Evidence for a Pre-latent Form of the Serpin Plasminogen Activator Inhibitor-1 with a Detached β-Strand 1C*

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Latency transition of plasminogen activator inhibitor-1 (PAI-1) occurs spontaneously in the absence of proteases and results in stabilization of the molecule through insertion of its reactive center loop (RCL) as a strand in β-sheet A and detachment of β-strand 1C (s1C) at the C-terminal hinge of the RCL. This is one of the largest structural rearrangements known for a folded protein domain without a concomitant change in covalent structure. Yet, the sequence of conformational changes during latency transition remains largely unknown. We have now mapped the epitope for the monoclonal antibody H4B3 to the cleft revealed upon s1C detachment and shown that H4B3 inactivates recombinant PAI-1 in a time-dependent manner. With fluorescence spectroscopy, we show that insertion of the RCL is accelerated in the presence of H4B3, demonstrating that the loss of activity is the result of latency transition. Considering that the epitope for H4B3 appears to be occluded by s1C in active PAI-1, this finding suggests the existence of a pre-latent conformation on the path from active to latent PAI-1 characterized by at least partial detachment of s1C. Functional characterization of mutated PAI-1 variants suggests that a salt-bridge between Arg273 and Asp224 may stabilize the pre-latent conformation.

The serpins constitute a family of proteins of which the majority are inhibitors of serine proteases (1). Of decisive importance for the inhibitory mechanism is the surface-exposed reactive center loop (RCL)5 between β-strands s5A and s1C (Fig. 1). The scissile P1–P1′ bond in the RCL is attacked by the serine protease, but at the enzyme-acyl intermediate stage, where the active site serine of the protease and the P1 residue of the serpin are linked by an ester bond, the N-terminal part of the RCL inserts as s4A thereby pulling the protease to the opposite pole of the serpin and distorting its active site. The required energy stems from stabilization of the serpin in the “relaxed” conformation with an inserted RCL, as compared with the “stressed,” active conformation with a surface-exposed RCL. The conformational changes associated with the insertion of s4A in serpins are well described by x-ray crystal structure analyses and include rearrangements in a region around s1A, s2A, hD, and hE referred to as the flexible joint region (reviewed in Ref. 2).

Plasminogen activator inhibitor-1 (PAI-1) and antithrombin III are unique among serpins as they can spontaneously adopt the relaxed conformation without prior cleavage of the RCL. This is referred to as latency transition and involves insertion of the N-terminal part of the intact RCL as s4A while the C-terminal part and the connected s1C is stretched out on the surface of the molecule (3, 4). Thus, during latency transition, full insertion of s4A requires the detachment of s1C and passage of the RCL through the gate region between the s3C/s4C and s3B/hG loops (Fig. 1). For PAI-1, latency transition is accelerated by the monoclonal antibody 33B8, which recognizes an epitope overlapping with part of the flexible joint region and binds preferentially to relaxed forms of PAI-1 (5–7). Because latency transition is irreversible, the effect of 33B8 cannot be explained by stabilization of the latent conformation. It was therefore hypothesized that 33B8 accelerates latency transition by binding to and stabilizing an intermediate, pre-latent conformation existing on the path from active to latent PAI-1 (5, 7).

This pre-latent form would exist in an unfavorable equilibrium with the active form and be characterized by sufficient insertion of N-terminal residues from the RCL at the top of β-sheet A for the high affinity 33B8 epitope to form (5–7). The positioning of s1C at the C terminus of the RCL in the putative pre-latent PAI-1, however, remains elusive, although it has been sug-

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5 The abbreviations used are: RCL, reactive center loop; HBS, Heps-buffered saline; HRP, horseradish peroxidase; LMW-uPA, low molecular weight uPA; PAI-1, plasminogen activator inhibitor-1; s, strand in a β-sheet; uPA, urokinase-type plasminogen activator; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; Ni-NTA, nickel-nitriiatriacetic acid.
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Here, the time-dependent loss of PAI-1 activity was found to be accelerated by the monoclonal antibody H4B3. To elucidate the underlying mechanism we characterized its effect in detail by fluorescence spectroscopy and site-directed mutagenesis. Binding of H4B3 was found to induce the conversion of PAI-1 to a form in which the RCL was inserted, consistent with an antibody-mediated acceleration of latency transition similar to what has been described for 33B8. The access of H4B3 to its binding area was demonstrated to require at least partial detachment of s1C, suggesting that this is a structural feature of pre-latent PAI-1. To obtain further information about the course of events during latency transition, we have isolated a peptide from phage displayed libraries with affinity exclusively for the latent conformation of human recombinant PAI-1. The binding sites for this peptide and for the monoclonal antibody MAI-12 were found to overlap with the epitope for H4B3. Considering that neither the peptide nor MAI-12 accelerated latency transition a map of their binding sites allowed us to clarify the features of H4B3 binding that govern its PAI-1-neutralizing effect. We also performed a functional analysis of the generated panel of PAI-1 variants with substitutions in the mapped binding regions and thereby identified a new region important for the functional stability of PAI-1. Finally, the binding profiles of the isolated peptide and antibodies to glycosylated and non-glycosylated PAI-1 in both stressed and relaxed conformations provide information about the orientation of the N-linked glycosylation on Asn267 in latent PAI-1.

