB as described previously (34).

**Statistical Analysis**—The statistical significance of differences was evaluated using Student's *t* test. The two-tailed *p* values are indicated where necessary.

**RESULTS**

**Characterization of PAI-1 Inhibitory Monoclonal Antibodies**—In a PAI-1 neutralization assay, MA-44E4, MA-42A2F6, and MA-56A7C10 inhibited PAI-1 activity in a dose-dependent manner revealing that MA-56A7C10 is the most potent inhibitor (i.e. 80 ± 5% inhibition using a 2-fold molar excess of MA versus 21 ± 6% and 15 ± 6% for MA-42A2F6 and MA-44E4, respectively). At a 128-fold molar excess, PAI-1 inhibition exceeding 85% is observed for all three antibodies.

When evaluating the influence of MA-44E4, MA-42A2F6, and MA-56A7C10 on the reaction products generated during interaction between PAI-1 and t-PA, a significant ($p < 0.005$) transition to a nonreactive form was observed for all three monoclonal antibodies (Table I). Again, MA-56A7C10 was revealed as the most potent inhibitor of PAI-1 activity.

Moreover, when a substrate variant of PAI-1 (i.e. PAI-1-P12 (Ala at position 335 replaced by a Pro (35))) was incubated with these monoclonal antibodies prior to incubation with t-PA, the cleavage of the substrate form was significantly ($p < 0.0001$) hindered (i.e. 59 ± 0.8, 46 ± 0.9, and 70 ± 0.7% nonreactive material in the presence of MA-44E4, MA-42A2F6, and MA-56A7C10, respectively, *versus* 14 ± 1.4% nonreactive material in the absence of antibody).

**Affinity of Monoclonal Antibodies with PAI-1 from Different Species and Amino Acid Sequence Alignments**—MA-44E4 binds human PAI-1 with a $K_{D}$ of $4 \times 10^{-8}$ M$^{-1}$ but lacks affinity (i.e. $K_{D}$ at least 400-fold less) for porcine, murine, and rat PAI-1 (Table II). Alignment of the sequence of human PAI-1 between residue 81 and 197, the region previously suggested to harbor the major interaction sites for MA-44E4 (24), with the corresponding segment of the latter three species reveals the presence of only two charged residues, i.e. Asp$^{181}$ and Arg$^{186}$, that are not conserved in any of the other species. Consequently, we hypothesized that one or both of these residues play an essential role in the interaction between human PAI-1 and MA-44E4.

The $K_{D}$ values of MA-42A2F6 and MA-56A7C10 for human PAI-1 are $3.8 \times 10^{-9}$ and $1.3 \times 10^{-9}$ m$^{-1}$, respectively (Table II). The affinity constants of both antibodies for porcine PAI-1 are reduced 6- and 9-fold, respectively, and no binding to murine or rat PAI-1 was observed. In the stretch of residues in human PAI-1 C-terminal from position 327 (24), only one charged amino acid in human PAI-1, Glu$^{350}$, differs from the corresponding residues in murine and rat PAI-1, whereas it is conserved in porcine PAI-1. Therefore, we hypothesized that Glu$^{350}$ is involved in the interaction of PAI-1 with MA-42A2F6 and MA-56A7C10.

Localization of residues Asp$^{181}$, Arg$^{186}$, and Glu$^{350}$ in the three-dimensional structure of human PAI-1 (17) reveals that they are all surface-exposed in a region close to or in the distal hinge of the reactive site loop. To investigate whether these residues contribute to the epitope of MA-44E4, MA-42A2F6, or MA-56A7C10, it was decided to produce alanine mutants at these positions (i.e. PAI-1-D181A, Asp$^{181}$ to Ala; PAI-1-R186A, Arg$^{186}$ to Ala; PAI-1-E350A, Glu$^{350}$ to Ala). Nine other charged residues (i.e. His$^{185}$, Arg$^{187}$, His$^{190}$, Lys$^{191}$, Glu$^{242}$, Lys$^{243}$, Glu$^{244}$, Asp$^{255}$, and Arg$^{356}$) were found within a radius of 20 Å of the Asp$^{181}$, Arg$^{186}$, Glu$^{350}$ cluster in the three-dimensional structure of active human PAI-1. To study the possible contribution of these residues to the epitopes, the corresponding alanine mutants were generated (PAI-1-H185A, His$^{185}$ to Ala; PAI-1-R187A, Arg$^{187}$ to Ala; PAI-1-H190A, His$^{190}$ to Ala; PAI-1-K191A, Lys$^{191}$ to Ala; PAI-1-E242A, Lys$^{242}$ to Ala; PAI-1-K243A, Lys$^{243}$ to Ala; PAI-1-E244A, Glu$^{244}$ to Ala; PAI-1-D355A, Asp$^{355}$ to Ala; and PAI-1-R356A, Arg$^{356}$ to Ala). To further investigate the impact of several alanine substitutions four double mutants (PAI-1-H185A/R186A, PAI-1-R186A/R187A, PAI-1-H190A/K191A, PAI-1-D355A/R356A), two triple mutants (PAI-1-H185A/R186A/R187A, PAI-1-E242A/K243A/E244A), and one quadruple mutant (PAI-1-E242A/K243A/E244A/E350A) were also created, expressed, and isolated.

