Plasminogen activator inhibitor 1 (PAI-1) binds to the somatomedin B (SMB) domain of vitronectin (VN), a domain present in at least seven other proteins. In this study, we investigate the PAI-1 binding activity of these SMB homologs and attempt to more specifically localize the PAI-1 binding site within this domain. SMB VN and several of its homologs were expressed in Escherichia coli, purified, and tested for PAI-1 binding activity in a competitive ligand binding assay. Although recombinant SMB VN was fully active in this assay, none of the homologs bound to PAI-1 or competed with VN for PAI-1 binding. These inactive homologs are structurally related to SMB VN, having 33–45% sequence identity and containing all 8 cysteines at conserved positions. Thus, homolog-scanning experiments were conducted by exchanging progressively larger portions of the NH2- or COOH-terminal regions of active SMB VN with the corresponding regions of the inactive homologs. These experiments revealed that the minimum PAI-1-binding sequence was present in the central region (residues 12–30) of SMB VN. Alanine scanning mutagenesis further demonstrated that each of the 8 cysteines as well as Gly12, Asp22, Leu24, Try27, Tyr28, and Asp34 were critical for PAI-1 binding and were required to stabilize PAI-1 activity. These results indicate that the PAI-1 binding motif is localized to residues 12–30 of SMB VN and suggest that this motif is anchored in the active conformation by disulfide bonds.

Urokinase-type and tissue-type (t-PA) plasminogen activators catalyze the conversion of the zymogen plasminogen into its active form plasmin (1, 2). Plasmin is the primary enzyme of the fibrinductive system and may also function in ovulation, embryonic development, inflammation, wound healing, angiogenesis, and neoplasia. The activities of t-PA and urokinase-type plasminogen activator in vivo are regulated by plasminogen activator inhibitor-1 (PAI-1) (3), a member of the serine protease inhibitor (Serpin) family (4). Although PAI-1 exists in both an active and a latent conformation (5–8), it appears to be synthesized in the active form but is conformationally unstable in solution and rapidly decays into the more stable but inactive latent form (3). Recent studies suggest that the active to latent transition is caused by the insertion of the reactive center loop into β-sheet A (9). For example, variants of PAI-1 constructed to reduce the rate of exchange between the reactive center loop and β-strands 3C/4C have a significantly lower rate of transition into the latent form (i.e., they are more stable; Ref. 10). Furthermore, Berkenpas et al. (11) used random mutagenesis to demonstrate that a combination of mutations greatly prolongs the half-life of active PAI-1. Besides possibly reducing strand 4A insertion, these mutations result in markedly increased thermal stability of the resulting active recombinant inhibitor relative to that of active native PAI-1.

Interestingly, the binding of PAI-1 to vitronectin (VN) significantly stabilizes this labile inhibitor in its active conformation (12–15) and may alter its specificity (16). Although VN binds to active PAI-1 with high affinity (12, 13, 16), little specific binding of the inactive latent form of PAI-1 to VN could be demonstrated (13, 18, 19). VN itself is a major component of plasma and also is present in many tissues (reviewed in Ref. 20). It appears to regulate a variety of processes including cell adhesion, complement activation, and fibrinolysis. This 75-kDa adhesive glycoprotein is composed of an NH2-terminal somatomedin B domain (SMB), a region containing a number of heparin-like repeats, and the heparin binding domain. Residues 1–40 of the SMB domain have been shown to contain the primary high affinity binding site in VN for active PAI-1 (14, 21). The amino acid sequence of SMB predicts an intricate compact structure because it contains 8 cysteines that probably anchor the PAI-1 binding motif in its active configuration. Like intact VN, the isolated SMB domain has been reported to stabilize PAI-1 (14).

Several other proteins were recently identified that also contain SMB-like domains (22, 23). Interestingly, megakaryocyte-stimulating factor (MSF) (22), the plasma cell membrane glycoprotein, PC-1 (24), autotaxin (ATX) (25), and the tumor cell surface antigen, gp130 (18, 26), all have two tandem copies of a SMB-like domain at their NH2 termini. SMB homologs also were identified in placental-specific protein 11 (PP11) (27), the T cell-specific protein, Tc3-30 (28), and the Drosophila scavenger receptor, dSR-CI (29). There is no information in the literature to indicate whether these SMB homologs also bind to PAI-1 with high affinity. Moreover, the structural requirements for the interaction between the SMB domain of VN (SMB VN) and PAI-1 are not known, and the actual PAI-1 binding motif within SMB VN has not been defined.