EXPERIMENTAL PROCEDURES

PAI-1—We will refer to amino acid residues in PAI-1 by the numbering system of Andreasen et al. (10) starting at Ser1-Ala2-Val3. Recombinant PAI-1(W177F) was prepared from Escherichia coli expression cultures as described previously (11). Other recombinant PAI-1 variants were expressed with an N-terminal His6 tag and purified from E. coli expression cultures as described previously (12), except that expression cultures were incubated at 30 °C to minimize latency transition of the produced PAI-1. The cDNA for murine PAI-1 was PCR amplified and inserted into the expression vector pT7-PL as described for the human PAI-1 cDNA (12). The DNA coding for a PAI-1 murine/human chimera consisting of the murine sequence from the N terminus to residue 122, the conserved human and murine sequence between residues 123 and 142, and the human sequence from residue 143 to the C terminus was constructed using overlap extension PCR (13). The inverse human/murine chimera was also constructed. The stable, active PAI-114–1B variant carries the four amino acid substitutions described by Berkenpas et al. (14). PAI-1 cleaved at the P4-P3 peptide bond was prepared by incubating 425 μg/ml PAI-1 with 12.5 μg/ml elastase (Roche) in HBS (10 mM Hepes, 140 mM NaCl, pH 7.4). Elastase activity was irreversibly inhibited after 5 min at 37 °C by adding 4 mM Pefablock SC (Roche). The same conditions were used to prepare the uPA:PAI-1 complex, except that the concentration of PAI-1 was reduced to 225 μg/ml and elastase was replaced with 500 μg/ml uPA (Wakamoto Pharmaceutical Co., Japan). Natural glycosylated human PAI-1 was purified by immunofinity chromatography from serum-free conditioned medium of dexamethasone-treated HT-1080 cells (15). Recombinant glycosylated human PAI-1, PAI-1(N211Q), and PAI-1(N267Q) were expressed by transiently transfected HEK293T cells and purified from the conditioned medium by immunofinity chromatography (16). Latent PAI-1 from HT-1080 and HEK293T cells was activated by denaturation with 4 M guanidinium chloride and dialysis against HBS (15).

Antibodies—The monoclonal anti-PAI-1 antibodies were described previously as follows: mAb-1 (17, 18), mAb-2 (17, 19), mAb-5 (20, 21), mAb-6 (19), mAb-7 (16), 33B8 (5–7), H4B3 (16, 22), and MAI-12 (also known as MA7D4B7) (16, 23–26). Rabbit polyclonal anti-PAI-1 antibodies were those described (27). A horseradish peroxidase (HRP)-conjugated monoclonal anti-M13 phage antibody was from Amersham Biosciences.

Measurements of the Specific Inhibitory Activity of PAI-1—To measure the specific inhibitory activity of PAI-1, i.e. the fraction of PAI-1 capable of inhibiting the proteolytic activity of uPA,
PAI-1 was serially diluted in HBS supplemented with 0.25% gelatin (HBS-G), resulting in PAI-1 concentrations between 0.01 and 20 μg/ml (0.22 nm and 0.45 μM). One volume of 0.25 μg/ml (4.6 nm) uPA in HBS-G was added to each well, followed by incubation for at least 5 min. The remaining uPA activity was determined by adding the chromogenic substrate S-2444 (pyro-Glu-Gly-Arg-p-nitroanilide, Chromogenix, Sweden) for 30 min and measuring the absorbance at 405 nm. The specific inhibitory activity of PAI-1 was calculated from the amount of PAI-1 that inhibited 50% of the uPA.

**Determining the Rate of PAI-1 Latency Transition**—PAI-1 was incubated at 37 °C in HBS-G at a concentration of 20 μg/ml. At various time points, the specific inhibitory activity of PAI-1 was determined as described above and the half-life for latency transition was calculated from semilogarithmic plots of the specific inhibitory activity versus time. The effect of H4B3 and paionin-3 on latency transition was assessed in a similar assay with the following modifications. The temperature was lowered to 23 °C to reduce the rate of latency transition; the concentration of PAI-1 was 5 μg/ml (112 nm); H4B3 (200 nm) or paionin-3 (10 μM) were present during the incubation; and the concentration of uPA was 0.1 μg/ml (1.9 nm).

**Fluorescence Labeling of Recombinant PAI-1**—Labeling of the P9-Cys in PAI-1(S340C) with N-(2-(iodoacetoyl)ethyl)-N-methylamino-7-nitrobenz-2-oxa-1,3-diazole (Molecular Probes) and the P9-Cys in PAI-1(W177F/S340C) with N,N′,N″,N‴-dimethyl-N′-(acetyl)-N″-methyl(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylene diamine (Molecular Probes) created the fluorescent molecules PAI-1_{ps-NBD} and PAI-1(W177F)_{ps-NBD} and was carried out essentially as described (11). The labeling efficiency was ~0.8–1.0 mol of probe/mol of PAI-1. Incorporation of the fluorescent probes at this position on PAI-1 was shown previously to have no adverse effects on PAI-1 activity for either variant (11, 29). Latent PAI-1, which typically accumulated during the labeling reaction, was subsequently removed by affinity chromatography on immobilized β-anhydrotrypsin as described (11). Latent PAI-1_{ps-NBD} was prepared by incubation of a 0.5 μM concentration at 37 °C for 24 h in a buffer containing 30 mM Heps, 135 mM NaCl, 1 mM EDTA, 0.1% PEG 8000, pH 7.4, with the addition of 0.02% NaN₃. Complete conversion to the latent state was verified by the absence of complex formation when the latent preparation was reacted with a molar excess of uPA and analyzed by SDS-PAGE.

**Fluorescence Emission Spectroscopy**—Emission spectra of the binding interactions between labeled PAI-1 variants and H4B3 were carried out in a SPEX-3 spectrophotometer equipped with a Peltier temperature controller maintaining the measurement and incubation temperatures at 25 °C. Fluorescence experiments were carried out using semi-micro (0.2 × 1.0-cm) quartz cuvettes and in a reaction buffer containing 30 mM Heps, 135 mM NaCl, 1 mM EDTA, 0.1% PEG 8000, pH 7.4. The excitation wavelength used for studying the fluorescence of PAI-1_{ps-NBD} was 480 nm, and the emission spectra were scanned from 500 to 650 nm using a bandwidth of 5 nm for both the excitation and emission beams. Emission spectra for PAI-1_{ps-NBD} (50 nm) were recorded prior to and after the addition of H4B3 (500 nm) and a 40–60-min incubation at 25 °C. Results are presented as the averaged spectra of three to nine independent acquisitions. All individual emission spectra were collected as averages of three emission scans using a 0.5-s integration over a 1.0-nm step resolution and corrected for background fluorescence in the absence of PAI-1_{ps-NBD} and dilution effects, which were typically less than 2%. Spectra of latent PAI-1_{ps-NBD} (50 nm) were collected as described above, whereas reactions of PAI-1_{ps-NBD} (50 nm) with uPA (0.5 μM) were recorded after a 10-min incubation with the protease.

