Mapping of a Conformational Epitope on Plasminogen Activator Inhibitor-1 by Random Mutagenesis

IMPLICATIONS FOR SERPIN FUNCTION

The mechanism for the conversion of plasminogen activator inhibitor-1 (PAI-1) from the active to the latent conformation is not well understood. Recently, a monoclonal antibody, 33B8, was described that rapidly converts PAI-1 to the latent conformation (Verhamme, I., Kvassman, J. O., Day, D., Debrock, S., Vleugels, N., Declerck, P. J., and Shore, J. D. (1999) J. Biol. Chem. 274, 17511–17517). In an attempt to understand this interaction, and more broadly to understand the mechanism of the natural transition of PAI-1 to the latent conformation, we have used random mutagenesis to identify the 33B8 epitope in PAI-1. This site involves at least 8 amino acids scattered over more than two-thirds of the linear sequence that form a compact epitope on the PAI-1 three-dimensional structure. Surface plasmon resonance studies indicate a high affinity interaction between latent PAI-1 and 33B8 that is ~100-fold higher than comparable binding to active PAI-1. Structural modeling results together with surface plasmon resonance analysis of parental and site-directed PAI-1 mutants with disrupted 33B8 binding suggest the existence of a specific PAI-1 intermediate structure that is stabilized by 33B8 binding. These analyses strongly suggest that this intermediate form of PAI-1 has a partial insertion of the reactive center loop into β-sheet A, and together, these data have significant implications for the general serpin mechanism of proteinase inhibition.

Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor (serpin) gene family and is the principal inhibitor of the plasminogen activators tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) in vivo (1). In healthy individuals, PAI-1 is found at low levels in the plasma (~10 ng/ml), but it is elevated significantly in a number of diseases, including atherosclerosis (2), deep vein thrombosis (3), and non-insulin dependent diabetes mellitus (4). PAI-1 stabilizes both arterial and venous thrombi, contributing, respectively, to coronary arterial occlusion in post-myocardial infarction (5) and venous thrombosis following post-operative recovery from orthopedic surgery (6). Plasma PAI-1 is also elevated in postmenopausal women, and has been proposed to contribute to the relative increased incidence of cardiovascular disease in this population (7). The role of PAI-1 in atherosclerosis is less well defined, but PAI-1 is found in high concentrations in vascular plaque (8), and is involved in the in vitro regulation of tissue proteolysis and smooth muscle cell migration (9).

Whereas PAI-1 elevation is associated with disease progression, PAI-1 inhibition is associated with improvement in a number of pathophysiologic processes. PAI-1 null mice are viable and have normal coagulation and normal bleeding time (10). However, when subjected to vascular injury, PAI-1 null mice develop unstable thrombi that lyse spontaneously, indicating that revascularization of occluded arteries is improved significantly in the absence of PAI-1 (11). With respect to pathological fibrosis, PAI-1 null mice are protected from the development of vascular plaque (12) and pulmonary fibrosis (13, 14). These studies, together with the aforementioned clinical observations, suggest that PAI-1 inhibition may restore endogenous stimulation of fibrinolysis by plasminogen activation, re-establishing a critical defense mechanism for the prevention of intravascular thrombosis and tissue fibrosis.

Structurally, PAI-1 is a metastable protein exhibiting conformational plasticity, and exists in active, latent, and cleaved forms (15). The conversion of PAI-1 from the active to the latent conformation appears to be unique among serpins in that it occurs spontaneously at a relatively rapid rate. And while this transition has been known for more than 15 years (16), and the latent structure has been known for 10 years (17), the mechanism of the conversion from the active to the latent conformation is not well understood.

To gain a better understanding of the structural basis for the latent transition we have analyzed the interaction of a murine monoclonal antibody, 33B8, with PAI-1 (18). This antibody was raised against the tPA-PAI-1 complex, and has previously been shown to rapidly inactivate PAI-1 by accelerating the conversion of PAI-1 to the latent conformation (19). The reported rate of inactivation by this antibody is ~4000-fold faster than the spontaneous conversion of PAI-1 to the latent structure. Based on these studies it was suggested that exposure of the antibody epitope depended on an unfavorable equilibrium in PAI-1 that might involve an intermediate PAI-1 structure with partial insertion of the RCL into β-sheet A. Therefore, to see if this epitope requires partial RCL insertion into β-sheet A we have used a random PAI-1 mutant library (20) to identify this bind-
Conformational Epitope on Plasminogen Activator Inhibitor-1