In the present study, we express and purify the SMB domains of VN and several of its homologs and examine their PAI-1 binding activities. We show that the recombinant SMB domain of MSF does not bind to PAI-1. Based on this observation, we developed a domain swapping (i.e., homolog-scanning)
approach and used it to localize the PAI-1 binding site to the central region of SMB\textsubscript{VN}. The essential residues for PAI-1 binding in SMB\textsubscript{VN} were further identified by site-directed mutagenesis, and a consensus PAI-1 binding motif was derived. These findings provide insights into the unique structure of SMB and suggest that this domain may mediate the binding of other proteins to cell surfaces and extracellular matrices.

**EXPERIMENTAL PROCEDURES**

**Materials—**Bovine serum albumin, casein, Triton X-100, Tween 80, Tris base, phenylmethylsulfonyl fluoride, dithiothreitol, and ampicillin were obtained from Sigma, Isopropyl-\(\beta\)-thiogalactoside (IPTG) from Calbiochem (La Jolla, CA). Reagents for SDS-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. XAR-5 x-ray film was from Eastman Kodak. Oligonucleotides and Taq-DNA polymerase were from Life Technologies Inc. Restriction enzymes and bacteriophage T4 ligase were from Boehringer Mannheim. PAI-1 was purified from the conditioned medium of a transformed human lung fibroblast cell line (SV40 WI38 VA13 2RA) using standard chromatographic techniques in the absence of SDS (6). Analysis of the final preparation by SDS-PAGE and staining with silver nitrate revealed a single protein with a molecular mass of 50,000 kDa. Activation of purified PAI-1 with 4 M guanidine hydrochloride was performed as described previously (5).

DNA Cloning and Mutagenesis—For protein expression, DNA was cloned into the bluelight vector (Novagen) for mammalian expression (7) or into the pET22b expression vector. The DNA for the SMB domain was amplified from VN cDNA (30) by using the S′-oligonucleotide primer, S′-GCCATGGAACTCAGTTGTAAAGGGCGGTGTTTTGTGAAAG-

| Nco | Xho | I digestion |
|-----|-----|-------------|
| TTGGCGCGTCGCAATC-3 | -GCCATGGAACTCAGTTGTAAAGGGCGGTGTTTTGTGAAAG-TTTTGAACGGGGGCGGGAATGTGATTGCGACGCGC-3 | 5′-GCCATGGAACTCAGTTGTAAAGGGCGGTGTTTTGTGAAAG-

Double-stranded plasmid DNA sequencing was performed using the T7 enzyme (Sequenase version 2.0, US Biochemical Corp). SDS-PAGE was performed in slab gels according to Laemmli (32). The upper stacking gel contained 4% acrylamide, whereas the lower resolving gel contained 15% acrylamide. Chemical reduction of samples was accomplished by adding dithiothreitol to the sample to a final concentration of 50 mM and boiling it for 3 min. The Rainbow-colored protein (low range) from Amersham Corp. was used as a molecular weight standard for SDS-PAGE. Reverse phase HPLC was performed using a C-18 column on a Waters 501 Unit. The active fraction was directly used for mass spectrometry analysis. In these experiments, the peptides were recovered in approximately 24% acetonitrile and 0.1% trifluoroacetic acid. Mass spectrometry was performed by the Mass Spectrometry Core Facility (The Scripps Research Institute, La Jolla, CA).

**RESULTS**

**Expression and Analysis of Recombinant SMB—**The SMB\textsubscript{VN} domain (residues 1–41) and the domain from one of its homologs (the SMB\textsubscript{MSF} domain; residues 42–83; see Table I) were cloned into the pET22b vector by fusing their NH\textsubscript{2} termini to the pEB signal peptide and their COOH termini to a stretch of 6 histidines. The expressed proteins contained the pEB signal peptide and were thus directed to the periplasm of E. coli, facilitating proper folding of these cysteine-rich peptides. The recombinant peptides were purified from the periplasm of E. coli on a nickel-charged column. The majority of purified SMB\textsubscript{VN} peptide migrated as a single band when analyzed by reducing SDS-PAGE with an apparent molecular mass of approximately 8 kDa (data not shown). However, varying amounts of SMB\textsubscript{VN} dimers and trimers were also apparent. The molecular mass of SMB\textsubscript{VN} was also determined by mass spectrometry after further purification by HPLC, and the measured molecular mass was identical to the calculated molecular mass of 6 kDa (i.e., 5180 Da for SMB plus 822 Da for the 6 histidines). These results indicate that the signal peptide was cleaved correctly. The purified peptide was recognized by monoclonal antibodies raised against human VN purified from plasma.\(^2\) These results suggest that the recombinant peptide was properly folded. Under identical conditions, SDS-PAGE revealed that the majority of the SMB\textsubscript{MSF} peptide migrated in bands of approximate molecular masses 13 and 20 kDa (data not shown), corresponding to the molecular mass of dimerized and trimerized SMB\textsubscript{MSF}. Comparison of the sequences of SMB\textsubscript{VN} and SMB\textsubscript{MSF} (Table I) predicted that they would have similar structures because the 8 cysteines in each peptide are identically positioned, they share greater than 40% homology, and most of the charged residues are conserved (Table I).

