Modulation of Serpin Reaction through Stabilization of Transient Intermediate by Ligands Bound to α-Helix F

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Mechanism-based inhibition of proteinases by serpins involves enzyme acylation and fast insertion of the reactive center loop (RCL) into the central β-sheet of the serpin, resulting in mechanical inactivation of the proteinase. We examined the effects of ligands specific to α-helix F (αHF) of plasminogen activator inhibitor-1 (PAI-1) on the stoichiometry of inhibition (SI) and limiting rate constant (klim) of RCL insertion for reactions with β-trypsin, tissue-type plasminogen activator (tPA), and urokinase. The somatomedin B domain of vitronectin (SMBD) did not affect SI for any proteinase or klim for tPA but decreased the klim for β-trypsin. In contrast to SMBD, monoclonal antibodies MA-55F4C12 and MA-33H1F7, the epitopes of which are located at the opposite side of αHF, decreased klim and increased SI for every enzyme. These effects were enhanced in the presence of SMBD. RCL insertion for β-trypsin and tPA is limited by different subsequent steps of PAI-1 mechanism as follows: enzyme acylation and formation of a loop-displaced acyl complex (LDA), respectively. Stabilization of LDA through the disruption of the exosite interactions between PAI-1 and tPA induced an increase in the klim but did not affect the SI. Thus it is unlikely that LDA contributes significantly to the outcome of the serpin reaction. These results demonstrate that the rate of RCL insertion is not necessarily correlated with SI and indicate that an intermediate, different from LDA, which forms during the late steps of PAI-1 mechanism, and could be stabilized by ligands specific to αHF, controls bifurcation between the inhibitory and the substrate pathways.

Serpin (1) plasminogen activator inhibitor-1 (PAI-1),2 the major endogenous inhibitor of tissue- (tPA) and urokinase-type (uPA) plasminogen activators (2–5) is a well characterized thrombotic risk factor. High levels of PAI-1 inhibit the fibrinolytic system by preventing the formation of plasmin from plasminogen and promote thrombosis (6, 7). An elevated concentration of PAI-1 correlates with an increased risk of ischemic arterial disease (8) as follows: atherosclerosis, angina pectoris (9–14), and myocardial infarction (15–22), as well as with deep vein thrombosis and disseminated intravascular coagulation (23–25). Because complete inactivation of PAI-1 is not life-threatening (26–28), the development of new approaches to the therapeutic neutralization of PAI-1 is a subject of many studies (for review see Refs. 29, 30).

Like other serpins, PAI-1 (Scheme 1, I) (31) inactivates a target proteinase (E) through a unique conformational mechanism (Fig. 1). In this mechanism, the formation of the acyl-envelope intermediate (E–I) triggers the spontaneous insertion of a reactive center loop (RCL) as a strand 4 into the central β-sheet A, the 70 Å translation of the acyl-envelope to the opposite pole of the PAI-1 molecule, and enzyme inactivation because of the mechanical distortion of its catalytic site (Fig. 1) (32–34). Despite the fact that the structures of both the Michaelis complex (Scheme 1, E·I) (35) and the final inhibitory complex (E–I') (34, 36) are known for several serpins and proteinases, it is not clear what transient intermediates form on the reaction pathway from E·I to E–I, as well as how bifurcation is regulated between the inhibitory and substrate pathways of the serpin reaction (Scheme 1) (31).

The binding of proteinase to the initial docking site at the top of the serpin molecule (Fig. 1) results in the Michaelis complex E·I (Scheme 1), cleavage of the scissile bond of the RCL, and acylation of the enzyme (E–I). The formation of a loop-displaced acyl complex (LDA (E-I'; Scheme 1)) was proposed as a limiting step for the reaction between PAI-1 and tPA (31). Because LDA forms prior to RCL insertion, enzyme translocation requires disruption of exosite interactions at the PAI-1/tPA interface and dissociation of the primed part of cleaved RCL from the active site of the enzyme (31). In contrast to tPA, β-trypsin lacks exosite interactions with PAI-1. As a result, the rate of RCL insertion for the reaction of PAI-1 with β-trypsin is limited by formation of the acyl-envelope E–I (31). Thus, because of the lack of exosite interactions at the proteinase/serin interface, the limiting rate of RCL insertion (klim) for the reaction of PAI-1 with β-trypsin is 30 times higher than that for tPA. The substrate pathway (Scheme 1), where proteinase completes the normal catalytic cycle and the hydrolysis of the acyl-envelope results in an inactive cleaved PAI-1 with inserted RCL...
reaction with activated protein C (46). On the other hand, the binding of Vn results in an increase in both the rate and SI for the reaction of PAI-1 with thrombin (43, 47, 48). Moreover, Vn dramatically enhances the fraction of the substrate reaction between tPA or uPA and complexes of PAI-1 with mAbs (MA-55F4C12, mAb-2) directed against αHF (44, 45, 49). Therefore, the binding of Vn and anti-αHF mAbs to PAI-1 affects steps of the serpin mechanism, which contribute to distribution between substrate and inhibitory reactions.

To the best of our knowledge the contribution of the 44-amino acid N-terminal SMBD to the effects of Vn on the PAI-1 mechanism has not been studied thus far. To address this question, we have evaluated the effects of recombinant SMBD on the kinetics of RCL insertion and stoichiometry of the reaction of uPA and tPA with PAI-1 or its complex with anti-αHF mAb MA-55F4C12, and compared the results with the data obtained previously for the whole Vn isolated from human plasma (45). According to the Scheme 1, bifurcation between inhibitory and substrate pathways is controlled by LDA; however, disruption of the pattern of the exosite interactions at the PAI-1/tPA interface, which changes significantly $k_{lim}$ for the reaction of PAI-1 with tPA, does not affect SI (31, 50, 51). Therefore, a step of the serpin mechanism, which is likely different from stabilization/destabilization of LDA, contributes to the outcome of PAI-1 reaction.