Table I

| Mutant | Residue | Mutagenic primers |
|--------|---------|-------------------|
| 1      | N87D    | 5'-GGAGCTCAGCAGTGGACATGAGGATGAGAT-3' |
| 2      | K85E    | 5'-GGCTCAAGCAGTTAGAGGATGAGATCAG-3' |
| 3      | D98G    | 5'-CATGACAAAGGACATGAGGATGAGAT-3'    |
| 4      | Q174R   | 5'-GCTGCTTATCTCTAAGGAGAGGAGACTCTCCTCC-3' |
| 5      | G230V   | 5'-CTGATGACACTACCTACGTTGACACCTATTATGTTC-3' |
| 6      | T225S   | 5'-CCCAGGAACTCCCTCCAGATG-3'         |
| 7      | N329I   | 5'-GTAAGATCGAGTTGAGTTGAGGACATTG-3'  |
| 8      | S381R   | 5'-GTAAGATGAGNGGACCGGTG-3'          |

General DNA Techniques—DNA manipulation techniques were carried out according to standard procedures and following the manufacturer's instructions. Plasmid DNA was isolated using QIAprep Spin Miniprep kit from Qiagen Inc. (Valencia, CA). DNA sequencing was done with the BigDye™ Terminator Cycle sequencing ready reaction kit on a ABI PRISM™ 310 genetic analyzer from Applied Biosystems (Foster City, CA). PCR was performed using the Mastercycler® Personal 5332 from Eppendorf Scientific, Inc. (Westbury, NY). DNA sequences were analyzed with the Vector NTI™ Suite 6.0 molecular biology software for Windows™.

Site-directed Mutagenesis—Nine individual site-directed human PAI-1 mutants were constructed using either the Transformer™ or QuikChange™ site-directed mutagenesis kits purchased from Clontech Laboratories, Inc. or Stratagene (La Jolla, CA), respectively. The pEX-human-PAI-1 plasmid DNA (20) was used as the template to generate the required mutations. The following “mutagenic” and “selective” primers were designed to make the desired replacements (Table I). All mutations were confirmed by sequencing throughout the entire PAI-1 coding region of mutant plasmids isolated from at least four separate clones of each individual mutant.

Immunoscreening for 33B8 Binding Negative Variants—The expression AEIX(lox) library of random PAI-1 mutants with >2 x 10^6 independent clones, constructed and described by Berkenpas (20), was screened for mutant PAI-1 molecules that were negative for 33B8 binding. Briefly, 200 µl of exponentially growing host cells of E. coli BL21(DE3) resuspended in 10 mM MgSO4 at an A600nm of 2.0 were infected with ~10000 plaque forming units of AEIX(lox) PAI-1 library and plated. After 5.5 h of growth at 37 °C, the plates were overlaid with nitrocellulose filters previously coated with TPA at 10 µg/ml, blocked with 1% BSA, 5% milk in Tris-buffered saline and saturated with 10 mM D-galactoside, carbenicillin, tetracycline, and kanamycin were from Bethesda Research Laboratories. Inc. or Stratagene (La Jolla, CA), respectively. The pEX-human-PAI-1 plasmid DNA (20) was used as the template to generate the required mutations. The following “mutagenic” and “selective” primers were designed to make the desired replacements (Table I). All mutations were confirmed by sequencing throughout the entire PAI-1 coding region of mutant plasmids isolated from at least four separate clones of each individual mutant.

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isopropyl-1-thio-β-D-galactoside. The plates were then incubated for an additional hour at 37 °C. During this time lyrically infected cells expressing PAI-1 mutants release the protein into the plaques, where functionally active inhibitor binds to PA. The filters were then removed from the plates and washed in Tris-buffered saline containing 0.5% SDS to remove any PAI-1 that was not covalently bound to PA. (21). The filters were then incubated with 33B8 as the primary antibody, and filter-bound PAI-1-33B8 complexes were detected immunologically with goat anti-mouse horseradish peroxidase-conjugated immunoglobulins and ECL. The dark spots revealed on ECL Hyperfilms indicated plaques positive for 33B8 binding. The filters were then washed with water and Tris-buffered saline several times, and then incubated with rabbit anti-PAI-1 polyclonal antibodies as a primary antibody and goat anti-rabbit alkaline phosphatase-conjugated antibodies as a secondary. The filters were then developed in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate with dark purple spots corresponding to plaques expressing functionally active PAI-1 molecules. To select 33B8 binding negative plaques, ECL hyperfilms were overlaid with the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate developed nitrocellulose filters, and phage negative for 33B8 binding but positive for binding to the polyclonal antibody were picked from relevant bacterial plates. These isolated phage were then subjected to two sequential rounds of double screening to obtain a pure phage culture for each parental mutant.

