Importance of the Hinge Region between α-Helix F and the Main Part of Serpins, Based upon Identification of the Epitope of Plasminogen Activator Inhibitor Type 1 Neutralizing Antibodies*

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The serpin plasminogen activator inhibitor type 1 (PAI-1) is an important protein in the regulation of fibrinolysis and inhibits its target proteinases through formation of a covalent complex. In the present study, we have identified the epitope of two PAI-1-neutralizing monoclonal antibodies (MA-33H1F7 and MA-55F4C12). Based upon differential cross-reactivity data of these monoclonals with PAI-1 from different species and on a sequence alignment between these PAI-1s, combined with the three-dimensional structure, we predicted that the residues Glu128, Val129, Glu130, Arg131, and Lys154 (at the hinge region between α-helix F and the main part of the PAI-1-molecule) might form the major site of interaction. Therefore a variety of alanine mutants were generated and evaluated for their affinity toward both monoclonal antibodies. The affinity constants of MA-55F4C12 and MA-33H1F7 for PAI-1 were 2.7 ± 1.6 × 10^9 M⁻¹ and 5.4 ± 1.7 × 10⁸ M⁻¹, respectively, but decreased between 15- and 270-fold upon mutation of Lys154 to Ala or Glu128, Val129, Glu130, Arg131 to Ala-Ala-Ala-Ala. The combined mutations (PAI-1-EVER/K), however, resulted in an absence of binding to either of the antibodies. Both antibodies bound to PAI-1-wt/t-PA complexes with a similar affinity as to PAI-1-wt (Kᵦ = 4.5 × 10⁹ M⁻¹). The epitope localization reveals the molecular basis for the neutralizing properties of both monoclonal antibodies. In addition, it provides new insights into the validity of various models that have been proposed for the serpin/proteinase complex, excluding full insertion of the reactive-site loop.

Plasminogen activator inhibitor type 1 (PAI-1), 1 a member of the serine proteinase inhibitor (serpin) superfamily (1–4) is an important protein in the regulation of fibrinolysis. PAI-1 is the most important physiological inhibitor of tissue-type plasminogen activator (t-PA) in plasma (5).

PAI-1 is unique among the serpins because of its functional and conformational flexibility. The active conformation of PAI-1 inhibits its target proteinases by the formation of a stable, inactive complex. After the formation of an initial, reversible Michaelis-like complex, the proteinase cleaves the active site of PAI-1 and forms a stable, covalent complex resulting in the inactivation of the proteinase (6, 7). The structure of a covalent complex between PAI-1 and t-PA in particular, or between a serpin and its target proteinase in general, is presently unknown. However, two different models have been proposed. According to one model, the proteinase moves after the initial attack to the opposite pole of the serpin, thereby resulting in a complete insertion of the N-terminal side of the reactive-site loop (7–9). Alternatively, it was hypothesized that movement of the proteinase following the initial proteinase/serpin interaction is less extended, yielding a complex in which the N-terminal side of the reactive-site loop is only partially inserted (10, 11), accompanied by a distortion of the catalytic triad of the proteinase (6) and stabilized by multiple interactions between serpin and proteinase.

Although PAI-1 is synthesized as an active molecule, it converts spontaneously to an inactive, latent form that can be partially reactivated by denaturing agents (12). In this latent conformation, the active site is inaccessible for the target proteinases as a result of the insertion of the N-terminal side of the reactive-site loop in β-sheet A of the PAI-1 molecule (13). In addition, a third conformation with substrate properties has been identified (14–16). This form of PAI-1 reacts with t-PA or u-PA, resulting in a cleavage of the active site of PAI-1 but without the formation of a covalent complex (17).

Previously, we have characterized a panel of monoclonal antibodies that neutralize PAI-1 activity by converting the active pathway into the non-inhibitory substrate pathway (18). For two of these antibodies, MA-55F4C12 and MA-33H1F7, the binding region was found to be located remote of the reactive-site loop, within a region comprising residues at positions 128–156 (19). Within the three-dimensional structure of PAI-1, these residues cover α-helix F (residues 128–145) and part of the turn connecting α-helix F and β-strand s3A (residues 146–156).