Effects of ligands, with opposite binding sites relative to αHF (recombinant SMBD and anti-αHF mAbs MA-55F4C12 and MA-33H1F7) (52, 53), on kinetics and the outcome of the reactions of PAI-1 with β-trypsin, tPA, and uPA were analyzed in order to identify steps of the serpin reaction, which could be targeted for PAI-1 neutralization through intermolecular mechanisms. Because RCL insertion for the reactions of PAI-1 with tPA and β-trypsin is limited by different steps of the mechanism (31), the effects of mAbs and SMBD on the kinetics and outcome of the reactions with these enzymes were measured and compared. Finally, the possible contribution of the exosite interactions to the effects of the ligands was evaluated. SI values for the reactions of a mutant variant of PAI-1 (glutamate to alanine mutations at the positions 4$^4$ and 5$^5$ of the RCL) with tPA and β-trypsin were measured and compared with the data obtained for wtPAI-1. The results obtained in this study strongly support the major contribution of the SMBD to the effects of Vn on the PAI-1/proteinase reaction and indicate formation of a new transition intermediate (αHF intermediate), which appears at the late steps of the serpin mechanism and probably controls bifurcation between the inhibitory and substrate pathways of the reaction.

**EXPERIMENTAL PROCEDURES**

**Proteins and Reagents**—Bovine trypsin and analytical grade buffer reagents were from Sigma. Human plasma Vn was from Promega (Madison, WI). SMBD was obtained and characterized as described previously (53). Human recombinant PAI-1 and its S338C (P9 Cys) mutant variant were purified, labeled with an NBD group, and characterized as described elsewhere (54). NBD P9 E350A/E351A PAI-1, which was obtained and characterized as described elsewhere (50), was provided by Dr. C. Ibarra. The activity of PAI-1 was determined by titrating the
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inhibitor with proteinase. Concentrations of wtPAI-1 were calculated from absorbance at 280 nm, using an extinction coefficient ($e_{280}$) of 0.93 ml mg$^{-1}$ cm$^{-1}$ and an $M_r$ of 43,000 (55). Human recombinant tPA (activase) and uPA were provided by Genentech (San Francisco, CA) and by Abbott, respectively. Two-chain tPA was obtained from the single-chain enzyme by treatment with immobilized plasmin (56). Mouse mAbs MA-554C12 and MA-33H1F7 were selected from the panel raised against the human PAI-1-tPA complex (57). The concentrations of tPA, uPA, and mAbs were calculated from absorbance values at 280 nm, using $M_r$ values of 63,500, 54,000, and 150,000, and $e_{280}$ of 1.90, 1.36, and 1.3 ml mg$^{-1}$ cm$^{-1}$, respectively. All experiments were performed using a 0.05 mM phosphate buffer (pH 7.4 or 6.2).

Effects of SMBD on Kinetics of RCL Insertion Because of the Reaction of Proteinases with NBD P9 PAI-1 and Its Complexes with mAbs—Stopped flow fluorimetry was employed to examine the effects of SMBD on the rate of RCL insertion because of the reaction between proteinases and NBD P9 PAI-1 (10–20 nM) or its complexes with mAbs (45). A micro-volume stopped flow reaction analyzer SF-61 DX2 double mixing stopped flow system (Hi-Tech Scientific) or SX-18MV (Applied Photophysics Ltd.) (58), equipped with a fluorescence detector and a thermostatted cell, were used to monitor the changes in NBD fluorescence because of the reaction of proteinases with NBD P9 PAI-1 and its complexes with mAbs and SMBD. Briefly, SMBD (30–80 nM) was added either to NBD P9 PAI-1 (15–30 nM) or to its preformed complex with mAb (1.5–2.0-fold molar excess over NBD P9 PAI-1) at least 10 min prior to data collection. Traces of changes in NBD fluorescence emission because of the reaction with proteinase were measured. Measurements were carried out in 50 mM phosphate buffer, pH 7.4, at 25 °C. The data were analyzed using SigmaPlot 8.0 (SPSS Inc.) for Windows employing the nonlinear least squares Levenberg-Marquardt algorithm. Changes in NBD fluorescence emission (excitation at 480 nm, detection through 500 nm cutoff filter) with time were measured. A single exponential equation ($F_t = A + B(1 - e^{-k_{obs}t})$), where $F_t$ is fluorescence emission at $t$ seconds; $A$ is the fluorescence at $t = 0$; $B$ is the amplitude of the fluorescence change, and $k_{obs}$ is the observed first-order rate constant) was fit to the data using either KinAssist (Hi-Tech Scientific) or SigmaPlot 8.0 software. The quality of the fit was estimated by visual analysis of plots of the residuals (deviation of the fitted function from the data). The values of $k_{obs}$ (average of 5–10 measurements; standard error (S.E.) less than 10%) were measured at concentration of proteinase at least 5-fold higher than that of NBD P9 PAI-1 or its complexes. The values of $k_{obs}$ obtained at different concentrations of uPA, tPA or β-trypsin were plotted against proteinase concentration using SigmaPlot 8.0, and plots were fit by a hyperbolic equation $k_{obs} = k_{lim} \times (E)/(K_m + [E]); k_{lim}$ is the limiting rate constant of RCL insertion, and $K_m$ is the proteinase concentration ($E$) at half-saturation ($k_{obs} = k_{lim}/2$). Correlation coefficients ($r^2$) were used to estimate goodness of fitting ($r^2$ were ≥0.98 for all the data).