**Binding of Monoclonal Antibodies to Different Conformations of Human PAI-1**—MA-44E4 reacts with active, latent, and substrate conformations of human PAI-1 with comparable affinity (Table III). For MA-42A2F6 and MA-56A7C10, the affinity constants indicate that both antibodies react preferentially with PAI-1 in the active conformation. The affinity of the latter antibodies for the substrate conformation is reduced 9- and 5-fold, respectively, and the affinity for the latent form is decreased 56- and 25-fold, respectively. For both antibodies the affinity constants for the stable variant is similar to that for human PAI-1.

**Characterization of the PAI-1 Mutants**—Table IV summarizes the conformational distribution of all PAI-1 mutants studied in comparison with PAI-1-wt. For 7 of 13 single mutants (Table IV), inhibitory activity toward t-PA and the ratios between the various conformations were similar to those for PAI-1-wt, i.e. 68–85% occurred in the active form, 9.4–22% in the latent form, and 3.0–12% in the substrate conformation. However, alanine mutations at positions 187, 190, 191, 193, 355, and 356 caused a reduction in PAI-1 activity ($\leq 52\%$) together with an increase of nonreactive PAI-1 (latency $\geq 41\%$). As expected, the destabilizing effect of the latter mutations was also observed if the mutations were combined with adjacent alanine mutations (Table IV). These data are fully compatible with previous studies demonstrating that the charged residues in these regions contribute to the stability of PAI-1 (22, 36). Moreover, in previous studies (22, 36) it was shown that the nonreactive form of these mutants is the latent form. The destabilizing effect of some alanine mutations compromised the interpretation of the affinity data of MA-42A2F6 and MA-56A7C10 toward the respective mutants, because both monoclonal antibodies exhibit a reduced affinity for the latent conformation. Therefore, identical mutations were introduced in PAI-1-stab. Comparison of the conformational distribution of the corresponding mutants introduced in PAI-1-wt or PAI-1-wt.
The affinity of MA-44E4 is completely lost by any combination for PAI-1-wt, Table V. The data in Table V demonstrate that bind to PAI-1-H185A/R186A/R187A (p = 0.0005) and PAI-1-R187A (p = 0.0002) of MA-44E4, whereas the single mutations at positions 185 and 187 had a less pronounced effect on the affinity of MA-44E4. In contrast, MA-44E4 did not that for PAI-1-wt, indicating that this residue is not involved in the interaction with MA-44E4. In view of data obtained in previous studies (24) excluding the major contribution of residues beyond position 187 for MA-44E4, and in view of the modest reduction (6-7-fold) in affinity of the double mutants H190A/K191A and D355A/R356A, the residues at these positions (190, 191, 355, and 356) should not be considered the dominant residues in the epitope of MA-44E4.

Localization of the Residues Contributing to the Epitope of MA-44E4 Based on Changes in Affinity for PAI-1 Mutants—The affinities of MA-42A2F6 and MA-56A7C10 for PAI-1-wt were 4 \times 10^8 and 1 \times 10^9 M^{-1} respectively. From the data shown in Table VI it can be deduced that the affinity of MA-42A2F6 and MA-56A7C10 for PAI-1-wt is reduced 8-fold compared with that of MA-44E4.