Kinetic experiments following the time-dependent fluorescence change in NBD fluorescence were carried out with PAI-1_{ps-NBD} (100 nm) or PAI-1(W177F)_{ps-NBD} (100 nm) and H4B3 (500 nm). Single emission spectra were collected rapidly to limit acquisition time by using modified excitation and emission bandwidths of 10 and 5 nm, respectively, while maintaining the 0.5-s integration over a 1.0-nm step resolution. Integrated fluorescence data were subsequently normalized as ΔF_{max}/ΔF_{obs}, where ΔF_{obs} is the observed integrated fluorescence change at each indicated time point and ΔF_{max} represents the total change in fluorescence following reaction with a 2-fold molar excess of uPA. Integrated fluorescence results are presented as the average of two independent experiments.

**H4B3 Binding to PAI-1 Captured on Vitronectin**—Maxisorp wells were coated with vitronectin (0.5 μg/ml in 100 mM NaHCO₃/Na₂CO₃, pH 9.6) and blocked with HBS supplemented with 1% bovine serum albumin (HBS-B). Active PAI-1, purified on immobilized β-anhydrotrypsin as described (11), or latent PAI-1 at the indicated concentrations were incubated in the wells and detected with either H4B3 (1 μg/ml in HBS-B) and a HRP-conjugated rabbit anti-mouse serum (DAKO, diluted 2,000-fold in HBS-B), or a polyclonal rabbit anti-PAI-1 antibody and a HRP-conjugated swine anti-rabbit serum (Dako, diluted 2,000-fold in HBS-B).

**Antibody Epitope Mapping**—Monoclonal anti-PAI-1 antibody H4B3 or MAI-12 was coated in 96-well plates (5 μg/ml in 100 mM NaHCO₃/Na₂CO₃, pH 9.6) followed by blocking with HBS supplemented with 5% skimmed milk powder (HBS-M). Dilution series of PAI-1 variants, reactive center-cleaved PAI-1, or PAI-1-uPA complex (0.01–150 nm in HBS-M) were incubated for 1 h and bound PAI-1 was detected with a rabbit polyclonal anti-PAI-1 antibody as described above. All assays were done in triplicate and the obtained binding curves fitted to a one-site binding hyperbola using Prism 4.01 (GraphPad Software Inc., San Diego, CA) to calculate the EC_{50} value.

**Isolation and Identification of PAI-1-binding Peptides by Screening of Phage-displayed Peptide Libraries**—PAI-1-binding peptides were selected from phage-displayed peptide repertoires in the formats X₇₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋_-
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erol. After extensive washing, bound phage was eluted with an HCl/glycine buffer, pH 2.2, and neutralized (30). The eluted phage was propagated in E. coli TG-1 cells and concentrated from the culture supernatant by precipitation with NaCl and polyethylene glycol. Peptides displayed on individual phage were identified by DNA sequencing as described previously (31).

ELISAs for Measuring PAI-1-Phage Binding—Before the ELISA, PAI-1 variants other than PAI-1(W177F) and PAI-1(W177F/P9-NBD) were incubated for 16–20 h at 37 °C to obtain latent material. In one type of ELISA, His6-tagged PAI-1 expressed from E. coli was immobilized in wells of Ni-NTA HisSorb Strips (Qiagen, Germany). PAI-1 at the indicated concentrations was immobilized and incubated with ~10^9 colony-forming units/ml of phage in HBS supplemented with 0.2% bovine serum albumin in the absence or presence of monoclonal antibodies as stated for each experiment. The wells were washed with HBS supplemented with 0.05% Tween 20 (HBS-T) and bound phage was detected with a HRP-conjugated anti-M13 monoclonal antibody (Amersham Biosciences; diluted 5,000-fold in HBS-B). After a final wash, wells were developed by adding 0.5 mg/ml ortho-phenylenediamine (KemEnTech, Denmark) in 50 mM citric acid, pH 5, supplemented with 0.03% H_2O_2. When suitable color had developed, the reactions were quenched with 1 volume of 1 M H_2SO_4 and the plates read at 492 nm in a microplate reader.

In a second type of ELISA, PAI-1 expressed from E. coli, HEK293T cells, or HT-1080 cells was immobilized on the solid phase by the use of monoclonal antibodies. Antibody (5 μg/ml in 100 mM NaHCO_3/Na_2CO_3, pH 9.6) was coated in wells of a 96-well Maxisorp plate (Nunc, Denmark) followed by blocking with HBS-M. PAI-1 at the indicated concentrations and phage (~10^9 colony-forming units/ml) were incubated for 1 h each in the antibody-coated wells, and bound phage was detected as described above. To ensure that equal amounts of the individual PAI-1 variants were bound to the wells, a parallel ELISA was performed with 1 μg/ml polyclonal rabbit anti-PAI-1 antibody instead of phage and with HRP-conjugated swine anti-rabbit serum (DAKO, Denmark, diluted 2,000-fold in HBS-B) instead of anti-M13 antibody.

SDS-PAGE—PAI-1 variants (150 μg/ml in HBS) were incubated for 15 min at 37 °C in the presence or absence of H4B3 (300 μg/ml) before addition of 300 μg/ml LMW-uPA (Abbott, Denmark). The reaction products were separated by SDS-PAGE (12% acrylamide, 1.5 μg PAI-1 per lane) and stained with Coomassie Blue.