Effects of SMBD on Stoichiometry of Inhibition for the Reactions of Proteinases with NBD P9 PAI-1 and Their Complexes

with mAb—The SI is the number of moles of PAI-1 (or its complex with ligand(s)) required for the inactivation of 1 mol of proteinase. The SI values for reactions of β-trypsin, tPA, or uPA were determined directly by titration of NBD P9 PAI-1 or its binary (with SMBD or mAb) or ternary (with both SMBD and mAb) complexes with proteinase using either Photon Technology International or Varian Cary Eclipse fluorescence spectrophotometer as described previously (59). Briefly, the known amount of NBD P9 PAI-1 (or its complex with ligand(s)) was titrated with small aliquots (less than 2% of NBD P9 PAI-1 volume) of proteinase. An increase in fluorescence emission because of the reaction of NBD P9 PAI-1 with the enzyme was measured and plotted against proteinase concentration. Starting from the equivalence point, the addition of proteinase did not induce changes in the NBD fluorescence. The values of SI (average of 3–5 measurements) were calculated from the amounts of proteinase required to reach the equivalence point as the ratios between the number of moles of NBD P9 PAI-1 present in solution and the number of moles of proteinase required for its complete titration.

Effects of SMBD on Stoichiometry of Inhibition of uPA with wtPAI-1 and Their Complexes with mAb—The effect of the SMBD of Vn on the SI for the reaction of uPA with wtPAI-1 and its complexes with MA-554C12 or MA-33H1F7 was determined from a decrease in the fluorescence emission of uPA/p-aminobenzamidine (PAB) complex because of the displacement of PAB by PAI-1 (58, 60). The progress of the reaction between a mixture of uPA (50–100 nM) with PAB (100 μM) and increasing the amounts of wtPAI-1 or its complexes with SMBD, mAbs, or both ligands (25–500 nM) was monitored using micro-volume stopped flow reaction analyzers SF-61 DX2 (double mixing stopped flow system; Hi-Tech Scientific) or SX-18MV (Applied Photophysics Ltd.) (58). The SI was calculated from the dependences of a fraction of PAB displaced from the active site of uPA by wtPAI-1 (a decrease in PAB fluorescence, % of maximal) on the ratio of moles of wtPAI-1 (or its complexes) reacting with unchanged amount of uPA. The SI was equal to the value of [PAI-1]/[uPA] corresponding to complete displacement of PAB from uPA active site.

Stoichiometry of Inhibition of Proteinases with wtPAI-1 and Its Complexes with mAbs Estimated from SDS-PAGE—The values of the SI for the reactions of proteinases with wtPAI-1 and its complexes with mAbs and SMBD were also estimated directly from the results of SDS-PAGE (4–12% gradient gel; Invitrogen) as described previously (59). The SMBD and mAb were taken in 1.5–3.0-fold molar excess over wtPAI-1. The SI was calculated as an average of 3–5 measurements ± S.E.

RESULTS

SMBD Affects Differently the Kinetics of RCL Insertion for Reactions of NBD P9 PAI-1 with tPA and β-Trypsin—The effects of recombinant SMBD and human plasma Vn on the reactions of PAI-1 with β-trypsin and tPA were studied in order to determine what step of the reaction (Scheme 1) is affected by the ligands, and to elucidate the mechanism of modulation of PAI-1 activity, as well as to determine contribution of SMBD to the effects of whole Vn. The insertion of RCL, which is the central event of the reaction between PAI-1 and proteinase (Fig.
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1), was monitored through the increase in the fluorescence emission of the NBD group attached to a cysteine residue in S338C mutant variant of PAI-1 (NBD P9 PAI-1) (54). The binding of SMBD did not affect NBD fluorescence emission under any experimental conditions employed in this study. Thus, an increase in NBD fluorescence emission resulting from the reaction of proteinase with NBD P9 PAI-1 or its complexes with the ligands was employed for the evaluation of the kinetic parameters of RCL insertion and measurements of SI. The observed first-order rate constants ($k_{\text{obs}}$) of the RCL insertion were measured using stopped flow fluorimetry as described under “Experimental Procedures.” The dependence of $k_{\text{obs}}$ on the enzyme concentration (Fig. 2A) demonstrated that binding of the SMBD to NBD P9 PAI-1 results in a decrease in the limiting rate of RCL insertion ($k_{\text{lim}}$) for the reaction with β-trypsin. In contrast, the SMBD did not affect the $k_{\text{lim}}$ for the reaction with tPA (Fig. 2B). It was proposed that exosite interactions formed at the proteinase/PAI-1 interface in the Michaelis complex (EI, Scheme 1) affects the $k_{\text{lim}}$ (31). As a result, the $k_{\text{lim}}$ for tPA (2.8 s$^{-1}$; Table 1), which forms several exosite interactions with PAI-1, is 30 times lower than that for β-trypsin (86.0 s$^{-1}$; Table 1), which presumably has none. “North to South Pole” translocation of the proteinase (Fig. 1) because of RCL insertion requires breaking all of the exosite interactions. Thus, the RCL insertion for tPA is limited by the formation of the LDA (E-I; Scheme 1) (31). On the other hand, because of lack of exosite interactions between serpin and proteinase, the rate of RCL insertion for the reaction of β-trypsin is limited by the acylation of the enzyme (formation of E~I’), the step of the mechanism that precedes LDA formation (Scheme 1) (31). Therefore, a decrease in the $k_{\text{lim}}$ induced by binding of the SMBD observed for the reaction with β-trypsin (Table 1) could reflect changes in the limiting step of the reaction. However, in contrast to β-trypsin, SMBD did not affect the limiting rate of RCL insertion for tPA (Fig. 2A; Table 1). Therefore, the limiting step for RCL insertion for the reaction of tPA with PAI-1 or its complexes with SMBD (Table 1) or Vn (45) is the same (formation of the LDA; E-I’; Scheme 1) (31). In contrast to physiological pH 7.4, at a pH of 6.2 SMBD induces a more than 3-fold decrease in the $k_{\text{lim}}$ for tPA (from 8.1 ± 0.5 (51) to 2.6 ± 0.2 s$^{-1}$ (Table 1)). Thus at low pH, SMBD affects tPA reaction in a manner similar to that observed for uPA at pH 7.4 (a decrease in $k_{\text{lim}}$ from 23.0 ± 2.2 (45) to 6.5 ± 0.2 s$^{-1}$). Therefore, under conditions when the pattern of the exosite interactions between PAI-1 and tPA is disrupted (51), RCL insertion becomes limited by a step of the mechanism, which is different from LDA formation.

