Vitronectin Functions as a Cofactor for Rapid Inhibition of Activated Protein C by Plasminogen Activator Inhibitor-1

IMPLICATIONS FOR THE MECHANISM OF PROFIBRINOLYTIC ACTION OF ACTIVATED PROTEIN C*

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Activated protein C (APC) is a natural anticoagulant in plasma that down-regulates the coagulation cascade by degrading factors Va and VIIIa. In addition to its anticoagulant function, APC is also known to possess a profibrinolytic property. This property of APC has been attributed to its ability to neutralize PAI-1, thereby increasing the concentration of tissue plasminogen activator in plasma leading to up-regulation of the fibrinolytic cascade. This hypothesis, however, has not been well established, since the concentration of PAI-1 in plasma is low, and its reactivity with APC is very slow in a purified system. Here we demonstrate that vitronectin enhances the reactivity of PAI-1 with APC ~300-fold making PAI-1 the most efficient inhibitor of APC thus far reported ($k_\text{cat} = 1.8 \times 10^3 \text{M}^{-1} \text{s}^{-1}$). We further show that PAI-1 inhibition of the Glu192$\rightarrow$Gln mutant of APC is enhanced ~40-fold, independent of vitronectin, suggesting that vitronectin partially overcomes the inhibitory interaction of PAI-1 with Glu192. Additionally, we show that PAI-1 inhibition of the Lys37-Pro38-Lys39$\rightarrow$Pro-Gln-Glu mutant of APC is severely impaired, suggesting that, similar to tissue plasminogen activator, the basic 39-loop of APC plays a critical role in the reaction. Together, these results suggest that vitronectin functions as a cofactor to promote the profibrinolytic activity of APC.

Activated protein C (APC)$\dagger$ is a vitamin K-dependent serine proteinase in plasma that exhibits both anticoagulant (2, 3) and profibrinolytic (4–8) properties. The mechanism of anticoagulant function of APC is extensively studied. It is established that APC inhibits thrombin generation by limited proteolytic degradation of two essential procoagulant cofactors, Va and VIIIa (2, 3, 9). The physiological significance of APC as an antithrombotic factor is documented by the observation that inherited and acquired deficiency of protein C is associated with venous thromboembolic disease (10, 11). In contrast to its anticoagulant function, the mechanism by which APC exerts its profibrinolytic function is not well established. Several types of whole blood clot lysis assays in vitro have indicated that APC functions as a profibrinolytic factor in a plasminogen activator inhibitor-1 (PAI-1)-dependent manner (6–8). Based on these results, it has been hypothesized that neutralization of this inhibitor by APC increases the concentration of plasminogen activators in plasma leading to up-regulation of the fibrinolytic cascade (6). In support of this mechanism, incubation of active APC, but not diisopropyl fluorophosphate-inactivated APC, with the endothelial cell culture conditioned media or with PAI-1 partially purified from platelets resulted in SDS-stable APC-PAI-1 complexes (7). This has suggested that APC interacts with PAI-1 as other serine proteinases interact with their target serine proteinase inhibitors of the serpin superfamily (12).