RESULTS

The Monoclonal Antibody H4B3 Neutralizes PAI-1 by Accelerating Latency Transition—Incubating either human or murine PAI-1 with H4B3 accelerated the conversion of PAI-1 to an inactive form unable to inhibit the proteolytic activity of uPA (Fig. 2A). The half-life of inactivation mediated by 200 nM H4B3 at 23 °C was 26 ± 4 and 18 ± 2 min (n = 3) for human and murine PAI-1, respectively, compared with >300 min for both in the absence of antibody. Similar to the reported observations using the monoclonal antibody 33B8 (7), the rate of H4B3-induced activity loss was concentration dependent as the half-life

FIGURE 2. The monoclonal antibody H4B3 stimulates latency transition of wild-type PAI-1, but not the stable W177F mutant. A, time course of human (closed symbols) and murine (open symbols) PAI-1 (112 nM) activity loss at 23 °C in the absence (triangles) or presence (circles) of H4B3 (200 nM). The activities are normalized to the value at time 0. B, fluorescence emission spectra of H4B3-treated PAI-1(W177F/P9-NBD). Fluorescence emission spectra of PAI-1(W177F/P9-NBD) (50 nM) were recorded before (solid line) and after (dash-dot-dash line) incubation with H4B3 (500 nM) at 35 °C. Fluorescence spectra of 50 nM latent PAI-1(W177F/P9-NBD) (dashed line) or 50 nM PAI-1(W177F/P9-NBD) in complex with uPA (dash-dot-dot-dash line) are included. All fluorescence spectra were acquired with an excitation wavelength at 480 nm and represent the averaged spectra of three to nine independent experimental acquisitions. C, emission spectra of 100 nM PAI-1(W177F/P9-NBD) (closed circles) or PAI-1(W177F)P9-NBD (open circles) are shown. The reaction progress curve for PAI-1(W177F/P9-NBD) was fit by a single exponential function to obtain an observed rate for loop insertion in the presence of H4B3 of 0.14 ± 0.02 min⁻¹ (solid line) and a linear fit of the data for PAI-1(W177F)P9-NBD is shown as a dashed line.
in the presence of 100 nm H4B3 was 39 ± 3 and 33 ± 9 min (r = 3) for human and murine PAI-1, respectively (data not shown). For human PAI-1, these values correspond to rate constants for latency transition of <0.002, 0.018, and 0.027 min⁻¹ in the presence of 0, 100, and 200 nM H4B3, respectively.

To determine whether H4B3 neutralizes PAI-1 by accelerating the latency transition in a manner analogous to that found for the monoclonal antibody 33B8 (7), we took advantage of previous work demonstrating that labeling of the P9 position of PAI-1 (Ser140) with the environmentally sensitive fluorescent NBD probe produces a PAI-1 variant (PAI-1P9-NBD) in which the RCL insertion occurring during latency transition may be followed by fluorescence spectroscopy (29). Full insertion of the P9-NBD-labeled RCL as s4A thus results in a sizable fluorescence increase, which is consistent with transfer of the probe from a solvent exposed to a more hydrophobic environment. Fig. 2B illustrates the fluorescence emission spectrum of the PAI-1P9-NBD variant prior to, and following the binding of a molar excess of H4B3 for 40–60 min. Association of PAI-1P9-NBD and H4B3 resulted in a maximal enhancement of the fluorescence for the NBD probe by ~35% with an associated 12-nm blue shift in the peak emission from 539 nm in the absence of H4B3 to 527 nm in its presence. A comparable enhancement in fluorescence and blue-shifted peak emission was observed for the spectrum of an equimolar amount of latent PAI-1P9-NBD and also with PAI-1P9-NBD in complex with uPA (Fig. 2B). No change in the fluorescence spectra of active PAI-1P9-NBD alone was observed following incubations for up to 3 h (data not shown). That the NBD probe on the RCL of PAI-1P9-NBD becomes transferred into a hydrophobic environment, which is equivalent to that found with the loop-inserted species of PAI-1 such as latent and uPA-complexed PAI-1P9-NBD upon the binding of H4B3 is therefore consistent with a H4B3-accelerated latency transition of PAI-1. Time course experiments following the rate of full loop insertion indicated that the H4B3-induced enhancement in fluorescence was complete in 30–35 min with no additional changes in the fluorescence spectrum observed following incubations of up to 60 min with 500 nM H4B3 (Fig. 2C). When using this concentration of H4B3, the observed rate for the antibody-induced latency was 0.14 ± 0.02 min⁻¹, a rate much higher than that expected in the absence of antibody (~0.0005 min⁻¹ (29)). Taking the increased antibody concentration into account, this value is in good agreement with the rate constants for activity loss described above.

The mutation W177F in PAI-1 is located in the breach region at the point of initial RCL insertion and decreases the rate of latency transition (11). It was hypothesized that the mutation significantly reduces the probability of partial insertion of the RCL and thereby delays latency transition (11). We therefore predicted that the rate of H4B3-accelerated RCL insertion should be greatly reduced with PAI-1(W177F) compared with wild-type PAI-1 if H4B3 prefers binding to a pre-latent conformation. The PAI-1(W177F)P9-NBD variant was completely resistant to H4B3-induced latency transition during the 60-min time course (Fig. 2C). Incubations of PAI-1(W177F)P9-NBD for up to 5 h only showed slight increases in NBD fluorescence after 2–3 h. Based on the extrapolated fluorescence change over 5 h, we estimate that the rate of H4B3-accelerated RCL insertion with PAI-1(W177F)P9-NBD is at least 100-fold lower compared with the initial fluorescence change of PAI-1P9-NBD. These data are thus consistent with H4B3 accelerating latency transition by binding to a pre-latent conformation of PAI-1.