**Functional Analysis of Mutant PAI-1 Molecules**—PAI-1 mutants from purified phage were automatically subcloned in pEX plasmids using the Cre/lox P system (Novagen), and expressed in E. coli BL21(DE3) or E. coli BL21(DE3) Tuner host strains along with wild-type PAI-1. The cell-free extracts were prepared from the cells grown to an optical density of 0.5–1.5 and then induced by isopropyl-β-D-galactoside (1 mM) for 2 h. Pellets from 1.5 ml of culture were resuspended in 0.4 ml of 0.05 M KPi buffer (pH 5.1), containing 0.001 M EDTA, 0.15 M NaCl, and 0.02% sodium azide, then sonicated using a microtip on an Ultrasonic processor XL from Misonix Inc. (Farmingdale, NY) for 1 min with a 50% pulse in an ice slurry. Homogenates were then centrifuged for 15 min at 14,000 rpm, and supernatant fractions were resuspended in 0.4 ml of 0.05 M KPi buffer, containing 0.15 M NaCl, 0.01% BSA, 0.0001% Tween 20, 0.02% sodium azide at room temperature at concentrations of 0.016–40 mg/ml of protein in a volume of 100 μl and analyzed for functional activity as measured by inhibitory activity against plasminogen activators (uPA or tPA) using the single step chromogenic substrate assay as described above. (20, 22). The specific inhibitory activity of PAI-1 was calculated from the amount of PAI-1 that had to be added to inhibit 50% of the 1.5 nM uPA in the reaction with the chromogenic substrate. The total soluble protein contents in CE were determined by BCA (Pierce). The level of expression of PAI-1 in the CE was calculated as the percent ratio of active PAI-1 detected in the activity assay to the total soluble protein contents of each individual mutant.

**Immunoblot Assays**—To evaluate the 33B8 binding negative phenotype of selected PAI-1 mutants, aliquots of CE of E. coli BL21(DE3) Tuner containing at least 5 ng of PAI-1, were separated by SDS-PAGE in 4–20% Tris glycine gradient gels under reducing conditions; electro-transferred to nitrocellulose membranes, blocked with 1% BSA, 5% milk, Tris-buffered saline, and then probed with either 33B8 or rabbit anti-PAI-1 polyclonal antibodies as the primary antibodies. The formed complexes were detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit immunoglobulins by the ECL method according to the same methods used for library screening (described above).

**Surface Plasmon Resonance (SPR)**—The binding of purified PAI-1 proteins and PAI-1 proteins in E. coli BL21(DE3) Tuner CE to 33B8 was analyzed by SPR using a BIACore™ 3000 optical biosensor from BIACore AB. This method detects binding interactions in real time by measuring changes in refractive index at a biospecific surface, allowing the evaluation of association and dissociation constants. A mouse monoclonal antibody 31C9 served as the control. This antibody is also directed toward PAI-1, but does not cause PAI-1 to convert to the latent conformation and binds to the active, latent, and cleaved forms of PAI-1 with similar affinity. Control reactions were also analyzed with α mouse Fcy fragments. All antibodies were coupled to an N-hydroxy succinimidyl-β-(dimethylaminopropyl)carbodiimide-activated CM-5 research grade sensor chip surface, to yield ~1000 resonance units response.

Purified wild-type PAI-1 in its active, or latent conformations, and E. coli CE containing functionally active PAI-1 were diluted to a concentration of 62 nM were analyzed in HBS-P buffer (pH 7.4) (BIACore AB). Samples were injected for 120–600 s at a flow rate of 10–20 μl/min followed by 10 min dissociation. Regeneration of the sensor chip surface was done with 20 μl of 0.01 M glycine buffer (pH 1.5). All measurements were performed at 25 °C, and BIACore 3000 Bioevaluation software program package (BIACore AB) was used for analysis of data.