Localization of the Residues Contributing to the Epitopes of MA-42A2F6 and MA-56A7C10 Based on Changes in Affinity for PAI-1 Mutants—The affinities of MA-42A2F6 and MA-56A7C10 for PAI-1-wt were 4 \times 10^8 and 1 \times 10^9 M^{-1} respectively. From the data shown in Table VI it can be deduced that the affinity of MA-42A2F6 and MA-56A7C10 for PAI-1-wt is reduced 8-fold compared with that of MA-44E4.

Table II

| PAI-1 variant | Kₐ (M⁻¹) |
|--------------|-----------|
| PAI-1-wt     |           |
| Active       | MA-44E4   | 82 ± 2.1 |
|              | MA-42A2F6 | 78 ± 5.3 |
|              | MA-56A7C10| 76 ± 2.8 |
|              |           | 8 ± 0.2  |

Table III

| PAI-1 variant | Kₐ (M⁻¹) |
|--------------|-----------|
|              |           |
|              | Active    | MA-44E4   | 4.2 ± 1.2 \times 10^8 |
|              |           | MA-42A2F6 | 3.8 ± 0.9 \times 10^8 |
|              |           | MA-56A7C10| 6.6 ± 0.0 \times 10^8 |

Table IV

| PAI-1 variant | Conformation' |
|--------------|---------------|
|              | Active        | Latent |
|              | MA-44E4       | MA-42A2F6 | MA-56A7C10 |
|              |             |           |            |
|              |             |           |            |
|              |             |           |            |

The amino acids are represented using one-letter codes. The charged amino acids in human PAI-1 are indicated in red. The residues in porcine, rat, and murine PAI-1 that differ from the corresponding position in human PAI-1 are indicated in cyan blue. Charged residues hypothesized to explain the differential species reactivity are indicated.

* Mean ± S.D. (n = 2–6); NB, no binding (Kₐ ≤ 10⁶ M⁻¹).

** Affinity constants (Kₐ) of MA-44E4 and of MA-42A2F6 and MA-56A7C10, combined with the amino acid (AA) sequence alignment between AA²⁸⁷ and AA³⁵⁷ and between AA²⁵⁷ and AA³⁷³.

### Table II

| PAI-1 variant | Kₐ (M⁻¹) |
|--------------|-----------|
|              |           |
|              |           |
|              |           |

### Table III

| PAI-1 variant | Kₐ (M⁻¹) |
|--------------|-----------|
|              |           |
|              |           |
|              |           |

### Table IV

| PAI-1 variant | Conformation' |
|--------------|---------------|
|              | Active        | Latent |
|              | MA-44E4       | MA-42A2F6 | MA-56A7C10 |
|              |             |           |            |
|              |             |           |            |
|              |             |           |            |
Asp355, and/or Arg356.

K191A and PAI-1-stab-D355A/R356A, the data do not allow an
interpretation of the changes in affinities observed for the various
alanine mutant variants of PAI-1 (Table VI) was possible,
cannot be interpreted unambiguously, because of their in-
creased latency.

To determine whether the reduced affinity (versus that of
PAI-1-wt) of both antibodies for the latent PAI-1 mutants is a
result of the increased latency rather than of the mutation, the
affinities of MA-42A2F6 and MA-56A7C10 were determined for the
corresponding mutant-stabs in PAI-1-stab (Table VI). The mut-
ant PAI-1-stab-E350A served as a control. The relatively un-
changed affinities of both antibodies for PAI-1-stab-H185A/
R186A/R187A and PAI-1-stab-D193A (versus PAI-1-stab)
indicate that these residues do not contribute to the epitope of
these antibodies. On the other hand, the data further confirm
the possible role of residue Glu350 (p < 0.0001 versus value for
PAI-1-stab). Because of the latency of PAI-1-stab-H190A/
K191A and PAI-1-stab-D355A/R356A, the data do not allow an
unambiguous interpretation for the role of His190, Lys191,
Asp350, and/or Arg356.

To confirm that MA-42A2F6 and MA-56A7C10 interact si-
multaneously with Glu242, Lys243, Glu244, and Glu350, the
corresponding mutations were combined in the mutant PAI-1-
E242A/K243A/E244A/E350A. The data in Table VI reveal that
MA-42A2F6 and MA-56A7C10 react 122- and 76-fold less, re-
spectively, with PAI-1-E242A/K243A/E244A/E350A (p <
0.002 versus value for PAI-1-wt).