Mapping the Epitopes for Monoclonal Antibodies H4B3 and MAI-12—The epitope for H4B3 has been demonstrated to include Glu214 (16) and the epitope for MAI-12 has been shown to involve residues between positions 237 and 381 (25). Guided by these reports, we employed a panel of PAI-1 variants with site-directed mutations of residues in and around the cleft exposed by s1C detachment in latent PAI-1, all in their latent conformation, to determine the epitopes for these antibodies in detail. In addition to alanine scanning mutagenesis, Ala241 and Glu380 located in the bottom of the s1C cleft of latent PAI-1 were replaced by the bulkier residues valine or leucine and the oppositely charged residue lysine, respectively. When replacing a residue in PAI-1 with alanine increased the EC50 value for antibody binding to latent PAI-1 more than 10-fold, i.e. when the concentration of latent PAI-1 required to reach the half-maximal signal in ELISA was more than 10-fold higher than the EC50 value for latent wild-type PAI-1, the mutated residue was considered part of the epitope (Table 1). According to this distinction, the H4B3 epitope includes Tyr212, Glu214, Tyr243, Glu244, and Arg279, whereas the MAI-12 epitope includes Thr216, Tyr222, Tyr243, Glu244, Lys245, Arg279, and Glu380 (Table 1). These residues are in the immediate vicinity of the s1C cleft in latent PAI-1, and occluding this cleft by either the A241V or A241L mutation impaired binding of both antibodies. Noteworthy, H4B3 is raised against murine PAI-1 in PAI-1-deficient mice (22) and binds with ~50-fold higher apparent affinity to murine than to human PAI-1 (Table 1). The decreased affinity of H4B3 to human PAI-1 as compared with murine PAI-1 was almost completely counteracted by mutating Tyr222 in human PAI-1 to the corresponding Glu found in murine PAI-1 (Table 1). Thus, the epitope for H4B3 in murine PAI-1 includes Glu222.

In agreement with previous reports (24), MAI-12 binds with indistinguishable affinity to active and latent PAI-1. In contrast, H4B3 bound strongly to latent PAI-1 but showed no detectable binding to the stabilized active PAI-1 variants PAI-114–1B (14) or PAI-1(W177F) (11) (Table 1). Furthermore, the apparent affinity of H4B3 was markedly decreased when PAI-1 was either cleaved in the RCL by elastase or in complex with uPA (Table 1). Taken together, these observations suggest that optimal binding of H4B3 depended on the detachment of s1C because this is a characteristic of only latent PAI-1. To further elucidate this point, we captured either active or latent PAI-1 on vitronectin, which binds with ~200-fold higher affinity to the active form compared with the latent form (33), and measured H4B3 binding in an ELISA. Detection of PAI-1 with a polyclonal antibody confirmed that a higher density of PAI-1 was captured on vitronectin when the active form of PAI-1 was used (Fig. 3). Nevertheless, when incubating with H4B3, binding was only evident when the latent form of PAI-1 was used (Fig. 3). This strongly suggests that H4B3 does not recognize active PAI-1, and that the detachment of s1C is a stringent requirement for H4B3 binding.
monoclonal antibody and not coated directly on plastic during selections. Thirteen different sequence motifs of formats CX₅C or CX₁₀C with the recurring core sequence WPR(Y/W) were selected. Among these, the sequences CLTWPRYLC and CRE-QISWPRYYC were the most frequent and the corresponding phase bound indistinguishably to PAI-1 when used as reagents in ELISAs (data not shown). The latter of these will be referred to as paimonin-3 in the following. We found that paimonin-3 synthesized as a peptide with an N-terminal KKGA extension inhibited paimonin-3 phase binding (IC₅₀ = 270 ± 157 nm, n = 3), whereas a peptide targeting the flexible joint region of PAI-1 (34) had no effect (Fig. 4A).

When incubating PAI-1 at 37 °C, a condition where our preparation of PAI-1 is converted to the latent form with a half-life of about an hour (12), there was a gradual increase in the binding of paimonin-3 phase to PAI-1, as measured by a sandwich ELISA (Fig. 4B). The binding approached its final, maximal value with a rate indistinguishable from the rate of activity loss due to latency transition. Binding of paimonin-3 phase to different conformational forms of recombinant PAI-1 immobilized by their N-terminal His₆ tag were measured by ELISA. In this assay, paimonin-3 phase bound strongly to the latent form of PAI-1, whereas the binding to active, elastase-cleaved or uPA-complexed PAI-1 was strongly impaired (Fig. 4C). These data demonstrate that paimonin-3 binds the latent conformation of PAI-1 and that insertion of s4A by itself does not allow binding.

**Effects of Monoclonal Antibodies on Paimonin-3 Binding**—The monoclonal anti-PAI-1 antibodies H4B3 and MAI-12 completely abolished paimonin-3 phase binding to latent PAI-1 captured by the engineered N-terminal His₆ tag (Fig. 5). In the same assay, mAb-1, mAb-5, and mAb-6 did not affect binding, whereas 33B8 stimulated binding of paimonin-3 to initially active PAI-1 and PAI-1₁₄₋₁₇ (Fig. 5). As described above, both H4B3 and MAI-12 bind close to the s1C cleft, whereas 33B8 binds the hD/s2A loop and the top of s3A (5, 6); mAb-1 binds the hI/s5A loop and hC (18); mAb-5 binds the s3A/s4C loop (20); and mAb-6 binds hF and the hF/s3A loop (19). Considering the latency inducing effect of 33B8 (7), its ability to stimulate paimonin-3 binding is in agreement with the preferential binding of paimonin-3 to the latent conformation of PAI-1 (Fig. 4). That both MAI-12 and H4B3 competes with paimonin-3 phase for binding implies a peptide-binding site close to their epitopes.

**Mapping the Binding Site for Paimonin-3**—In ELISAs where His₆-tagged PAI-1 captured on immobilized Ni-NTA was incubated with paimonin-3 phase, there was no measurable binding to the latent forms of murine PAI-1 or a human/murine PAI-1 chimera, composed of the human PAI-1 sequence from the N terminus to the species-conserved part of α-helix F followed by the murine PAI-1 sequence to the C terminus (data not shown). In contrast, the latent form of the inverse chimera bound indistinguishably from latent human PAI-1 (Fig. 6). These observations advocate a binding site for paimonin-3 in the C-terminal part of PAI-1 that is revealed upon latency transition and differs between human and murine PAI-1. In this part of the molecule, the most pronounced structural effect of latency transition is the detachment of s1C (see Fig. 1) (3).