**RESULTS**

**Screening of the PAI-1 Random Mutant Library for 33B8 Binding-deficient Clones**—A screen of the PAI-1 random mutant library for clones with a specific 33B8 binding deficit was performed as described above. Fig. 1A shows 33B8 positive clones and Fig. 1B shows the same filter reacted with polyclonal anti-PAI-1 antibodies. Plaques positive for functional PAI-1 but negative for 33B8 binding were identified by overlapping of these screens, and the 33B8-binding negative clones (Fig. 1C, note the circles) were isolated as described above. From three-hundred thousand λEX (lox) phage subjected to primary screening, 22 phage exhibited a negative 33B8-binding phenotype in the first round of selection, and of these 10 isolates remained 33B8-binding negative following two more rounds of selection. These 10 independent clones were then subjected to Cre-mediated recombination and the pEX-PAI-1 mutant plasmids coding for the parental PAI-1 mutants were isolated. PAI-1 protein from each of these mutants was then expressed in E. coli and their 33B8 deficit confirmed by immunoblotting (Fig. 1D). Nine of the 10 parental PAI-1 mutants showed a complete loss of 33B8 binding in this assay and only one of the 10 parental mutants (p5) showed any residual binding to 33B8 by immunoblot. The functional activity of each of these mutants was also examined and as expected, because the

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**Table II**

| Parental Mutants | Amino Acid Substitution | Number of Mutations |
|------------------|-------------------------|---------------------|
| P9               | S37TD                   | 3                    |
| P2               | R115W                   | 3                    |
| P5               | S33IR                   | 2                    |
| P10              | D95G                    | 3                    |
| P3               | G23RD                   | 3                    |
| P1               | S13T                    | 3                    |
| P4               | E814V                   | 3                    |
| P8               | G23RD                   | 3                    |
| P6               | V1E                     | 2                    |
| P7               | Q124R                   | 2                    |
Fig. 3. Mapping of mutations identified in the parental clones on the three-dimensional structures of active and latent PAI-1. Mutations from each parental clone are shown in a different color. Panels A and B show different orientations of the PAI-1 structures, and the arrows indicate the location of the mutational cluster on each view.

Fig. 4. Analysis of site-directed PAI-1 mutants. Panels A and B show immunoblots of cell-free extracts of wild-type and site-directed PAI-1 mutants. Panel A was probed with 33B8 and panel B with anti-PAI-1 polyclonal antibodies. Panel C shows the inhibitory activity toward high molecular weight uPA of wild-type and each of the PAI-1 site-directed mutants in cell-free extracts. The inhibitory activity is expressed as the percentage of functionally active PAI-1 relative to the total soluble protein of the cell extracts.

screening assay selected only clones that produced active, soluble PAI-1, all of the clones effectively inhibited uPA (Fig. 1F). However, the amount of active PAI-1 produced by the different clones varied by ~20-fold.

DNA Sequence Analysis and Molecular Modeling—DNA sequence analysis of the entire PAI-1 coding sequence of each of the parental mutants identified 34 independent mutations in 30 different positions spread across the entire PAI-1 coding sequence (Fig. 2). This represents an average of 3.4 amino acid substitutions per variant with all clones having at least two mutations and one clone having six. Two of the mutations, K88E and S331R, were present in more than one identified clone with S331R being present in 4 of the 10 clones (Table II). This suggested that at least these 2 residues were part of the 33B8 epitope. By highlighting the positions of each of the 34 mutations on the three-dimensional structure of latent and active PAI-1, we identified a cluster of 8 mutations that suggested a potential surface of contact with 33B8 (Fig. 3). The residues of this putative epitope, Asn^87, Lys^88, Asp^89, Gln^174, Gly^230, Thr^232, Asn^329, and Ser^331, were spread over two-thirds of the PAI-1 linear sequence (244 amino acids), but encompassed only a small area on the surface of latent PAI-1 (~18 Å × 8 Å).

Furthermore, as would be expected for the 33B8 epitope, this area contained at least 1 mutation from each of the 10 identified clones including the K88E and S331R mutations that were present on multiple parental clones. Together these data suggested that these residues likely constituted a significant portion of the 33B8 binding epitope.