The data obtained demonstrated that SMBD does not affect the limiting rate of RCL insertion for interaction between PAI-1 and tPA at a neutral or slightly alkaline pH, when the reaction is limited by formation of LDA. In contrast, for the reactions with β-trypsin (limited by enzyme acylation (31)) or with uPA (or tPA at pH 6.2) SMBD induces significant decreases in the $k_{\text{lim}}$. SMBD (Fig. 3) binds far away from the initial docking site of the proteinase (Fig. 1), where enzyme acylation and the formation of LDA occur. Thus, the binding of SMBD most likely contributes to the stabilization of a transient intermediate, which is different from LDA (Scheme 1). Notably, the SMBD-binding site is located in close proximity to the epitopes of MA-55F4C12 and MA-33H1F7, and includes αHF (Fig. 3). SMBD interacts with the residues located at the side of αHF, which is opposite the epitopes of MA-55F4C12 and MA-33H1F7. Binding of these mAbs results in a decrease in the $k_{\text{lim}}$ for both tPA and uPA (59), which could reflect stabilization of the same transient intermediate. Previously we demonstrated additivity in the effects of Vn and MA-55F4C12 (mAb-2) on $k_{\text{lim}}$ for reactions of PAI-1 with tPA and uPA (45). However, to the best of our knowledge, there are no data on the effects of anti-αHF mAbs, Vn, or SMBD on the reaction of PAI-1 with β-trypsin, which is limited by acylation of the enzyme (Scheme 1) (31). Thus, at the next step, the effects of SMBD on kinetics of RCL insertion for the reaction of β-trypsin, tPA, and uPA with complexes of NBD PAI-9 and MA-55F4C12 and MA-33H1F7 were studied.

SMBD Decreases the Limiting Rate of RCL Insertion for Reactions of Proteinases with NBD PAI-1 Complexed with Anti-αHF mAb—Both mAbs employed in this study bind with low nanomolar affinity to both active and RCL inserted species of PAI-1 (wild type or NBD P9) (59, 61). Whereas the binding of MA-33H1F7 did not affect the fluorescence for NBD PAI-1, MA-55F4C12 induced ~20% enhancement in NBD fluorescence emission when bound to RCL inserted species of NBD PAI-1 (59). An increase in NBD fluorescence emission because of the binding of MA-55F4C12 was additive to the effect reporting the RCL insertion (54), i.e. a similar final NBD fluorescence was observed as a result of the reaction of the enzyme with an NBD PAI-1 MA-55F4C12 complex, and for the mAb bound to preformed RCL inserted species of NBD PAI-9 or PAI-1. Values of $k_{\text{obs}}$ were determined at different enzyme concentrations, and $k_{\text{lim}}$ and $K_m$ values were calculated from the dependences of $k_{\text{obs}}$ on the proteinase concentration (Fig. 4) as described under “Experimental Procedures.” Similar to the effects on reactions with uPA and tPA (59), MA-55F4C12 and MA-33H1F7 significantly decreased (7.6 and 6.9 times, respectively) the $k_{\text{lim}}$ for the reaction with β-trypsin (Table 2). Thus, in contrast to SMBD (Table 1) and Vn (45) anti-αHF mAbs similarly affect the $k_{\text{lim}}$ for tPA and β-trypsin, proteinases with different steps, which limit the rate of RCL insertion (31). On the other hand, SMBD, when bound to the PAI-1 mAb complex, always induced a decrease in the $k_{\text{lim}}$ for all three proteinases (Table 2). The effect of the SMBD on reactions of uPA and β-trypsin with NBD P9 PAI-1 MA-33H1F7 was less pronounced than that observed for MA-55F4C12, which could originate from slight differences in the epitopes of the two mAbs (61). The most significant change in the limiting rate of RCL insertion was observed for the reactions of PAI-1 mAb complexes with β-trypsin (Figs. 1 and 4), where binding of MA-55F4C12 and SMBD resulted in more than a 100-fold decrease in the $k_{\text{lim}}$ (from 86.0 to 0.72 s$^{-1}$ (Table 2)). To determine the contribution of the exosite interactions formed by deprotonated histidine residues (45), $k_{\text{lim}}$ and $K_m$ values for the reaction of tPA with NBD P9 PAI-1 mAb complexes were determined at pH 6.2 in the presence of SMBD. The values of $k_{\text{lim}}$ at pH 6.2 were several times higher than those observed at pH 7.4 (Table 2), indicating that formation of the exosite interactions at the PAI-1/tPA interface affects the $k_{\text{lim}}$ value for the reaction with binary (either SMBD or mAb is...
bound to PAI-1) and ternary (both SMBD and mAb are bound to PAI-1) complexes. These results support the hypothesis that SMBD and mAb, when bound to PAI-1 at both sides of αHF (Fig. 3), affect the step of PAI-1 mechanism, which follows dissociation of the acyl-enzyme from the initial docking site, and which results in the formation of LDA (Scheme 1). Moreover, the binding of MA-33H1F7 to αHF of PAI-1 (Fig. 3) results in a decrease in the $k_{\text{lim}}$ and a re-direction of the reaction with tPA and uPA from the inhibitory to the substrate pathway (Scheme 1) (45, 58). Thus, studies of the effects of SMBD on the SI for the reactions of PAI-1 and its complexes with anti-αHF mAbs with β-trypsin and tPA were carried out in order to determine a step of the PAI-1 mechanism, which is affected by ligands interacting with αHF.