To accurately define the binding site for paimonin-3 on PAI-1, we used site-directed mutagenesis, which showed that binding
of the paionin-3 phage to PAI-1 was decreased when the latter carried one of the substitutions E214A, A241V, or E380K, whereas the substitutions D224A, A241L, or R273A rendered paionin-3 binding undetectable. In contrast, the substitution E380A augmented the binding of paionin-3 (Fig. 6). None of the other tested mutations in PAI-1 listed in Table 1 affected paionin-3 binding. This agrees with a binding site for paionin-3 in the s1C cleft of human PAI-1. Importantly, saturating concentrations of the paionin-3 peptide (10 μM) had no effect on the rate of latency transition of recombinant human PAI-1 (data not shown).

FIGURE 4. Effect of PAI-1 conformation on the binding of paionin-3 phage. A, binding of paionin-3 phage to latent PAI-1 (10 nM) captured on mAb-6 in the presence of the paionin-3 peptide with an N-terminal KKGA extension (open circles) or a peptide binding the flexible joint region of PAI-1 (34) (closed symbols). The curves represent the mean ± S.D. for one of three experiments, each performed in triplicate. B, PAI-1 expressed from E. coli (20 μg/ml) was incubated at 37°C in HBS. At the time points indicated on the x axis, PAI-1 was analyzed for specific inhibitory activity (left y axis, closed circles) and ability to bind to paionin-3 phage, using a sandwich ELISA with 10 nM PAI-1 and anti-PAI-1 mAb-6 on the solid phase (right y axis, open circles). No detectable binding was obtained when paionin-3 phage was replaced with phage from the naive phage library. The curves represent the mean of two independent experiments with standard deviation indicated. C, different forms of His6-tagged PAI-1 (10 nM), either active, reactive center cleaved (cleaved), in complex with uPA (complex), latent, or active PAI-114–18 (14–18), were incubated in microtiter wells with immobilized Ni-NTA chelates and the binding of paionin-3 phage determined by ELISA (black bars). Signals from control wells without PAI-1 have been subtracted. The bars represent the mean ± S.D. for one of two experiments performed in triplicate with indistinguishable results.

FIGURE 5. Effect of monoclonal antibodies on the binding of paionin-3 to latent PAI-1. His6-tagged PAI-1 (10 nM), latent PAI-1 (white bars), active PAI-1 (hatched bars), or active PAI-114–18 (black bars) were incubated in microtiter wells with immobilized Ni-NTA chelates and the binding of paionin-3 phage in the presence or absence of monoclonal anti-PAI-1 antibodies (40 nM) was determined by ELISA. Signals from control wells without PAI-1 have been subtracted. The bars represent the mean ± S.D. for one of two experiments performed in triplicate with indistinguishable results.

FIGURE 6. Paionin-3 phage binding to PAI-1 variants. The binding of paionin-3 phage to PAI-1 variants was determined in an ELISA using immobilized Ni-NTA chelates for capture of His6-tagged PAI-1. Data from latent forms of wild-type PAI-1 (open circles), PAI-1(E214A) (closed circles), PAI-1(A241V) (open squares), PAI-1(E380K) (closed squares), PAI-1(E380A) (open triangles), and a human-murine PAI-1 chimera (closed triangles) are shown. The murine-human chimera of PAI-1 has the species-conserved N-terminal part of α-helix F as the boundary region. Paionin-3 phage did not bind to the following PAI-1 variants in this assay: murine PAI-1, the human-murine chimera of PAI-1, PAI-1(D224A), PAI-114–18, and active PAI-114–18. Signals from control wells without PAI-1 have been subtracted. The curves represent the mean ± S.D. for one of two experiments, each performed in triplicate.
Characterization of PAI-1 Variants with Mutations in the s1C Area—The specific inhibitory activity toward uPA and the functional half-life was determined for all PAI-1 variants. The mutations T186A, R189A, E380A, and E380K accelerated the spontaneous conversion of PAI-1 to an inactive, presumably latent form, whereas the mutations D224A and R273A stabilized the active conformation of PAI-1 (Table 1). All the generated PAI-1 variants were individually reacted with a 2-fold molar excess of uPA followed by SDS-PAGE analysis to separate and detect the SDS-stable uPA:PAI-1 complex and free PAI-1. As expected, there was an inverse correlation between the amount of uPA:PAI-1 complex from each PAI-1 variant and its functional stability as listed in Table 1. We observed that the mutations R189A, Y212A, and Y245A rendered PAI-1 slightly more susceptible to cleavage by uPA, detected as an additional band migrating faster than the band corresponding to intact PAI-1 (data not shown).

Effect of PAI-1 Glycosylation on the Binding of Paionin-3 and H4B3—Paionin-3 phage showed no detectable binding to latent glycosylated PAI-1 expressed from HT-1080 or HEK293T cells. However, the binding was restored when latent PAI-1 from HEK293T cells carried the N267Q mutation, which abolishes glycosylation at Asn267 (16). In contrast, there was no effect of an N211Q substitution, abolishing glycosylation at Asn211 (Fig. 7). There was no effect of either substitution on binding of paionin-3 phage to PAI-1 expressed from E. coli (data not shown). This suggests that the glycans on Asn267 shields the paionin-3 binding site.

H4B3 does not bind to latent PAI-1 expressed from mammalian cells but only to recombinant PAI-1 expressed from E. coli, suggesting that glycosylation shields its epitope (Table 1). To further elucidate this observation, we tested the neutralizing effect of H4B3 on four different forms of PAI-1: natural fully glycosylated PAI-1, expressed from HEK293 cells; PAI-1(N211Q) and PAI-1(N267Q), lacking the glycans on Asn211 and Asn267, respectively, both expressed from HEK293 cells; and PAI-1 lacking both glycosylations, expressed from E. coli. These were treated with H4B3, reacted with LMW-uPA, and the reaction products analyzed by SDS-PAGE (Fig. 8). In agreement with the acceleration of latency transition induced by H4B3, there was no complex formation involving antibody-treated non-glycosylated PAI-1 from E. coli. In contrast, complex formation involving fully glycosylated PAI-1 from HEK293 cells was not affected by H4B3. The same is true for PAI-1(N211Q), indicating that it is not the glycans on Asn211 that impair antibody binding. In the case of PAI-1(N267Q), however, H4B3 prevents complex formation with LMW-uPA, suggesting that the removal of the glycans on Asn267 allows antibody binding (Fig. 8).