In the present study, we hypothesized that evaluation of the differential species reactivity of these antibodies, combined with a linear sequence alignment of the region from 128 to 156 in PAI-1 of various species and with the relative three-dimensional localization of these residues, could provide unambiguously which amino acids are directly involved in the interaction with the neutralizing monoclonal antibodies. The obtained data demonstrate that the epitope is composed of five residues at maximum. The precise nature and the location of this nonlinear epitope have important implications for the structural basis of the neutralizing mechanism of the antibodies and for the model of the complex between serpins and their target.
proteinase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden), from Roche Molecular Biochemicals, and from New England Biolabs (Beverly, MA). T4 DNA ligase, Thermo scientific phosphatase were purchased from Roche Molecular Biochemicals. T4 kinase was from U. S. Biochemical Corp., Klenow polymerase was from Amersham Pharmacia Biotech, *PfuTurbo*® DNA polymerase was purchased from Stratagene. Synthetic oligonucleotides (for mutagenesis and DNA sequencing) were synthesized by Amersham Pharmacia Biotech. Hae III restriction enzyme was from Boehringer Ingelheim (Brussels, Belgium). Recombinant human PAI-1, porcine PAI-1, and rat PAI-1 were expressed in *Escherichia coli* strains WK6 and WK6 mutS (20) kindly provided by Corvas (Ghent, Belgium). PMc-PAI-1 was constructed as described before (21). The expression vector pGE20 containing a heat-inducible promoter, the pAcI plasmid encoding a thermolabile repressor, as well as the *E. coli* strains DH1 and MC1061, for cloning and expression respectively, were kindly provided by Innogenetics (Ghent, Belgium). M13K07 helper phage was obtained from Promega (Leiden, The Netherlands).

Luria Broth (LB) growth medium was purchased from Life Technologies, Inc. (Gent, Belgium). Tissue-type plasminogen activator (Activyse®) was a kind gift from Boehringer Ingelheim (Brussels, Belgium). Urokinase-type plasminogen activator (urokinase, Clauth®, Sanofi Winthrop) was a kind gift from Bournonville Pharma (Brussels, Belgium). Recombinant human PAI-1, porcine PAI-1, murine PAI-1, and rat PAI-1 were expressed in *E. coli* and were produced basically as described previously (22–24). Rabbit PAI-1 (recombinant, expressed in *Saccharomyces cerevisiae*) was a kind gift from Dr. Hofman (Merck Research Laboratories, West Point, PA) (25). Most chemical reagents were purchased from Sigma (St. Louis). Chromogenic substrate S-2403 was obtained from Nodia/Chromogenex (Antwerp, Belgium). SP Sepharose® Fast Flow and Heparin-Sepharose® CL 6B were purchased from Amersham Pharmacia Biotech. The E. coli monolocal antibodies MA-3H1F7 and MA-5P4C12 (directed against human PAI-1) were raised against the PAI-1-16-PA complex as described elsewhere and were found to inhibit PAI-1 by inducing the substrate pathway (18).

**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® cartridges (Macherey-Nagel). DNA sequencing was performed by Macrogen (Leiden, The Netherlands).

**Construction of PAI-1 Mutants**—The following synthetic oligonucleotides were designed to introduce the desired mutations by site-directed mutagenesis using the pMa/c method (22): A, 5′-CACGGCTTCG-GCCCAAGAAGTGCCG-3′ was used to mutate Lys 154 into Ala and simultaneously introduced a Hae III restriction site (CCGCGG); B, 5′-GAAATCGTGGCGCGGCGGGTGAAAAGTCC-3′ was used to mutate Glu128-Val129-Glu130-Arg131 into Ala-Ala-Ala-Ala and simultaneously introduced a SacII restriction site (CCGGCG). The introduction of the additional restriction site allowed the confirmation of the desired mutation by restriction enzyme analysis. pMA-PAI-1-K, pMA-PAI-1-EVER, and pMA-PAI-1-EVER/K were constructed using oligonucleotides A, B, and A + B, respectively. Randomly selected clones containing the pMA-PAI-1 construct were evaluated for the presence of the mutated sequence. Therefore, small scale DNA preparations from the pMA-PAI-1 constructs were analyzed for the presence of the mutated sequences by restriction enzyme analysis.