The PAI-1 binding activities of the recombinant peptides were tested in the competitive ligand binding assay (14). In this assay, a constant amount of PAI-1 was added to VN-coated microtiter wells in the presence of increasing amounts of the

\(^2\) G. Deng, G. Royle, S. Wang, K. Crain, and D. J. Loskutoff, unpublished observation.
recombinant SMB domains. After incubation, the amount of PAI-1 bound to the immobilized VN was determined by enzyme-linked immunosorbent assay. Fig. 1 shows that the purified recombinant SMBVN peptide competed with VN for PAI-1 binding, with half-maximal inhibition at approximately 0.04 mg/ml or 6.6 nM. This concentration compares favorably with the concentration of plasma-derived VN or SMB required to inhibit PAI-1 binding to VN (i.e., 2–3 nM for 50% inhibition) (14, 33). In contrast to these results with SMBVN, the related peptide (i.e., SMBMSF) had no detectable PAI-1 binding activity in the competitive ligand binding assay under the conditions employed. Because dimeric and trimeric SMBVN continue to bind PAI-1,2 these differences in activity do not appear to result from the different forms of the domain present in the two preparations. Thus, although structurally related to SMBVN, the SMBMSF homolog lacks the specific residues necessary to bind to PAI-1 with high affinity.

The PAI-1 Binding Site in SMBVN—To more precisely identify the PAI-1 binding site in SMBVN, a domain swapping strategy was adopted (Fig. 2). In these experiments, portions of the inactive SMBMSF domain were exchanged for the corresponding regions of active SMBVN. The sequence homology and conserved cysteines in these two peptides (Table I) suggest that domain swapping in this way will not lead to gross structural alterations in the resulting peptides. A two-step PCR method (34, 35) was used to create the fusion genes in these domain swapping experiments, and each of the resulting constructs was sequenced prior to use to verify that it contained the correct sequence. Again, all peptides were purified from the periplasmic fluid using a nickel column, and then each was tested for PAI-1 binding activity in the competitive ligand binding assay (Fig. 3). When residues 1–20 of SMBVN were replaced with the corresponding residues of SMBMSF, the resulting fusion peptide (i.e., SMBMSF1–20VN) no longer bound to PAI-1 (Fig. 3A). Negative results also were obtained when residues 1–11 were exchanged, but the hybrid peptide containing residues 1–12 of SMBMSF (SMBMSF1–12VN) showed no PAI-1 binding activity (Fig. 3A). Because the only difference between SMBMSF1–11VN and SMBMSF1–12VN is the substitution of Gly12 with a serine, this glycine must be critical for PAI-1 binding. Hybrid molecules containing residues 1–14, 1–16, and 1–18 of SMBMSF also were inactive. Thus, these initial studies establish that residues 1–11 at the NH2 terminus of SMBVN are not required for PAI-1 binding when replaced with the corresponding sequence from another SMB homolog. Residue 12 (Gly) appears to be essential for the PAI-1 binding activity of SMBVN.

**TABLE I**

| VNs       | 5 | 9 | 19 | 21 | 25 | 33 | 39 |
|-----------|---|---|----|----|----|----|----|
| human     | + | + | ++ | +  | +  | +  | +  |
| rabbit    | + | + | +  | +  | +  | +  | +  |
| mouse     | + | + | +  | +  | +  | +  | +  |
| pig       | + | + | +  | +  | +  | +  | +  |

**Homologs**

| Tc1-30 | 128 |
|--------|-----|
| PPl    | 26  |
| DSR-CI | 10  |
| Msf    | 21  |
| Msf exon 2 | 43 |
| PC-1   | 53  |
| PC-1 (23) | 94 |
| Autocax1 | 24 |
| Autocax2 | 24 |
| GPI30 (25) | 52 |
| GPI30 (25) | 96 |