Construction and Characterization of Site-directed Mutants—To see if the molecular modeling was indicating the correct localization of the epitope, all of the 8 putative epitope mutations (N87D, K88E, D89G, Q174R, G230V, T232S, N329I, and S331R) were constructed individually by site-directed mutagenesis and each was analyzed for 33B8 binding by Western blot and for inhibitory activity against uPA (Fig. 4). Data from these analyses indicated that 4 of the 8 point mutations significantly impaired 33B8 binding. These mutants, K88E, D89G, G230V, and S331R, appear to account for most if not all of the binding defect in 8 of the 10 parental clones (Table II). Of the remaining 4 point mutations, 2 of these, Q174R and T232S, were the only 2 mutations present in the parental clone p7 that has a severe binding deficit as judged by immunoblot analysis (Fig. 1D). Therefore, these 2 mutations must act together to disrupt 33B8 binding. The two remaining point mutations, N87D and N329I, were also present in the same parental clone, p9, which also contained 2 additional mutations. Therefore, to see if these 2 mutations also act together they were constructed in tandem, and this double mutant now showed a severe 33B8 binding defect (Fig. 4A). Functional analysis of the inhibitory activity of each of these site-directed mutants toward uPA was also performed and these data indicated that all of the mutants were active uPA inhibitors (Fig. 4C).

Together, these data demonstrate that these 8 residues must comprise at least part of the 33B8 binding epitope, their location in active and latent PAI-1 relative to one another is shown in Fig. 5. This analysis indicates the conformation of the identified epitope is quite different in the two conformations. It is also readily apparent from these structures that in latent PAI-1 the putative epitope is much more linear and compact than it is in active PAI-1. This result is consistent with earlier studies suggesting that latent PAI-1 bound the antibody with much higher affinity that did active PAI-1 (19).

SPR Analysis of PAI-1 Binding to 33B8—To better characterize the binding of PAI-1 in different conformations to 33B8 and to develop methods to quantify and characterize the binding defect in our point mutations, surface plasmon resonance experiments were performed. Fig. 6 shows the binding of purified active and latent wild-type PAI-1 to 33B8 (panel A) and to a control monoclonal antibody 31C9 (panel B). These data indicate that the latent form of PAI-1 binds to 33B8 with much higher affinity than active PAI-1 and that this difference in affinity is because of a lower rate of active PAI-1 association with the antibody and not to differences in the rate of dissociation. This is consistent with the observation that 33B8 converts active PAI-1 to the latent conformation and also with the relatively less accessible appearance of the putative epitope in the active structure.

Similar experiments with the parental clones and with the site-directed mutants were then performed and these data are shown in Fig. 7. These results demonstrate that all of the mutants show a significant and specific deficit in binding to 33B8 compared with wild-type PAI-1 (Fig. 7), whereas no difference in binding of the mutants and wild-type PAI-1 to the
31C9 control antibody was observed (not shown). However, the extent of the deficit and the type of defect vary for each mutant. For example, four of the single point mutants show either no binding (K88E, and G230V), or very large increases in the rates of dissociation (D89G and S331R). These are the same four point mutations that showed no reactivity in the immunoblots (Fig. 4). In contrast, 3 of the point mutations (N87D, T232S, and N329I) show clear reductions in their rates of association with 33B8 but only very modest changes in their dissociation rates, whereas the final point mutation (Q174R) shows modest reductions on both the association rates and the dissociation rates. In each of these latter cases, where the dissociation rate is not severely reduced, all 4 point mutations retained significant reactivity in the immunoblots. Together, these data support the conclusion that this site is the 33B8 binding epitope.

**DISCUSSION**

Together, our data demonstrate that the identified 8 residues must comprise at least a significant part of the 33B8 binding epitope on the surface of PAI-1. From our modeling of this epitope on the active and latent conformations (Fig. 5) it is readily apparent that in latent PAI-1 the putative epitope is much more linear and consolidated than it is in active PAI-1. The surface plasmon resonance experiments also show that the latent form of PAI-1 binds 33B8 with much higher apparent affinity than does active PAI-1, and that the difference in apparent affinity is because of a large reduction in the rate of active PAI-1 association with the antibody and not to differences in the rates of dissociation. This observation is consistent with earlier suggestions that active PAI-1 may be in equilibrium between a structure with the RCL fully exposed and an intermediate form with partial RCL insertion into β-sheet A (19, 23). If this equilibrium exists then there are two possible ways in which the PAI-1 mutants identified in this study could show reduced binding to 33B8. The first, which has already been discussed, is by directly disrupting the epitope, and the second is by stabilizing PAI-1 in a structure with low affinity for 33B8. In the latter case we would expect that these mutants would also show a reduced rate of conversion to the latent form because the putative intermediate should be on the pathway from active PAI-1 to the latent structure. To test this possibility we determined the rate at which each of the point mutants converted to the latent conformation. These data indicated that there was no correlation between the rate of PAI-1 conversion to the latent form and the 33B8 binding defect. Specifically, the three point mutations that show major reductions in their rates