Alternatively, mutants were created using a method based on the QuickChange® site-directed mutagenesis kit from Stratagene. Therefore, pmc-paI-1-wt was used as template to generate PMC-PAI-1-E128A (Glu128 to Ala), PMC-PAI-1-V129A (Val129 to Ala), PMC-PAI-1-E130A (Glu130 to Ala), and pMC-PAI-1-R131A (Arg131 to Ala), as described previously (22). The following oligonucleotides were used for random priming with dNTPs: 5′-TTGGATTTTTCCACGGTTGAGAGAGCCG-3′, 5′-GGTTGACTTTTCAGAGGTGGCGAGAGCCG-3′, 5′-CAGGAGTGGCAGGCGCCATCATC-3′, 5′-CAGAGGTGGAGGCAGCCAGATTCATCATC-3′, and 5′-CTGGAGATCGGCCAGGAGAGAGCCG-3′, were used as primer to introduce the E128A, V129A, E130A and R131A mutations, respectively. PCR was performed using 2.5 units of *Pfu Turbo*® DNA polymerase, 50 ng of template, 125 ng of each primer, 0.2 mmol of each dNTP in 50 μl of buffer containing 10 mm KCl, 10 mm (NH₄)₂SO₄, 20 mm Tris-HCl (pH 8.8), 2.0 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, 30 s; 68 °C, 12 min). Subsequently, the amplified DNA was subjected to a DpnI digestion prior to transformation of WRK E. coli strains DK1 and DK2 into competent E. coli DH5α and MC1061, respectively. For all mutants, large scale DNA preparations were made and subjected to nucleotide sequencing for confirmation of the mutations.

**Construction of Expression Plasmids**—pIGE20-PAI-1-wt was constructed as described before (21). SacI-XhoI fragments were recovered from pMa-PAI-1-K, pMa-PAI-1-EVER, pMa-PAI-1-EVER/K, pMa-PAI-1-EVER/K, pMa-PAI-1-E128A, pMa-PAI-1-V129A, pMa-PAI-1-E130A, and pMa-PAI-1-R131A and used for the wild-type fragment SacI-XhoI fragment in pGE20-PAI-1. The resulting pGE20-PAI-1-K, pGE20-PAI-1-EVER, pGE20-PAI-1-EVER/K, pGE20-PAI-1-EVER/K, pGE20-PAI-1-EVER/K, pGE20-PAI-1-EVER/K, pGE20-PAI-1-EVER/K, and pGE20-PAI-1-R/K constructs, used for the expression of the PAI-1 mutants, were entirely sequenced in the PAI-1 encoding region.

**Expression and Purification of PAI-1-wt and PAI-1 Mutants**—MC1061 *E. coli* competent cells were cotransformed with pAcI and pGE20-PAI-1-wt or one of the pGE20-PAI-1 mutant expression constructs. Clonal isolates were grown at 28 °C in LB medium with 50 μg/ml ampicillin, 50 μg/ml tetracycline, and 0.2 M NaCl. Overnight cultures were diluted 1:100 and grown to an absorbance (A600 nm) of 0.2. Then, PAI-1 expression was induced by increasing the temperature to 42 °C. Cells were harvested after 3 h by centrifugation for 20 min at 4000 × g at 4 °C and resuspended in 50 μM sodium acetate, pH 5.5, containing 2 mM glutathione, 0.5 μM leupeptin, 0.05 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, 0.7 μg/ml pepstatin A, 1 mM benzamidine-HCl, and 1 μg/ml antipain. The cell suspension was disrupted in a French® pressure cell (SLM Instruments Inc., Rochester, NY), and the cell lysate was cleared by ultracentrifugation for 20 min at 40,000 × g at 4 °C. The PAI-1-containing supernatant was collected and immediately subjected to purification.