DISCUSSION

The Monoclonal Antibody H4B3 Inactivates PAI-1 by a Novel Mechanism—Active PAI-1 has been suggested to be in equilibrium with a pre-latent form characterized by partial insertion of the RCL as the beginning of s4A. Irreversible latency transition would then occur through this pre-latent intermediate. The monoclonal antibody 33B8 accelerates latency transition, presumably by binding to and stabilizing the pre-latent form of PAI-1, thereby shifting the equilibrium in its favor and accelerating latency transition (7). The preferential binding of 33B8 to pre-latent rather than active PAI-1 is in agreement with the epitope for 33B8 being dependent on at least partial insertion of the RCL for optimal binding (5, 6). Besides partial RCL insertion, the structure of the pre-latent form remains elusive, but it has been suggested to have features in common with the $\delta$-conformation of the L55P $\alpha_{1}$-antichymotrypsin mutant (35). In particular, insertion of the up to four residues of an otherwise exposed RCL, perhaps linked to a subtle distortion of s1C (8, 9).

We have mapped the epitope recognized by the monoclonal anti-PAI-1 antibody H4B3 and found it to be exposed only after partial detachment of s1C (Fig. 9B). Although they recognize completely different epitopes, H4B3 and 33B8 both induce latency transition of PAI-1 measured as antibody-induced loss
of anti-proteolytic activity and as accelerated RCL insertion with antibody-treated PAI-1P9-NBD (Fig. 2 and Ref. 7). As mentioned, this effect of 33B8 has been ascribed to its stabilization of the pre-latent conformation of PAI-1 and we propose a similar mechanism for H4B3. Accordingly, reversible and partial detachment of s1C in the pre-latent fraction of the active PAI-1 molecules could allow H4B3 to occupy the s1C cleft thereby stabilizing the pre-latent form and accelerating latency transition. Replacing Trp177 in PAI-1 with Phe delays PAI-1 latency transition and therefore could be hypothesized to render PAI-1 less prone to adopt the pre-latent conformation (11). The proposed mechanism is therefore consistent with PAI-1(W177F) being inert to H4B3-mediated latency transition (Fig. 2C). Interestingly, partial RCL insertion and s1C detachment, requirements for 33B8 and H4B3 binding, respectively, are the defining characteristics of a modeled “locking” conformation of serpins supposed to exist in equilibrium with the conventional active conformation (36). This proposed locking conformation could resemble the pre-latent PAI-1 conformation(s) stabilized by either H4B3 or 33B8.

In plasma, most if not all PAI-1 (~1 nm) circulates in complex with vitronectin (37). Because H4B3-mediated neutralization of recombinant PAI-1 was evident at the lower limit for detection of PAI-1 activity in our assays (~5 nm PAI-1), and because it was not affected by the presence of vitronectin (data not shown), our data do not exclude that the pre-latent form of PAI-1 can form in vivo.

In contrast to H4B3, the monoclonal antibody MAI-12 binds with comparable affinity to latent, cleaved and uPA-complexed PAI-1 and its epitope is not shielded by the glycosylation at Asn267 (Table 1). Our mapping of the MAI-12 epitope by alanine-scanning mutagenesis (Fig. 9C) is in partial agreement with the study by Keijer et al. (25) who suggested that residues from between positions 237 and 381 constitute the epitope. Due to the proximity of the MAI-12 epitope to the C terminus of the RCL of PAI-1, our data are in agreement with the suggestion that MAI-12 sterically hinders the interaction between PAI-1 and its target proteases uPA and tissue-type plasminogen activator (23). It remains to be investigated whether the pre-latent form hypothesized above has the RCL in an active conformation capable of inserting into the active site of a target protease. However, when this form is stabilized by H4B3 one would assume that access of the protease to the RCL would be sterically hindered by the antibody.

The Paionin-3 Binding Site: Implications for Serpin Structure and Function—The peptide paionin-3 binds a cryptic site on PAI-1 involving the cleft revealed by s1C detachment (Fig. 9D). This agrees with our observation that paionin-3 does not bind to elastase-cleaved or uPA-complexed PAI-1, which both have s1C in place, but an inserted RCL (Fig. 4C). By site-directed
A Pre-latent Form of PAI-1 with a Detached s1C

mutagenesis we identified Glu\textsuperscript{214}, Asp\textsuperscript{224}, and Arg\textsuperscript{273} as being the most important residues for paionin-3 binding (Fig. 6). The relocation of the side chain of Glu\textsuperscript{380} to the bottom of the s1C cleft concomitantly with detachment of s1C (compare A and D in Fig. 9 (38)) was shown to be important for paionin-3 binding because the mutations E380A and E380K augmented and impaired the binding of paionin-3, respectively (Fig. 6). This argues that the side chain of Glu\textsuperscript{380} flanks paionin-3 in the s1C cleft. In line with this, the binding of paionin-3 was impaired when occluding the s1C cleft by replacing Ala\textsuperscript{241} in its bottom with the bulkier amino acid Val, and even more so when replacing it with Leu (Fig. 6). Paionin-3 seems to be a highly specific probe for conformations of non-glycosylated PAI-1 in which s1C is peeled away, i.e. the latent conformation. To that end, the observation that monoclonal antibody 33B8 exposes the paionin-3 binding site is in agreement with the latency accelerating effect of this antibody (Fig. 5 and Ref. 7).

The binding sites for H4B3 and paionin-3 are overlapping and both are shielded by glycans on Asn\textsuperscript{267} or by s1C. Despite these common features paionin-3 does not accelerate latency transition, which suggests that paionin-3 does not bind the pre-latent form of PAI-1. To this end it is noteworthy that mutating Glu\textsuperscript{380} to either an Ala or a Lys only has insignificant effects on H4B3 binding but dramatic effects on paionin-3 binding (Fig. 6 and Table 1). This indicates that paionin-3, but not H4B3, is in close proximity of Glu\textsuperscript{380} when binding PAI-1. Inspection of the aligned structures of active PAI-1\textsubscript{114–1B} and latent PAI-1 (38) shows that exposure of the side chain of Glu\textsuperscript{380} in latent PAI-1 requires complete detachment of s1C as well as the bending of the ensuing loop (Asp\textsuperscript{357}–Pro\textsuperscript{399}) that accompanies latency transition. Accordingly, only complete latency transition will expose the paionin-3 binding site. These observations are consistent with a model where the partial detachment of s1C in pre-latent PAI-1 is sufficient for exposure of the H4B3 binding site, but insufficient for exposure of the paionin-3 binding site. In effect, H4B3 but not paionin-3 can accelerate latency transition.