![Fig. 5. Structure of the identified 33B8 binding epitope in latent and active PAI-1.](image)

![Fig. 6. Real time affinity binding of active and latent PAI-1 to 33B8 (panel A) or to control monoclonal antibody 31C9 (panel B).](image)

![Fig. 7. Real time affinity binding of parental (panel A) and PAI-1 site-directed mutants (panel B) to 33B8 was measured by SPR as described in the legend to Fig. 6 except that the samples were cell-free extracts of wild-type, parental, or site-directed PAI-1 mutants, diluted to 62 nM PAI-1 in HBS-P buffer (pH 7.4).](image)
of association with 33B8 but only small changes in their dissociation rates (N87D, T232S, and N329H) were either as stable, or less stable than wild-type PAI-1 (data not shown). These results support the conclusion that the identified mutations map the 33B8 binding epitope, and that the loss of binding by the mutants is not because of effects of the mutations outside the epitope that shift the putative equilibrium of active PAI-1 away from a form with high affinity for 33B8.

Nonetheless our data do provide significant support for active PAI-1 being in equilibrium with an intermediate form. This comes from modeling of the 33B8 epitope on the structures of two different serpins with partial RCL insertion. The first is native antithrombin III (24, 25) and the second is a mutant of α1-antichymotrypsin in the δ conformation (26). These structures are shown in Fig. 8 along with the active and latent forms of PAI-1. The 8 residues identified in our studies are purple in each PAI-1 structure, as are residues at the homologous positions in antithrombin III and α1-antichymotrypsin. Examination of the four structures demonstrates that in active PAI-1 the putative epitope is less compact than in the other structures, and that one of the residues (Ser331) is essentially entirely blocked by residues of the RCL in active PAI-1 (red arrow, Fig. 8, A and E). In contrast, both latent PAI-1 (Fig. 8, D and H) and the mutant form of α1-antichymotrypsin (Fig. 8, C and G) show a compact coherent epitope with all the residues visible on the surface of the molecule. In the case of antithrombin III the epitope structure is intermediate between active PAI-1 (Asn329-δ-sheet A at least partially in-
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Summary: The first step in binding of the synthetic peptide would be to the open conformation that we propose. During the normal inhibition reaction, proteinase cleavage of the RCL to the point of an acylenzyme intermediate is coupled with a rapid conformational rearrangement of the serpin involving complete RCL insertion, which in turn causes a distortion of the catalytic center of the enzyme and consequent trapping of the proteinase (21, 29, 31, 32, 38–41). This mechanism, which was predicted in 1990 (38), was largely confirmed when the crystal structure of a serpin-proteinase complex was recently solved (42). This distortion prevents the efficient deacylation of the complex, leading to stable inhibition. However, the initial steps of RCL insertion are not well defined. For example, it has been suggested that anti thrombin III can undergo an allosteric switch between two conformations of its RCL, one that is partially inserted and one that is fully expelled from β-sheet A (43). Furthermore, this transition regulates the activity of antithrombin toward different proteinases (44, 45). These studies, together with the results described here, suggest that a dynamic equilibrium between partial RCL insertion and an uninserted form may be a common feature of serpins, and that this equilibrium may play an important role in regulating serpin inhibitory activity.

Finally, our results demonstrate a novel approach for identifying the binding epitope of a PAI-1 inactivator and for distinguishing its mechanism of action. It is likely that this approach will be useful for examining other PAI-1 ligands, and ultimately, this experimental design may be applicable to the characterization of binding epitopes in other metastable proteins, among which serpins are a predominant family that presents many challenges, yet offers unique opportunities for drug discovery.

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