All purification steps were performed at 4 °C and were carried out as described previously (22, 26) with minor modifications. Briefly, the supernatant was diluted (1:4) with 0.15 M KH₂PO₄-Na₂HPO₄, pH 6.5, and 2 mM glutathione (buffer P) including 0.2 mM NaCl and applied to a SP-Sepharose column (1.5 × 25 cm) equilibrated with buffer P, containing 0.2 mM NaCl. The column was washed with the same buffer containing 0.3 mM NaCl, and bound proteins were eluted with buffer P with a linear sodium chloride gradient (0.3–1.3 M). Fractions were evaluated for content and purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). PAI-1-containing fractions were pooled, dialyzed against P, and applied onto a heparin-Sepharose column (1.2 × 8 cm) equilibrated with buffer P. The column was washed with buffer P containing 0.2 mM NaCl. Bound proteins were eluted with buffer P with a linear sodium chloride gradient (0.2–1.3 M). Fractions containing PAI-1 were pooled and the concentration was determined photometrically at 280 nm using an absorbance coefficient A280nm of 10.

**Determination of the Conformational Distribution of PAI-1-wt and PAI-1 Mutants**—Samples of PAI-1-wt and PAI-1 mutants were diluted to a final concentration of 30 μg/ml in 0.045 mM KH₂PO₄-Na₂HPO₄, pH 6, containing 0.2 mM NaCl and incubated with a 2-fold molar excess of t-PA or u-PA at 37 °C for 30 min. The reaction products were analyzed by SDS-PAGE using 10–15% gels followed by Coomassie Brilliant Blue staining. Quantitation of the formed reaction products (complexed, non-reactive, and cleaved, corresponding to the presence of active, latent, and substrate conformations, respectively; Ref. 22) was done by densitometric scanning of the gels using ImageMaster® (Amersham Pharmacia Biotech).

**Determination of the Stability of PAI-1-wt and PAI-1 Mutants**—Samples of PAI-1-wt and PAI-1 mutants were diluted to a final concentration of 30 μg/ml in 0.045 mM KH₂PO₄-Na₂HPO₄, pH 6, containing 0.2 mM NaCl and incubated with a 2-fold molar excess of t-PA or u-PA at 37 °C for 30 min. The reaction products were analyzed by SDS-PAGE using 10–15% gels followed by Coomassie Brilliant Blue staining. Quantitation of the formed reaction products (complexed, non-reactive, and cleaved, corresponding to the presence of active, latent, and substrate conformations, respectively; Ref. 22) was done by densitometric scanning of the gels using ImageMaster® (Amersham Pharmacia Biotech).
Epitope of PAI-1 Neutralizing Antibodies

TABLE I
Affinity of MA-55F4C12 and MA-33H1F7, combined with the amino acid (AA) sequence alignment between AA128 and AA156 for different species of PAI-1

| PAI-1 species | Affinity (Kd) (nM) | Sequence alignment between AA128 and AA156 in PAI-1 |
|---------------|--------------------|-----------------------------------------------------|
|                | MA-55F4C12         | MA-33H1F7                                           |
| Human         | 2.7±1.610⁴         | 5.4±1.710⁵  E D A F I I N D W V K T H K G M S L L G K GA  |
| Porcine       | NB                 | 5.3±2.910⁵  E D A F I I N D W V K T H K G M S L L G K GA  |
| Rabbit        | NB                 | 2.9±1.310⁶  D V R A F I I N D V E R H T K G M S L L G E G A  |
| Rat           | 1.2±0.110²         | 8.4±1.510⁶  E D A F I I N D W V K T H K G M S L L A K G A  |
| Murine        | 1.2±0.110²         | 8.4±1.510⁶  E D A F I I N D W V K T H K G M S L L A K G A  |

1 The amino acid sequences are represented using one letter codes. The amino acids that differ from the corresponding AA in human PAI-1 are indicated in red (correlation with differences in immunological reactivity) or cyan blue (no correlation with differences in immunological reactivity).