To investigate which residues in the 242–244 and 355–356
regions play the most important role in the epitopes of MA-
42A2F6 and MA-56A7C10, the reactivity of these monoclonal
antibodies was determined for a set of PAI-1 variants in which
a single residue was substituted with alanine. The data in Table VI confirm the major contribution of Glu242, Lys243, and
Glu244 to the epitope of MA-56A7C10 (p < 0.005) and of Lys243
to the epitope of MA-42A2F6 (p < 0.0002). Because of the
intrinsic latency of PAI-1-D355A, the importance or the ab-
sence of contribution of Asp355 cannot be deduced unambigu-
ously. However, the importance of Arg356 to the epitope of
MA-56A7C10 can be confirmed. Moreover, because the double
mutant PAI-1-D355A/R356A reveals at least a 10-fold reduc-
tion (Kd ≤ 106 m−1) in binding to MA-56A7C10 compared with
the single mutant PAI-1-R356A (Kd = 5.4 ± 0.3 107 m−1), it is
most likely that Asp355 contributes to the epitope of
MA-56A7C10.

Effect of Monoclonal Antibodies on the Rate of PAI-1-Protein-
ase Complex Formation—The proposed epitope localization
indicates that monoclonal antibody binding might impair the
PAI-1-proteinase complex formation through interference with
the residues involved in the formation of the initial, reversible
complex. To test this hypothesis, the effect of the monoclonal
antibodies on the rate of PAI-1-proteinase complex formation
was studied. The dependencies of kobs on PAI-1 concentration
were linear for both proteinases (0–2.5 μM PAI-1), and the
values of k1 for the reactions with t-PA and u-PA (4.5 ± 0.3
and 1.9 ± 0.3 107 m−1 s−1, respectively) indicate that the initial
complex formation is diffusion-limited. Therefore, under the
conditions used, kobs for the displacement of PAB by PAI-1 was
35 ± 2 and 2.2 ± 0.4 s−1 for t-PA and u-PA, respectively (Fig.
1). However, the presence of monoclonal antibodies induced a
significant decrease in the rate of the PAB displacement (Fig.
1). The values of kobs were decreased by 500–900 and 4,500–
6,500-fold with MA-44E4 and MA-42A2F6 for u-PA and t-PA,
respectively, and the reaction was completely prevented (kobs <
10−10 s−1) by MA-56A7C10. The considerable reduction of the
rate constant for displacement of PAB is due to a considerable
decrease in the reactivity of proteinases to PAI-1 monoclonal
antibody complexes compared with free PAI-1.

DISCUSSION

In this study two non-overlapping epitopes of PAI-1 inhibi-
tory antibodies are identified in close proximity to and in part
comprising residues of the distal hinge of the reactive site loop.
For MA-44E4, an antibody that reacts equally well with the
active, latent, and substrate forms of human PAI-1-wt, our
current approach allowed us to identify unambiguously three
amino acids as the major molecular determinants in its (linear)
epitope, i.e. His186, Arg186, and Arg187. Although all these res-
ides appear to be involved in the interaction with the anti-
body, the contribution of Arg186 to the epitope far exceeds the
importance of its two adjacent residues, an observation that is
fully compatible with the sequence alignments versus species reactivity (Table II). The residues are located in the loop con-
necting s3C with s4C (designated loop 1 (20), are surface-
exposed, and are in proximity to the distal hinge of the reactive
site loop (Fig. 2).

In contrast to MA-44E4, both MA-42A2F6 and MA-56A7C10
exhibited a differential reactivity toward the different confor-
mations of human PAI-1. Therefore no straightforward inter-
pretation of the changes in affinities observed for the various
alanine mutant variants of PAI-1 (Table VI) was possible,
because of alterations in distribution of these conformations. Therefore, we also investigated the interactions of these monoclonal antibodies with corresponding stabilized mutants. Detailed characterization of any mutant used for epitope analysis is a necessity, because observed decreases in affinities may result from the indirect effects of mutations remote from the molecular basis for the observed differential affinity of these epitopes of antibodies with inhibitory properties toward PAI-1 that are distinct from the affinities for the active and latent conformation. This finding further substantiates the belief that (subtle) conformational differences in the initial conformation contribute to the underlying mechanism of the differences between inhibitory and cleavage pathways (12, 14–16).