**Consensus**

---C---C---G--------

C-CD-LC-YY--CC-D---C--

**FIG. 1.** PAI-1 binding activity of recombinant SMBVN and recombinant SMBMSF. Increasing concentrations of either SMBVN (□) or SMBMSF (□) were incubated with active PAI-1 (0.6 nM active PAI-1) on VN-coated wells as indicated under “Experimental Procedures.” Bound PAI-1 was then detected by incubating the washed plate first with rabbit anti-PAI-1 IgG and then with biotin-labeled goat anti-rabbit IgG and avidin phosphatase. The results are expressed as the percentages of PAI-1 that bound to VN-coated wells in the absence of competitors.
Similar experiments were performed to identify the COOH-terminal sequences necessary for PAI-1 binding. In these experiments, hybrid peptides were constructed by exchanging residues in the COOH terminus of SMBVN with corresponding regions of SMBMSF. Fig. 3 shows that swapping SMBVN residues 31–41 (i.e., SMBVN1–30MSF) or residues 29–41 (i.e., SMBVN1–28MSF) with SMBMSF sequences created hybrid peptides that retained PAI-1 binding activity, although the latter peptide had somewhat lower affinity for PAI-1. In contrast, replacing residues 26–41 created a hybrid peptide (SMBVN1–25MSF) that completely lacked PAI-1 binding activity. Thus, residues 31–40 at the COOH terminus of SMBVN are not required for PAI-1 binding when replaced with the corresponding region of another SMB homolog. Residue 29 is necessary for full PAI-1 binding activity. The importance of residue 28 cannot be established from these experiments because it is conserved in both molecules (see below).

Although the above results suggest that the PAI-1 binding site is located in the central region of SMBVN, it is also possible that the loss of activity in the inactive hybrid molecules reflects conformational changes in the molecule and not the loss of specific sequences. To distinguish between these possibilities, gain of function experiments were performed. In these experiments, the central region of SMBVN was inserted into the non-PAI-1 binding homolog SMBMSF in an attempt to convert it into a PAI-1 binder. When residues 12–30 of SMBVN were exchanged with the corresponding sequences of intact but inactive SMBMSF, the resulting peptide (i.e., SMBMSF1–11VN12–30MSF) was indeed converted into an active PAI-1-binding protein (Fig. 4). To further confirm these results, SMBVN12–30 was introduced into the first SMB domain of PC-1, another inactive SMB homolog, creating the hybrid protein SMBPC-11–11VN12–30PC-1. Again the resulting peptide demonstrated full PAI-1 binding activity (Fig. 4). These results indicate that the 19 residues in the central region of active SMBVN contain the sequences necessary to convert the non-PAI-1-binding homologs into fully active PAI-1-binding proteins.

The Cysteines in SMBVN Are Essential for PAI-1 Binding—Approximately 20% of the residues in SMBVN are cysteines. Because free cysteines were not detected in recombinant SMBVN domain using Ellman’s method (36),2 and because 10 mM N-ethylmaleimide did not interfere with the binding of SMBVN to PAI-1 (data not shown), the 8 cysteines appear to be arranged in disulfide bonds. Complete chemical reduction of VN2 or of SMB itself (33) abolished PAI-1 binding activity. Thus, the cysteines in SMBVN appear to be essential for PAI-1 binding. To determine whether all 8 cysteines were required for PAI-1 binding, we systematically changed each cysteine to alanine and then determined the PAI-1 binding activity of the
resultant mutant peptides. Recombinant peptides with Cys5, Cys9, Cys21, Cys31, or Cys39 replaced by alanine were completely inactive (Fig. 5A). However, the mutants with Cys5 or Cys25 replaced by alanine exhibited detectable PAI-1 binding activities, although their activities were significantly decreased compared to the control. These latter results raise the possibility that Cys5 and Cys25 may not be essential for PAI-1 binding because each could be converted into alanine without affecting the PAI-1 binding activity of the mutant peptide. The remaining three negatively charged residues in SMBVN (i.e., Asp22, Glu23, and Asp34) were then individually mutated to alanines. Interestingly, the Asp22 to alanine mutant (D22A) was completely inactive, and the D34A mutant had only partial activity (Fig. 5B). Mutation of Glu23 to Ala23 had no effect on PAI-1 binding. These results indicate that among the 12 charged residues in SMBVN, the two negatively charged aspartic acids appear to be directly involved in PAI-1 binding. Because these amino acids are conserved in SMBVN and SMBMSF, they were not revealed in the domain swapping experiments.