**SMBD Potentiates Neutralization of PAI-1 by αHF mAbs in Reactions with Proteinases**—The effects of SMBD on the SI for the reactions of β-trypsin, tPA, and uPA with PAI-1 and their complexes with mAbs were determined as described under “Experimental Procedures.” Recombinant SMBD alone did not affect the partition between substrate and inhibitory pathways because of the reaction of NBD P9 PAI-1 with β-trypsin (A) and tPA (B). The dependence of $k_{\text{obs}}$ on the concentration of proteinase for the reaction of NBD P9 PAI-1 (40 nM) (filled symbols) and its complex with SMBD (60 nM) (open symbols) with β-trypsin (circles) and tPA (squares) is shown. Values of $k_{\text{obs}}$ were obtained by fitting a single exponential equation to the time traces of an increase in NBD-fluorescence emission, resulting from the reaction of NBD P9 PAI-1 with proteinase. Lines represent the best fit ($r^2 > 0.98$) of a hyperbolic equation $k_{\text{obs}} = k_{\text{lim}}(\text{[enzyme]}+ [\text{enzyme}])$ to the data shown. The values of $k_{\text{lim}}$ (the limiting rate of RCL insertion) and $K_m$ (concentration of the enzyme at $k_{\text{obs}} = k_{\text{lim}}/2$) for the reactions of NBD P9 PAI-1-SMBD complexes with β-trypsin, tPA, and uPA are shown in Table 1.

**TABLE 1**

Effects of recombinant SMBD on kinetics of RCL insertion for the reactions of β-trypsin, tPA, and uPA with NBD P9 PAI-1

| Proteinase | $k_{\text{lim}}$, s$^{-1}$ | $K_m$, μM |
|------------|--------------------------|-----------|
| β-Trypsin  | 86.0 $\pm$ 10.0          | 34.6 $\pm$ 2.8 |
| tPA, pH 7.4| 63.3 $\pm$ 9.5           | 18.3 $\pm$ 2.3 |
| tPA, pH 6.2| 1.90 $\pm$ 0.07          | 1.97 $\pm$ 0.03 |
| uPA        | 0.11 $\pm$ 0.02          | 0.14 $\pm$ 0.01 |
| $K_m$, μM  | 8.1 $\pm$ 0.5$^a$        | 2.6 $\pm$ 0.2 $^a$ |

Values were taken from Ref. 51.

Values were taken from Ref. 45.

$^a$ Values were taken from Ref. 51.

$^b$ Values were taken from Ref. 45.

FIGURE 2. Different effects of SMBD on the kinetics of RCL insertion because of the reaction of NBD P9 PAI-1 with β-trypsin (A) and tPA (B). The dependence of $k_{\text{obs}}$ on the concentration of proteinase for the reaction of NBD P9 PAI-1 (40 nM) (filled symbols) and its complex with SMBD (60 nM) (open symbols) with β-trypsin (circles) and tPA (squares) is shown. Values of $k_{\text{obs}}$ were obtained by fitting a single exponential equation to the time traces of an increase in NBD-fluorescence emission, resulting from the reaction of NBD P9 PAI-1 with proteinase. Lines represent the best fit ($r^2 > 0.98$) of a hyperbolic equation $k_{\text{obs}} = k_{\text{lim}}(\text{[enzyme]}+ [\text{enzyme}])$ to the data shown. The values of $k_{\text{lim}}$ (the limiting rate of RCL insertion) and $K_m$ (concentration of the enzyme at $k_{\text{obs}} = k_{\text{lim}}/2$) for the reactions of NBD P9 PAI-1-SMBD complexes with β-trypsin, tPA, and uPA are shown in Table 1.

FIGURE 3. Position of residues contributing to the epitopes of anti-αHF mAbs (highlighted in red ellipse) (61) at the molecular model of PAI-1. SMBD complex (53) provide structural basis for additivity of the effects of these ligands on the PAI-1 mechanism. The epitopes of MA-55F4C12 and MA-33H1F7 (include residues 128EVER131 and Lys154, shown as sticks) are located at the bottom of the αHF (HF, shown in blue) and its connecting loop. The PAI-1/SMBD binding interface is located on the other site of αHF. The key interaction of PAI-1 and SMBD is the ionic bonding between Arg101 of PAI-1 and Asp12 of SMBD, and this interaction is further stabilized by Tyr9 and Phe13 of SMBD that stack with the side chain of Arg91. SMBD structure is stabilized by four pairs of disulfide bonds (purple sticks). The β-sheet A of PAI-1 is shown in yellow, and SMBD is in orange. The binding of mAb and SMBD to the both sides of αHF results in a significant decrease of the limiting rate of RCL insertion between strands 3 and 5 of β-sheet A (s3A and s5A, respectively), and in significant increase in the fraction of the substrate reaction (Scheme 1). The figure was prepared by open source software Pymol using structure coordinate Protein Data Bank 10C0.