2 Mean ± SD (n = 8 to 11); NB, no binding.

RESULTS
Affinity of MA-55F4C12 and MA-33H1F7 with PAI-1 from Different Species and Amino Acid Sequence Alignment—From the affinity constants (Table I), it can be deduced that MA-55F4C12 reacts similarly with human, rat, and murine PAI-1, but exhibits no affinity for porcine and rabbit PAI-1. For MA-33H1F7 a similar pattern was observed, except toward porcine PAI-1 for which some, but strongly reduced, reactivity (100-fold lower versus human PAI-1) was observed. Both antibodies react with active, latent, and substrate forms of PAI-1 with comparable affinity (data not shown), excluding the possibility that differences in species reactivity (or reactivity toward any of the mutants studied, see below) would arise from differences in relative amounts of the three conformations.

Sequence alignment between amino acid 128 and 156 of the PAI-1 s from the latter five species (Table I) shows that residues 128–130 and the residue at position 154 are conserved among human, rat, and murine PAI-1, whereas these particular residues differ from these in porcine and rabbit PAI-1. Consequently, we hypothesized that residues 128–130 and residue 154, or a combination of both, play a major role in the interaction between PAI-1 and the antibodies. Localization of these residues within the three-dimensional structure revealed that all are exposed at the surface and therefore are potential candidates to contribute to an epitope. Three-dimensional analysis in the region revealed that the residue at position 131 might also contribute to the possible epitope. It was therefore decided to produce a variety alanine mutants within this region of human PAI-1. Either all residues at position 128 to 131 and/or at position 154 were mutated, or single residues at positions 128, 129, 130, and 131, respectively, were mutated in the absence or presence of the mutation at position 154.

Expression and Characterization of the PAI-1 Mutants—All mutants exhibited inhibitory activity toward t-PA, although to a different extent and with different ratios between the various conformations (Table II, A). PAI-1-K exhibited functional activities similar to these of PAI-1-wt, whereas PAI-1-EVER and PAI-1-EVER/K acted more as a substrate. The activity of the single alanine mutants (PAI-1-E128, PAI-1-V, PAI-1-E130, and PAI-1-K) varied between 40% and 51% (data not shown). Both antibodies reacted with active, latent, and substrate forms of PAI-1 with comparable affinity (data not shown), excluding the possibility that differences in species reactivity (or reactivity toward any of the mutants studied, see below) would arise from differences in relative amounts of the three conformations.

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for PAI-1-K as for human PAI-1. The mutants PAI-1-EVER and PAI-1-EVER/K were significantly less stable (p < 0.0001, versus PAI-1-wt) (Table III).

**Affinity of MA-55F4C12 and MA-33H1F7 with PAI-1-wt, PAI-1 Mutants, and PAI-1 in Complex with t-PA—**

MA-55F4C12 and MA-33H1F7 exhibited an affinity of 3–5 × 10^9 M^-1 for PAI-1-wt (Table IV, A). Both antibodies exhibited a 70- to 180-fold decreased affinity for PAI-1-K. MA-33H1F7 exhibited a 13-fold decreased reactivity for PAI-1-EVER compared with PAI-1-wt, whereas MA-55F4C12 exhibited a 270-fold reduced affinity for this mutant. None of the mutants reacted with PAI-1-EVER/K, the variant that combined the EVER and K mutation (Table IV, B).

Even though most single mutations between residues 128 and 131 (Table IV, C) had only a marginal effect on the affinity, the affinity of MA-55F4C12 for PAI-1-E130 and PAI-1-R was decreased 35-fold. In contrast, combination of any single mutation in the region from 128 to 131 with the K154A mutation resulted in a pronounced decrease of affinity for either of the antibodies studied (Table IV, D). Indeed, MA-55F4C12 does not bind to PAI-1 if the mutation K154A is combined with the mutation E128A, E130A, or R131A. In addition the affinity of MA-55F4C12 for PAI-1-V/K is 20-fold reduced compared with that for PAI-1-K and 1500-fold compared with that for PAI-1-wt.