Besides the cysteines and the 2 aspartic acids, a number of other residues are conserved in SMBVN and SMBMSF. Thus the question of whether these residues are important in PAI-1 binding could not be addressed in the domain swapping experiments. These residues, along with a number of others in the central region, were therefore converted individually into alanine to examine their role in PAI-1 binding. Among these residues, Gly12, Leu24, and Tyr28 were essential because converting any of them into alanine resulted in the total loss of PAI-1 binding activity (Fig. 6). Another tyrosine, Tyr27, also appears to play a role in PAI-1 binding because the alanine mutant has lower affinity for PAI-1. Finally, a number of residues were identified (Table II) that did not appear to be essential for PAI-1 binding because each could be converted into alanine without affecting the PAI-1 binding activity of the resulting peptide. Table II lists all of the above mutants and summarizes their PAI-1 binding activities. In brief, a number of individual residues appear to be necessary for PAI-1 binding. These include the 8 cysteines, the polar (Tyr27 and Tyr28) and hydrophobic (Gly12 and Leu24) residues, and the two aspartic acids (Asp22 and Asp34).

Fig. 7 shows that biologically active and presumably properly folded SMBVN migrates as a monomer of 8 kDa when analyzed on reducing SDS-PAGE. However, the inactive, G12A mutant migrated as a ladder of progressively larger bands, with very little material migrating at 8 kDa. The mutants L24A and Y28A both migrated in a pattern similar to that of wild type SMBVN. These results indicate that substituting Gly12 with alanine results in the formation of inactive, reduc-
activity remaining in the sample was determined by using the t-PA binding assay (31). Because only active PAI-1 can bind to t-PA, this assay provides a simple approach for determining the half-life of PAI-1 under various conditions. After incubation, the bound PAI-1 is quantitated by enzyme-linked immunosorbent assay using anti-PAI-1 antibodies. These experiments demonstrate that the ability of the various SMBVN mutants to stabilize PAI-1 correlates with their ability to bind PAI-1 (Fig. 9). Under conditions of excess peptide, active SMBMSF1–11VN and SMBMSF1–11VN12–30MSF stabilized PAI-1 as well as SMBVN, whereas inactive SMBMSF1–12VN and SMBVN1–25MSF had no stabilizing activity (Fig. 9). The hybrid peptide constructed between the SMB domains of VN and PC-1 (i.e., SMBPC-1–11VN12–30PC-1) also exhibited full activity in stabilizing PAI-1. These results indicate that the structural requirements for stabilizing the biological activity of PAI-1 are the same as those required for PAI-1 binding.

### DISCUSSION

The interaction between PAI-1 and vitronectin is of potential physiological significance because it not only stabilizes the relatively labile inhibitor, it also appears to localize and concentrate this potent antifibrinolytic molecule in tissues during a variety of pathologic processes (reviewed in Ref. 23). The demonstration that PAI-1 and VN frequently co-localize in the atherosclerotic vessel wall (20, 38) is consistent with this idea. In addition to these changes, recent data suggest that the binding of PAI-1 to VN may also alter the specificity of the inhibitor by significantly increasing its affinity for thrombin (15, 39). These observations raise the possibility that VN is a cofactor for PAI-1 and suggest that efforts to delineate the residues in each molecule responsible for their interaction will provide insights into the role of these molecules in a variety of biological processes. In spite of this, relatively little is known about the structure and function of the individual domains that govern the PAI-1-VN interaction. In this regard, preliminary mutagenesis studies suggest that polar and/or hydrophobic residues at the amino-terminal half of PAI-1 are involved (39, 40). Although the carboxyl-terminal half of the inhibitor contains its reactive center, it does not appear to be involved in VN binding. We (14, 17) and others (33) have mapped the high affinity binding site for active PAI-1 in VN to the amino-
Analysis of the PAI-1 Binding Motif in Vitronectin