FIGURE 4. Effect of SMBD on the rate of RCL insertion because of the reaction of complex of NBD P9 PAI-1 and MA-55F4C12 with proteinases. Dependences of $k_{\text{lim}}$ on the enzyme concentration for reactions of β-trypsin (C), tPA (I), and uPA (Δ) with NBD P9 PAI-1 (60 nM) preincubated with MA-55F4C12 (90 nM) and SMBD (100 nM), and for the reaction of β-trypsin with NBD P9 PAI-1-MA-55F4C12 complex without SMBD (○). Values of $k_{\text{obs}}$ were calculated by fitting a single exponential equation to the time traces of the NBD-fluorescence emission, as described under “Experimental Procedures.” The solid lines are the best fits to a hyperbolic equation $k_{\text{obs}} = k_{\text{lim}}(\text{[enzyme]}+ [\text{enzyme}])$. The values of $k_{\text{lim}}$ and $K_m$ for reactions of proteinases with NBD P9 PAI-1 complexed with mAb and SMBD are shown in Table 2.
for the reactions of PAI-1 with any of the proteinases (Fig. 5). Therefore, although the interaction of SMBD with its binding site (Fig. 3) affects the kinetics of RCL insertion for the PAI-1 reaction with tPA in a manner similar to that observed for the formation of exosite interactions. However, in contrast to uPA and β-trypsin, SMBD does not affect the \( k_{\text{lim}} \) for tPA, which has the highest number of exosite interactions with PAI-1 (Fig. 2B). Thus, there is no additivity in the effects on \( k_{\text{lim}} \) between exosite interactions formed at PAI-1/tPA interface in the Michaelis complex and the effects of SMBD, which binds to α-helices F and E and strand 1 of β-sheet A (53) (Fig. 3). Because the rate of RCL insertion for the reactions of PAI-1 with β-trypsin and tPA is limited by different steps of the mechanism (enzyme acylation and LDA formation, respectively (31)), these data could indicate that SMBD affects a step of the reaction different from the formation of LDA (Scheme 1). Thus, the RCL insertion for the reaction of PAI-1 with tPA is affected by the presence of SMBD but not by the presence of SMBD alone. Therefore, the effect of anti-αHF mAbs on the outcome of the PAI-1 reaction with tPA does not depend significantly on the pattern of the exosite interactions at the PAI-1/proteinase interface. There was no significant difference between the SI values observed for wt PAI-1 and NBD PAI-1 (Tables 3 and 4), demonstrating that the Ser to Cys mutation at the P9 position of RCL and the following attachment of the NBD group did not interfere with the effects of mAbs and SMBD on the PAI-1 mechanism, i.e. the conclusions drawn from the results of the experiments carried out with NBD P9 PAI-1 could be expanded to wtPAI-1.

**DISCUSSION**

The binding of SMBD to PAI-1 does not affect the SI for the reaction with any of the three proteinases and induces a significant decrease in the \( k_{\text{lim}} \) for uPA and β-trypsin (Table 1). Such an effect could indicate the stabilization of the Michaelis complex (E-I; Scheme 1) through a mechanism similar to that observed for the formation of exosite interactions. However, in contrast to uPA and β-trypsin, SMBD does not affect the \( k_{\text{lim}} \) for tPA, which has the highest number of exosite interactions with PAI-1 (Fig. 2B). Thus, there is no additivity in the effects on \( k_{\text{lim}} \) between exosite interactions formed at PAI-1/tPA interface in the Michaelis complex and the effects of SMBD, which binds to α-helices F and E and strand 1 of β-sheet A (53) (Fig. 3). Because the rate of RCL insertion for the reactions of PAI-1 with β-trypsin and tPA is limited by different steps of the mechanism (enzyme acylation and LDA formation, respectively (31)), these data could indicate that SMBD affects a step of the reaction different from the formation of LDA (Scheme 1). Thus, the RCL insertion for the reaction of tPA with the PAI-1/SMBD complex is limited by the same step of the mechanism (formation of LDA E ~ I'; Scheme 1) (31) as the reaction with free PAI-1. LDA is formed as a result of breaking the exosite interactions at the PAI-1/tPA interface and the dissociation of the primed part of the cleaved RCL from the active site of tPA (31). Disruption of these exosite interactions because of a decrease in pH results in an increase in both the \( k_{\text{lim}} \) and \( K_m \) values (51). As expected, the binding of SMBD to PAI-1 at pH 6.2 induces a decrease in the \( k_{\text{lim}} \) for the reaction with tPA in a manner similar to that observed for uPA (Table 1), indicating that the rate of RCL

**Table 2**

| mAb ligand | MA-55F4C12 | MA-33H1F7 |
|------------|------------|------------|
| β-Trypsin  | k_{lim} s^{-1} | k_{lim} μM |
|            | 11.3 ± 0.8 | 0.072 ± 0.03 |
| tPA, pH 7.4| k_{lim} s^{-1} | k_{lim} μM |
|            | 2.4 ± 0.4 | 0.030 ± 0.05 |
| tPA, pH 6.2| k_{lim} s^{-1} | k_{lim} μM |
|            | 0.18 ± 0.06a | 0.012 ± 0.02 |
| uPA        | k_{lim} s^{-1} | k_{lim} μM |
|            | 0.04 ± 0.01 | 0.004 ± 0.01 |
|            | 0.01 ± 0.01 | 0.040 ± 0.04 |
|            | 0.53 ± 0.08 | 0.28 ± 0.04 |
|            | 0.53 ± 0.08 | 0.28 ± 0.04 |
|            | 0.03 ± 0.4a | 0.95 ± 0.15 |
|            | 0.76 ± 0.17 | 0.16 ± 0.05 |

* Values were taken from Ref. 45.
* Values were taken from Ref. 59.
* Values were taken from Ref. 51.


FIGURE 5. Effect of SMBD on distribution between inhibitory and substrate pathways for reactions of target proteinases with wtPA-I and its complexes with anti-αHF mAbs. A, SDS-PAGE analysis of products of the reaction between tPA or uPA and PAI-1, and its complex with MA-33H1F7 carried out with and without SMBD. PAI-1 (6 μl) was incubated for 5 min in 50 mM phosphate buffer (pH 7.4, room temperature) without ligands (lanes 1, 2, and 6); with 9 μM of MA-33H1F7 (lanes 3 and 7), with 9 μM of recombinant SMBD (lanes 4 and 8), with both 9 μM of MA-33H1F7, and 9 μM of recombinant SMBD (lanes 5 and 9). Lane 10 shows separation of molecular mass markers.