The Glycans at Asn\textsuperscript{267} Can Block Access to the s1C Cleft in Latent PAI-1—Natural PAI-1 is glycosylated on two of three potential sites for N-linked glycosylation, one of which is Asn\textsuperscript{267} close to s1C and the C terminus of the RCL (Fig. 9A) (16, 39). Lack of glycosylation at Asn\textsuperscript{267} does not affect the rate of latency transition but renders PAI-1 more susceptible to stimulation of latency transition by detergents, suggesting a stabilizing role of these glycans (16). Paionin-3 and H4B3 do not react with PAI-1 glycosylated at Asn\textsuperscript{267} (Fig. 7 and Table 1), suggesting that the bi-antennary glycan complex points toward the s1C cleft in latent PAI-1 and thereby shields the binding sites for these compounds. This could not have been inferred from the reported crystal structure of glycosylated PAI-1 in which only the three glycan moieties closest to Asn\textsuperscript{267} are visible and located lateral to s1B (Fig. 9A) (39). The distance between Asn\textsuperscript{267} and the s1C cleft is roughly 25 Å, which can easily be covered by the glycan complex (16). An alternative explanation would be that the glycans alter the conformational preferences of the peptide backbone in PAI-1 thereby impairing paionin-3 and/or H4B3 binding. However, aligning the structure of glycosylated PAI-1 (39) with a similar structure of non-glycosylated PAI-1 (40) reveals no major differences in the overall backbone trace in and around the paionin-3 and H4B3 binding sites. Thus, from structural studies, there are no evident conformational changes associated with glycosylation at Asn\textsuperscript{267} that could explain the binding profile of paionin-3 and H4B3.

In contrast to H4B3, the affinity of MAI-12 is slightly augmented by PAI-1 glycosylation (Table 1) (16). The overlap between the H4B3 and MAI-12 epitopes could suggest that the glycans are not fixed in the s1C cleft as this would be expected to hinder MAI-12 binding as well. Alternatively, the surface areas covered by the antibodies, i.e. the structural epitopes, are not overlapping to the same extent as the functional epitopes mapped by alanine-scanning mutagenesis. Such discrepancies arise when residues are important for the integrity of the structural epitope without being part of it. Mutating these residues to alanine will affect antibody binding and hence they will be scored as part of the functional epitope, although they are not part of the structural epitope (41).

Removal of Charges Around s4C and Tighter Tethering of s1C Accelerates and Decelerates, Respectively, Latency Transition—Functional analysis of the generated PAI-1 variants identified residues that could not be replaced by alanine without significant effects on the functional stability of PAI-1, measured as the rate of spontaneous loss of inhibitory activity, presumably representing latency transition (Table 1). The common denominator for the destabilizing mutations T186A, R189A, and E380A is s4C in the gate region of PAI-1, which the RCL must pass around during latency transition (Fig. 1) (3). Thr\textsuperscript{186} and Arg\textsuperscript{189} are part of s4C, whereas the side chain of Glu\textsuperscript{380} in active PAI-1\textsubscript{114–1B} is located side-by-side with the oppositely charged side chain of the s4C residue Lys\textsuperscript{193} (38). The destabilizing effect of replacing Thr\textsuperscript{186} with Ala agrees with its high degree of conservation throughout the serpin family where it, through hydrogen bonding, stabilizes the breach region at the top of β-sheet A (1). Arg\textsuperscript{189} and Lys\textsuperscript{193} belong to a previously described cluster of basic residues important for the functional stability of PAI-1 (42). Accordingly, we believe that the acceleration of latency transition induced by mutations R189A and E380A is caused by a facilitated passing of the RCL around the gate region. Mutating Arg\textsuperscript{273} to alanine decelerated latency transition, which suggests that this side chain contributes to the functional instability of PAI-1 (Table 1). To rationalize this effect of Arg\textsuperscript{273}, we propose two models that are not mutually exclusive. First, in active PAI-1\textsubscript{114–1B} Arg\textsuperscript{273} is located beneath the C-terminal hinge of the RCL suggesting that replacing this residue with alanine could allow a tighter tethering of the RCL and thus induce a more rigid hinge. In particular, removal of the side chain of Arg\textsuperscript{273} in s2C, which in active PAI-1\textsubscript{114–1B} may H-bond to the backbone carbonyl of the neighboring Pro\textsuperscript{272} (38), could raise the energy barrier associated with s1C detachment by allowing an additional β-linkage between Glu\textsuperscript{352} in s1C and Pro\textsuperscript{272} in s2C. Second, a salt-bridge between Asp\textsuperscript{224} and Arg\textsuperscript{273} is evident in the structure of latent PAI-1 but prohibited by the presence of s1C in the structure of active PAI-1\textsubscript{114–1B} (Fig. 9D) (38). This interaction could be established concomitantly with partial s1C detachment, stabilize the resulting pre-latent form and thereby be a contributing factor.
to latency transition. In line with this, latency transition is also decelerated by mutating Asp224 to alanine (Table 1) and by the mutations D224K and R273C (28, 43).

In conclusion, our data are consistent with a reversible, partial detachment of s1C from the active conformation of PAI-1 that precedes latency transition. Our mapping of the binding sites for H4B3 and MAI-12 provides a molecular basis for their neutralizing effect on PAI-1. In addition, the present study emphasizes the cleft occupied by s1C in active PAI-1 as a hot spot for ligand binding in latent PAI-1 where not only the glycans attached to Asn267 and s1C but also paimonin-3 and H4B3 can bind.

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