The affinity of MA-33H1F7 is lost completely only by a combination of the mutations E128A and K154A. Combination of K154A with mutation R131A results in 4-fold decrease of affinity compared with that for PAI-1-K. In contrast, combination of mutation E128A or V129A with mutation K154A does not result in a further decrease of the affinity compared with mutation K154A only.

Complex formation of PAI-1 with t-PA had no effect on the affinity of the antibodies for PAI-1 (Table IV, E).

### DISCUSSION

In this study the epitope of MA-55F4C12 and MA-33H1F7 was identified within a stretch of 29 amino acids in PAI-1 (region Glu^{128}–Ala^{156}), mainly based on the differential reactivity of these antibodies for PAI-1 from different species. In combination with the three-dimensional structure of PAI-1, this approach allowed to designate five amino acids (Glu^{128}, Val^{129}, Glu^{130}, Arg^{131}, and Lys^{154}) that are the major determinants for the interaction between PAI-1 and the antibodies. Thus, the epitope of the antibodies does not cover the complete α-helix F and turn connecting α-helix F and β-strand s3A, but is restricted to the hinge region between α-helix F and the main part of the PAI-1 molecule (Fig. 1).

To check the importance of the five residues, eleven alanine mutants were produced and characterized. The replacement of the bulky or charged residues (Glu^{128}, Val^{129}, Glu^{130}, Arg^{131}, and Lys^{154}) into small, non-charged alanines was predicted to destroy the epitope. This approach (“alanine-scan”) has already

### TABLE IV

| Variant | Residues mutated into an alanine | Kₐ^a | MA-55F4C12 | MA-33H1F7 |
|---------|----------------------------------|------|------------|------------|
| A       | PAI-1-wt                         |      | 2.7 ± 1.6 10^9 | 5.4 ± 1.7 10^9 |
| B       | PAI-1-EVER/K                     | 128V129E130R131 + K154 | 9.9 ± 6.0 10^6 | 4.2 ± 4.4 10^6 |
| C       | PAI-1-EVER                      |      | 4.0 ± 3.1 10^7 | 3.0 ± 2.0 10^7 |
| D       | PAI-1-EVER/K                     | 128V129E130R131 + K154 | 7.4 ± 2.0 10^7 | 1.3 ± 3.0 10^8 |
| E       | PAI-1/E130                        |      | 8.5 ± 0.8 10^9 | 1.8 ± 0.2 10^9 |
| F       | PAI-1-R                          |      | 4.9 ± 1.9 10^9 | 4.1 ± 2.4 10^9 |
| G       | PAI-1-V/K                        | 128E130 + K154 | 1.8 ± 0.9 10^9 | 2.0 ± 0.7 10^7 |
| H       | PAI-1-V/K                        |      | 7.4 ± 2.0 10^7 | 1.3 ± 3.0 10^8 |
| I       | PAI-1-E130/K                     | 128E130 + K154 | 1.8 ± 0.9 10^9 | 2.0 ± 0.7 10^7 |
| J       | PAI-1-R/K                        | 128E130 + K154 | 7.4 ± 2.0 10^7 | 1.3 ± 3.0 10^8 |
| K       | PAI-1/E130/K                     | 128E130 + K154 | 7.4 ± 2.0 10^7 | 1.3 ± 3.0 10^8 |
| L       | PAI-1/E130                        |      | 8.5 ± 0.8 10^9 | 1.8 ± 0.2 10^9 |
| M       | PAI-1-R/K                        |      | 4.9 ± 1.9 10^9 | 4.1 ± 2.4 10^9 |

^a^ Mean ± S.D. (n = 4–11); NB, no binding.
been used before to detect epitopes or functional determinants (30, 31). It should be noted that mutation of residue Arg131 was included primarily based on its location in the three-dimensional structure.