B, titration of uPA,PAB with wtPA-I and its complexes with SMBD and MA-55F4C12. Stoichiometry of inhibition, the number of moles of PAI-1, or its complexes with ligand(s) required for inactivation of 1 mol of uPA was measured using displacement of PAB from the active site of uPA because of formation of the inhibitory complex with PAI-1 (E-I*; Scheme 1). Increasing amounts of wtPA-I (E-I*; Scheme 1) or its complexes with recombinant SMBD (E-I*; Scheme 1), both SMBD and MA-55F4C12 (E-I*; Scheme 1), were added to uPA (0.1 μM) preincubated with 100 μM of PAB as described under “Experimental Procedures.” The inactivation of uPA was detected through a decrease in the fluorescence emission that corresponds to free PAB. The SI was determined as the minimal value of [PAI-1]/[uPA] ratio resulting in complete inactivation of uPA. The values of SI are shown in Table 3.

insertion becomes limited by a step of the mechanism, which follows LDA formation. Results obtained in this and other studies demonstrate that the stabilization/distabilization of the LDA (Scheme 1) through the formation/disturbance of the exosite interactions, while affecting k_{lim}, does not change the distribution between the substrate and inhibitory pathways of the PAI-1 reaction (Scheme 1). Indeed only small (if any) changes in the SI were observed for the reactions of proteinases with mutant variants of PAI-1, with mutations localized at the serpin/proteinase interface (positions P1’ (31) and P5’ (50)), as well as for the reaction between PAI-1 and tPA at low pH (51). Because SMBD and whole Vn affect the reactions with proteinases similarly, formation of the exosite-like interactions between enzyme interacting with PAI-1 at the initial docking site (Fig. 1) and 72-kDa Vn or 5-kDa SMBD bound at the opposite end of PAI-1 molecule (Fig. 3) is unlikely. Thus, a decrease in k_{lim} induced by Vn and SMBD probably reflects stabilization of either LDA (a decrease in k_{lim} and k_{int}); Scheme 1) or another intermediate, which is formed after LDA.

The binding of MA-55F4C12 and MA-33H1F7 to their epitopes located at the side of αHF, which is opposite to the SMBD-binding site (Fig. 3), delays RCL insertion for all three proteinases, and obstructs the serpin mechanism, re-directing the reaction from the inhibitory to the substrate pathway (Tables 2–4) (45, 59). Because anti-αHF mAbs affect the reaction of both tPA and β-trypsin in a similar manner, one could suggest that binding of these mAbs results in a change of the limiting step for RCL insertion for both enzymes because of stabilization of a transient intermediate, which is favorable for the substrate pathway. The close proximity between binding sites of mAbs and SMBD (Fig. 3) provides the structural basis for additivity in the effects of both ligands on the PAI-1 reaction, especially if RCL insertion requires reversible displacement of αHF (62, 63). Although residues Arg^{131}, Ile^{135}, and Asp^{138} of αHF are in close contact with SMBD (53), residues Glu^{128}-Val-Glu-Arg^{131} and Lys^{154} contribute to the epitopes of MA-55F4C12 and MA-33H7F1 (61). Indeed, the binding of SMBD results in a further significant increase in the fraction of the substrate reaction (Tables 3 and 4) and a decrease in the k_{lim} (Table 2).

Therefore, the binding of both mAb and SMBD promotes greater restriction of the flexibility of αHF and β-sheet A and results in further stabilization of the intermediate and an increase in the fraction of the substrate reaction. It has been shown that several ligands such as two mAbs (59, 64), mAb-Fab and Vn (44, 45, 49), or even three mAbs (65) not only bind to the same PAI-1 molecule but also affect additively the kinetics of RCL insertion and the stoichiometry of the reaction between PAI-1 and the enzyme. Thus, based on the results of this study, and data obtained previously (45), additivity in the effects of SMBD and Fab or single chain Fv fragment (66) of antibody is anticipated.

### Table 3: Effects of recombinant SMBD on the SI for the reactions of β-trypsin with wild type and NBD P9 PAI-1, tPA, uPA with wtPA-I, and its complexes with MA-55F4C12 and MA-33H1F7

| mAb ligand | MA-55F4C12 | MA-33H1F7 |
|------------|------------|------------|
| None       |            |            |
| SMBD       |            |            |
| None       | SMBD       |            |
| None       | None       |            |

SI represents the number of moles of PAI-1 or its binary and ternary complexes with SMBD and the mAb required for inactivation of 1 mol of proteinase.

*This was estimated by SDS-PAGE of products of reaction of recombinant wtPA-I and its complexes with mAb and SMBD with proteinase.

*This was determined by the titration of fixed amount of complexes of NBD P9 PAI-1 with ligands by proteinase as described previously (45, 59).

*This was calculated from a decrease in fluorescence emission of uPA/PAB due to displacement of PAB with PAI-1 resulting from the reaction with wtPA-I and as described under “Experimental Procedures.”
TABLE 4
Effects of recombinant SMBD and human plasma Vn on the SI for the reaction of tPA with NBD P9 PAI-1, NBD P9 E350A/E351A PAI-1, and their complexes with MA-55F4C12 and MA-33H1F7
SI, representing the number of moles of PAI-1 or its binary and ternary complexes with SMBD and mAb required for inactivation of 1 mol of proteinase was determined by titration of fixed amount of complexes of NBD P9 PAI-1 variants with ligands by proteinase as described previously (45, 59).

| mAb ligand               | MA-55F4C12 |                   | MA-33H1F7 |                   |
|-------------------------|------------|-------------------|-----------|-------------------|
|                         | None       | SMBD              | Vn        | None              | SMBD              | Vn        |
| NBD P9 PAI-1            | 4.2 ± 0.3* | 9.7 ± 0.6         | 19 ± 2**  | 2.1 ± 0.3         | 2.9 ± 0.4         | 6.2 ± 0.7 |
| NBD P9 E350A/E351A PAI-1| 4.1 ± 0.5  | 11 ± 2            | 16 ± 2    | 2.2 ± 0.2         | 4.2 ± 0.5         | 8.5 ± 1.2 |

*Values were taken from Ref. 45.