Despite the evidence that APC interacts with PAI-1 by a typical serine proteinase-serpin reaction mechanism, no kinetic studies exist in the literature to indicate that human APC can react with PAI-1 in a homologous system. This is probably due to the fact that prolonged incubation of human APC with high concentrations of PAI-1 results in a minimal decline in the activity of the proteinase. In support of this, results of an in vitro clot lysis assay using purified components of the APC anticoagulant pathway have disputed whether this mechanism can contribute to the profibrinolytic property of human APC in vivo (8). Such results have raised the possibility that other factor(s) in blood may be involved in APC up-regulation of the fibrinolytic cascade (8). In the current study, vitronectin, an abundant plasma and platelet glycoprotein (13–15), has been identified as the cofactor that dramatically improves the ability of APC to react with PAI-1. It is shown that vitronectin enhances the reactivity of APC with PAI-1 ~300-fold, revealing PAI-1 to be the most efficient inhibitor of APC reported. The possible mechanism of the APC-vitronectin-PAI-1 interaction was studied by analyzing the kinetics of PAI-1 inactivation of two mutants of APC, in one of which the three basic Lys37-Lys38-Lys39 residues of APC were replaced with Pro-Gln-Glu (KKK/PQE) and in the other Glu192$\rightarrow$Gln (E192Q). The reactivity of KKK/PQE with PAI-1 was dramatically impaired, although the extent of the cofactor effect of vitronectin was not affected. On the other hand, the E192Q mutant of APC reacted efficiently with PAI-1 independent of vitronectin. These results suggest that similar to plasminogen activators, the basic 39-loop of APC is critical for a productive interaction with PAI-1. In contrast, Glu192$\rightarrow$Gln restricts the reactivity of APC with PAI-1 and vitronectin appears to overcome this inhibitory interaction. The possible physiological signifi-
Vitronectin as a Cofactor in PAI-1 Inhibition of APC

RESULTS AND DISCUSSION

APC is an important anticoagulant plasma proteinase that shuts down thrombin generation by limited proteolysis of factors Va and VIIIa, essential cofactors of the extrinsic and intrinsic pathways of the clotting cascade, respectively (2, 21). In addition to down-regulation of coagulation, in vitro (4) and in vivo (5, 6), studies have indicated that APC promotes fibrinolysis by neutralizing PAI-1. This property of APC has been hypothesized to increase the concentration of plasminogen activators in plasma leading to up-regulation of the fibrinolytic cascade (5, 6). Although the profibrinolytic property of APC has been relatively well documented by several types of in vitro whole blood clot lysis assays (6–8), there has been no study in a purified system of the kinetics of the APC-PAI-1 interaction with human proteins. This is due to the fact that human APC reacts with PAI-1 very slowly. As shown in Table I, a second-order inhibition rate constant of $-6 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ for PAI-1 inhibition of APC was determined. This same value was obtained if PAI-1 inhibition of APC was monitored in the presence of 220 nm protein S and 0–50 μM PC/PS or PC/PS/PE vesicles (data not shown). These results are consistent with other data in the literature (8). Therefore, unlike the potent profibrinolytic effect observed for APC in vivo (4), in whole blood clot lysis assays (6), and in endothelial cell culture supernatants (5), kinetic data in the purified system suggest that human APC neutralization of PAI-1 by itself may not significantly contribute to up-regulation of the fibrinolytic cascade. Alternatively, there may be other factor(s) in plasma or in endothelial cell culture supernatants that enhances the ability of APC to interact with PAI-1.

PAI-1 is a serpin, which similar to other inhibitory serpins, inactivates its target proteinases by binding to their active sites through an exposed reactive center loop and undergoes a conformational change that traps the enzymes in inactive, stable complexes (22). Previously, it has been demonstrated that incubation of active APC, but not diisopropyl fluorophosphate-inactivated APC, with endothelial cell culture conditioned media (6, 7) results in SDS-stable APC-PAI-1 complexes, suggesting that PAI-1 interacts with APC by a similar mechanism. Unlike other inhibitory serpins, however, PAI-1 in plasma exists in both active and latent forms (23, 24). The active form of PAI-1 has a half-life, under the conditions used to vitronectin (24). Vitronectin is a glycoprotein that is known to bind PAI-1 with a high affinity to stabilize the active conformation of the serpin (24, 25). Noting the abundance of vitronectin in plasma or platelets (13) and in endothelial cell culture supernatants (26), we hypothesized that vitronectin may function as a cofactor to enhance the reactivity of APC with PAI-1, potentially accounting for its profibrinolytic activity.