Comparison of the affinity constants in Table IV, part D versus those in part C and those for PAI-1-K, indicate that for MA-55F4C12 all five residues act cooperatively in the binding to the antibody and constitute the epitope. A similar comparative analysis for MA-33H1F7 reveals that the residues Glu128 and Val129 most likely do not contribute significantly, thereby suggesting that the epitope of MA-33H1F7 is predominantly composed of three residues (Lys154/Glu130/Arg131), positioned virtually linearly in the three-dimensional structure.

Even though some of the mutants in the EVER region (Table II) exhibit an increased substrate behavior, further supporting the role of this region in the inhibitor versus substrate properties of serpins, we, as well as others (34), have no straightforward explanation for these modified biochemical properties. It could be speculated that replacement of the charged residues by alaines and subsequent loss of interaction within the N-terminal portion of α-helix F, might result in a repositioning of α-helix F relative to the main β-sheet A (e.g. closer to β-sheet A, closer to the s3A-s5A opening) thereby impairing the shutter-like movement in β-sheet A, required for the inhibitory properties of a serpin.

Localization of the epitope has also implications for the mechanism of complex formation between PAI-1 and its target proteinase. Structures of the serpin/proteinase complexes are not elucidated yet, but several models have been proposed for such covalent complexes (7–11). According to one model (Fig. 2C), the proteinase moves 180° after the initial complex formation to form a stable covalent complex in which the reactive-site loop is completely inserted (7–9). It is obvious that the epitope of MA-55F4C12 and MA-33H1F7 is not accessible in this model (Fig. 2D). According to another model (Fig. 2A), the proteinase moves only to a limited extent such that the reactive-site loop is only partially inserted (10, 11). In the latter model the epitope clearly remains accessible since the proteinase is located rather remote from the epitope (Fig. 2B). According to the affinity constants (Table IV), the antibodies MA-55F4C12 and MA-33H1F7 bind to PAI-1 in complex with t-PA with an affinity similar to that for free PAI-1, demonstrating that the epitope is neither disturbed nor covered by complex formation. Taken together these findings rule out the model in which full insertion is proposed, but are compatible with the models in which partial insertion is proposed (Fig. 2A) (10, 11). Interestingly, this conclusion is also in agreement with a recent study.
(35) in which localization of a neoeptenic epitope in anti-thrombin-III/thrombin complexes indicates that in this complex, the most, residue P14 to P6 of the reactive-site loop can be inserted in \( \beta \)-sheet A.

To the best of our knowledge, this is the first report describing the elucidation of an epitope of a serpin-neutralizing monoclonal antibody down to five residues, being non-linear ("conformational") and remote of the active site. The identified epitope is different from the epitopes of other monoclonal antibodies with an inhibitory effect on a serpin. Asakura et al. (36) identified an epitope in the reactive-site loop of anti-antithrombin III (P8–P13), whereas Keijer et al. (37) suggested that the binding region of an inhibitory antibody was situated between amino acid residues 320 and 379 of PAI-1, a region containing the reactive-site loop. According to Björquist et al. (38), a panel of PAI-1-inhibiting antibodies can be divided into at least three groups, representing three non-overlapping epitopes. None of the suggested epitopes were located at the hinge region of \( \alpha \)-helix F, although the epitope of CLB-2CS is located near this area (residues 128–145) (39). Interestingly, other antibodies have been described with an epitope partially covering the same area (residues 125–132), that did not exert any inhibitory effect on PAI-1 (40). This further confirms that the functional properties of the currently described antibodies are due to the simultaneous interaction with residues Glu\(^{126} \)–Arg\(^{131} \) and with residue Lys\(^{154} \).

In conclusion, this study describes the localization of a conformational epitope of two anti-PAI-1 inhibitory antibodies, thereby providing a molecular explanation for their neutralizing mechanism. The precise localization of the epitope also has implications for the model of the complex between serpins and their target proteinases, i.e., only a model in which the N-terminal side of the reactive-site loop of serpins is partially inserted is compatible with the current observation.

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