Disruption of the exosite interactions because of Glu to Ala mutations at the positions P4’ and P5’ of RCL, which results in an increase in \( k_{lim} \) (50), did not affect the SI for the reactions of tPA with complexes of PAI-1 with mAb, SMBD (Vn), or both ligands (Table 4). Thus, formation of the Michaelis complex, the acylation of the enzyme, and breaking of exosite interactions, resulting in LDA (Scheme 1), which occur at the initial docking site (Fig. 1), probably do not affect the partitioning between the inhibitory and the substrate reaction. As a result, exosite interactions as well as LDA are unlikely targets for intermolecular re-direction of the serpin reaction to the substrate pathway. Therefore, the most effective approaches to the neutralization of PAI-1 through intermolecular mechanisms aiming the initial docking site would be using of ligands, which compete with proteinases for PAI-1. An example of successful use of mAbs, which block formation of the Michaelis complex between PAI-1 and tPA or uPA, was described elsewhere (58, 65, 67).

The smallest of the three proteinases, β-trypsin, demonstrated the lowest SI and highest \( k_{lim} \) for PAI-1-mAb complexes (Tables 2 and 3). The correlation between a decrease in the size (\( M_e \)) of the enzyme and a decrease in SI and an increase in \( k_{lim} \) supports the complete insertion of the RCL and translocation of the proteinase to the opposite pole of the PAI-1 molecule rather than the reversible dissociation of the mAb. Such a mechanism was proposed for anti-α-helix F mAb CLB-2C8 (67), which binds to both the active and RCL-inserted forms of PAI-1 with sub-nanomolar affinity (65). Low \( k_{off} \) values (10⁻³–10⁻⁴ s⁻¹ (59, 61, 65)) make the dissociation of anti-α-helix F mAb during the course of RCL insertion unlikely. Indeed, the results of surface plasmon resonance studies of the interaction of PAI-1 bound to immobilized CLB-2C8 with tPA have shown fast binding and dissociation (\( k_{off} > 0.01 \) s⁻¹) of tPA, whereas no dissociation of PAI-1 from the mAb was reported (67). On the other hand, the epitope of the mAb could be reversibly perturbed as a result of tremendous conformational changes accompanying RCL insertion (32, 36, 68–70) and possible movements of α-helix F (62, 63). To the best of our knowledge there is no molecular model of a PAI-1-mAb complex that could provide an insight into the possible mechanism of RCL insertion. Models of both active PAI-1 (71, 72), and its complex with SMBD (Fig. 3) (53) were obtained from studies of a “stable variant” of PAI-1, which contains K154T stabilizing mutation (73). Unfortunately, lysine 154 is a part of epitopes of anti-α-helix F mAbs (MA-55F4C12, MA-33H1F7 (61), and mAb-2 (44)), and its mutation in the stable variant of PAI-1 affects the interaction with the mAb (44).

In contrast to anti-α-helix F mAbs, the affinity of Vn (and SMBD) to the active and RCL-inserted PAI-1 differs up to 3 orders of magnitude (74). Therefore, it is unlikely that SMBD is still bound to the final inhibitory complex or to the cleaved PAI-1 under conditions used in this study. On the other hand, because SMBD (Vn) affects both the \( k_{lim} \) and SI for the reaction of PAI-1-mAb complexes with proteinases, dissociation most likely occurs during the very late steps of RCL insertion.

To explain the effects of SMBD (L1) and mAbs (L2) on the reactions of proteinases (E) with PAI-1 (I), we propose a transient αHF-intermediate \( E(I’LILI2) \) (Scheme 2), which is stabilized when mAb, SMBD, or both ligands are bound to αHF. \( E(I’LILI2) \) forms at the step of the reaction following formation of LDA (E(I’LILI2)) and prior to completing RCL insertion as a strand 4 to the β-sheet A of PAI-1 (Fig. 1). The limiting step of RCL insertion because of the reaction of tPA with the PAI-1-SMBD complex depends on a pattern of exosite interactions at the tPA/PAI-1 interface. As a result, at a pH of 7.4 and 6.2 the rate of the RCL insertion because of the reaction of tPA with PAI-1-SMBD complex is limited by the formation of different intermediates (Scheme 2) \( E(I’LILI2) \) and \( E(I”LILI2) \), respectively. Stabilization of αHF-intermediate by mAbs and SMBD (Vn), which affects RCL insertion, results in an increase in \( k_{k} \) (Scheme 2) and redirection of the reaction toward the substrate pathway. The localization of mAbs and SMBD-binding sites, together with the known importance of αHF in the serpin mechanism (63, 75), supports the restriction of movements of αHF and β-sheet A during the RCL insertion as an origin of additivity in the effects of SMBD (Vn) and mAbs on the kinetics and outcome of PAI-1/proteinase reactions. Thus, the stabilization of αHF-intermediate(s) via intermolecular interactions...
mechanisms resulting in complete re-direction of the PAI-1/protease reaction to the substrate pathway could be considered as a strategy for PAI-1 neutralization. Because the conformational changes of PAI-1, induced by RCL insertion, result in a dramatic decrease in affinity of Vn to PAI-1 (74), SMBD and its analogs, which selectively recognize the active conformation of PAI-1, could be employed for design of PAI-1 inhibitors (71). Thus, the αHF-intermediate (Scheme 2) is a convenient target for in vivo neutralization of PAI-1 through the use of rationally designed multivalent inhibitors composed of ligands interacting with mAb epitopes and the SMBD-binding site of PAI-1. On the other hand, in contrast to the effects on interaction of PAI-1 with uPA and SMBD-binding site of PAI-1, recombinant SMBD affects the reaction of tPA and uPA with Vn molecule. However, the data reported here and in our previous study (45) clearly demonstrate that the conformations of both recombinant SMBD and SMBD as a part of whole human plasma Vn (produced by Promega) are specific for active PAI-1 and are likely the same, and agree with the recent results on the pattern of disulfide bonds in the active conformation of SMBD (76).

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