Many of these proteins are unknown, and they have not been characterized with respect to PAI-1 binding. Thus, in our initial experiments, we prepared a number of recombinant homologs and directly compared their PAI-1 binding activity with that of recombinant SMB VN (Fig. 1). Although SMB VN bound to PAI-1 and competed with VN for PAI-1 binding, the homologs did not (Figs. 1, 3, and 4). Thus, although there is striking structural and sequence homology between these proteins, their ability to bind PAI-1 is not shared. The structural similarity of these molecules suggested that we might be able to systematically exchange portions of active SMB VN with corresponding regions of the inactive homologs without disrupting their cysteine pairing or tertiary structure. Analysis of the activity of the resulting hybrid peptides should therefore provide initial insights into the regions of SMB VN required for PAI-1 binding. This strategy is outlined in Fig. 2, and Figs. 3, 4, and 8 indicate that it was successful. For example, exchanging the NH2- or COOH-terminal halves of each molecule created hybrid molecules that lacked PAI-1 binding activity (Fig. 3). Thus, the PAI-1 binding motif does not appear to be included entirely in either half of the molecule. Exchanging progressively larger regions from both ends of the molecule revealed that the PAI-1 binding site was in fact localized to the central region of the molecule (i.e., to residues 12–30) and thus spanned both halves (Figs. 3, 4, and 8). The fact that residues 12–30 alone convert non-PAI-1-binding proteins into binders (Figs. 4, 8), demonstrates that a specific PAI-1 binding motif is contained within the central region of the molecule. Moreover, these gain of function experiments argue against the possibility that the absence of activity in many of the mutants is due to conformational changes in the molecule.

Critical residues for PAI-1 binding in the central region were further delineated by alanine scanning (Fig. 5; Table II) and include the nonpolar residue, Gly22, the charged residues, Asp22 and Asp34, a hydrophobic residue, Leu34, and the polar residues, Tyr27 and Tyr28. Comparison of the SMB domains present in human (30), mouse (43), rabbit (44), and porcine (Table I) VN reveals that these residues are conserved in all of these molecules (Table I). The human and mouse (43), as well as rabbit3 VNs are all PAI-1-binding proteins. Although there is no published data to indicate that porcine VN also binds PAI-1, it seems likely because it contains the critical residues required for PAI-1 binding (see below). Examination of Table I also reveals that a number of residues are not conserved in these VNs. For example, in the NH2-terminal half of the molecule, residues Gly7, Glu11, and N14VK of human SMB VN are not conserved in the other VNs (Table I). Similarly, in the COOH-terminal region, Ser36, Thr33, and T36AE are not conserved. Because each of these VNs bind PAI-1 with high affinity, the nonconserved residues are not essential for PAI-1 binding. This conclusion is consistent with the results from our mutagenesis studies (Fig. 5; Table II). In general, the residues identified in our mutagenesis studies as being critical for PAI-1 binding are conserved in the various VNs, whereas those that were not essential were not conserved. In any case, these results suggest the following consensus sequence for the PAI-1 binding motif in SMB VN: -C5-C6-C9-G12--C19-C22-D21- L24-C25-Y27-Y28-C31-C32-D34-C39- (see Table I). The fact that all of the VNs contain this sequence and bind to PAI-1 and that the homologs lack this sequence and do not bind to PAI-1 supports the conclusion that these residues are essential for PAI-1 binding. It should be noted that we also determined the PAI-1 binding activity of a number of the other homologs, including TCI-30, PP11, PC-1 (94–134), and ATX1. These SMB homologs also lacked PAI-1 binding activity (data not shown).

3 D. Seifert, unpublished observation.
Although VN and SMBVN stabilize PAI-1 in its active form (14, 18, 37), the minimum structural requirement in SMBVN for this PAI-1 stabilizing activity has not been defined. Data from the current study show that the ability of SMBVN mutants to stabilize PAI-1 correlates with their ability to bind PAI-1 (Fig. 9). The mutant with the highest affinity for PAI-1 (i.e., SMBMSF1–11VN) stabilized PAI-1 nearly as well as SMBVN, whereas SMBMSF1–12VN, which had little affinity for PAI-1, showed no stabilization. These results provide evidence for the hypothesis that SMBVN stabilizes PAI-1 by directly binding to it and preserving it in the active conformation.

In summary, our data suggest that the actual PAI-1 binding site in SMBVN is located in the central region of the molecule and is composed of several residues presented on the surface of this compact structure. Although the positively charged residues do not appear to be essential for PAI-1 binding, the negatively charged residues are critical for the high affinity PAI-1–VN interaction. The finding that the polar residues, Tyr27 and Tyr28, and hydrophobic residue, Leu24, were important for PAI-1 binding is consistent with the suggestion from studies of PAI-1 mutants (39, 40) that such molecules are involved. In this model, the two negatively charged aspartic acids may provide for the initial protein docking in the interaction or may be necessary for the proper folding of SMBVN.

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