Even though located in the proximity of previously reported epitopes of antibodies with inhibitory properties toward PAI-1 or other serpins, the currently identified epitopes are clearly distinct. Using overlapping peptides, Perrie et al. (44) demonstrated that ESPI-12 recognized an epitope spanning residues 342–349 of PAI-1 (P5-P3′), including the bait peptide bond, thereby explaining the inhibitory properties of the antibody through a direct interaction with the reactive center residue. The binding region of MAI-12 (same as MA-7D4B7 (45)) has been suggested as being situated between amino acid residues 320 and 379 of PAI-1 (46) and is overlapping or possibly identical to that of ESPI-12 (47). Several monoclonal antibodies inducing substrate behavior of PAI-1 were mapped to the region of α-helix F (30, 48–51). The epitope of CB5B10 was localized in α-helix E and the turn connecting helix E and strand s1A (52). Epitope mapping of the inhibitory anti-rat PAI-1 MA-124K1 revealed the major contribution of residues Glu312 and Glu220 localized on strand s1B and s2B (53). For the monoclonal antibodies I-201 (noninhibitory) and M-5 (inhibitory), the dominant residues were found to be Gln36 (located in helix C) and Asp381 (ts3As4C), respectively (49). On the other
hand, Asakura et al. (54, 55) identified the epitope of an inhibitory antibody in the reactive site loop of antithrombin III (i.e., between residues 382 and 386, which corresponds to the positions P8–P13 in the proximal hinge of the reactive site loop). Thus, none of the previously reported epitopes appears to be similar to the epitopes of the currently studied antibodies.

Previously, the surface-exposed and highly flexible loop 1 (19, 20) has been the subject of various structure-function studies and has been shown to play an important role in the conformational changes that accompany the conversion from the active to latent form (21, 22). It has been suggested subsequently that loop 1 might be a target region to modulate PAI-1 activity (22). The current localization of the epitope for MA-44E4 provides the first proof that targeting this particular region results in the inactivation of PAI-1. The latter hypothesis is therefore likely to form the molecular basis for the inhibitory effect of MA-44E4.

Previously, the residues Asp355 (P9̄), Glu350 (P4̄), and Glu351 (P5̄) have been suggested to be involved in secondary interactions between PAI-1 and t-PA, which stabilize the initially formed, reversible complex (56, 57). Consequently, binding of MA-42A2F6 or MA-56A7C10 to the distal hinge of the reactive site loop comprising these residues is likely to provide a molecular explanation for the PAI-1 inhibitory properties of these antibodies. Indeed, binding of the antibodies might prevent the target proteinase from forming an initial complex with PAI-1 because of a direct competition for this binding site.

The results of PAB displacement experiments clearly underscore this particular mechanism because both antibodies interfere with this initial complex formation. It is worth mentioning that MA-56A7C10 virtually completely blocks PAB displacement from the proteinase active site, providing convincing evidence for the hypothesized molecular mechanism of inactivation in combination with the particular epitope localization. MA-42A2F6 has a considerable effect but not as pronounced as that of MA-56A7C10. This difference could be due either to the lower affinity (~3.4-fold) or to differences in the relative contribution of residues 242–244 and the residues in the distal hinge region. The observed effect of MA-44E4 is most likely because of an indirect steric hindrance.

In conclusion, we have assigned the major determinants for non-overlapping epitopes of PAI-1 inhibitory antibodies in the vicinity of or comprising residues of the distal hinge of the reactive site loop, thereby revealing the molecular basis for the differential exposure of one of the identified epitopes in the different conformations of PAI-1. Furthermore, the localization of the epitopes elucidates two previously unknown mechanisms for the modulation of PAI-1 activity and opens new perspectives for the rational development of PAI-1 inhibitory compounds.

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The Distal Hinge of the Reactive Site Loop and Its Proximity: A TARGET TO MODULATE PLASMINOGEN ACTIVATOR INHIBITOR-1 ACTIVITY
Ann-Pascale Bijnens, Ann Gils, Jan M. Stassen, Andrey A. Komissarov, Isabelle Knockaert, Els Brouwers, Joseph D. Shore and Paul J. Declerck

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