### Table I

| Coefficient | Vitronectin | Fold acceleration |
|-------------|-------------|-------------------|
| APC         | $6 \times 10^5$ | 100 |
| APC E192Q   | $3 \times 10^4$ | 300 |
| APC KKK/PQE | $4 \times 10^4$ | 250 |

**Vitronectin as a Cofactor in PAI-1 Inhibition of APC**

The second-order inhibition rate constants ($k_2$ in $\text{M}^{-1} \text{s}^{-1}$) in the absence of vitronectin were determined from the linear slope of the plot of the $k_2$ values versus PAI-1 concentrations (50–2000 nM) as described under "Experimental Procedures." In the presence of vitronectin (600 nM), the maximal inhibition rate constant ($k$) and $K_i$ values were determined by fitting the saturable dependence of $k$ values on PAI-1 concentrations (6–100 nM) to a hyperbolic equation as shown in Fig. 24 and Scheme 1. The $k_2$ values ($m^+1$) were then calculated from the ratio of $k$ to the $K_i$ values. All values are the average of at least three independent measurements ± S.E.

**Experimental Procedures**

**Proteins and Reagents—**Methodologies for expression, purification, and activation of the wild type and Glu$^{192} \rightarrow$ Gln (E192Q) mutant of human protein C were described previously (16). The protein C mutant in which three basic residues, Lys$^{77}$-Lys$^{82}$-Lys$^{89}$, of 39-loop were replaced with Pro-Gln-Glu (KKK/PQE, residues found at the identical region of thrombin) was prepared by the same procedures. All derivatives were expressed in HEP-293 cells and purified to homogeneity on an immunoaffinity column using the Ca$^{2+}$-dependent monoclonal antibody, HPC4, as described previously (17). Protein C (100 μg) was incubated with thrombin (10 μg) in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4 (TBS buffer) containing 5 mM EDTA for 2 h at 37 °C. Activated protein C was separated from thrombin by a fast protein liquid chromatography Mono Q column developed with 40-mL linear gradient from 0.1 to 1.0 M NaCl, 0.02 M Tris-HCl, pH 7.4 fully using a V$\theta$. Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). The pseudo-first-order inactivation rate constants ($k$) were calculated by fitting the time-dependent change of the APC activity to a first-order rate equation of 220 nM protein S and 0–50 μM PC/PS or PC/PS/PE vesicles as described previously (18). The chromogenic substrate for amidolytic analysis (SpPCa) was purchased from American Diagnostica (Greenwich, CT).

**Kinetic Methods—**The PAI-1 inactivation rates of APC derivatives were measured under pseudo-first-order rate conditions by a discontinuous assay method as described previously (19). Briefly, 2 nM APC was incubated at room temperature with 50–2000 nM PAI-1 in 50–μL reactions in TBS buffer containing 2.5 mM Ca$^{2+}$, 0.1 mg/ml bovine serum albumin and 0.1% polyethylene glycol 8000. At the end of the incubation time (5–60 min) 50 μl of SpPCa was added to a final concentration of 0.5 mM. The remaining activity of APC was measured from the rate of chromogenic substrate hydrolysis at 405 nm using a V$\theta$. Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA). The pseudo-first-order inactivation rate constants ($k$) were calculated from the slope of the linear plot of the $k$ values versus PAI-1 concentrations as described previously (19). In all assays, less than 10% chromatographic substrate was utilized.

For the inactivation studies in the presence of vitronectin, first, the cofactor concentration dependence of PAI-1 inactivation of APC was studied by incubating 2 nM APC with 50 nM PAI-1 and 0–2.5 μM vitronectin at room temperature in 50–μL reactions in the same buffer system described above. At the end of the incubation time (5 min), 50 μl of 1 mM SpPCa in TBS buffer was added, and the $k$ values at different APC concentrations were determined as described above. Detailed kinetic studies in the presence of an optimal concentration of vitronectin (600 nM) were then carried out by incubating APC (2 nM) with varying concentrations of PAI-1 (6.25–100 nM) for 2.5–20 min in the same TBS buffer system. The $k$ values were calculated as described above and plotted versus PAI-1 concentrations. The maximal inhibition rate constant ($k$) and $K_i$ values were determined by fitting the saturable dependence of $k$ values on PAI-1 concentrations to a hyperbolic equation as described previously (20).

**Determination of Inhibition Stoichiometry—**The inhibition stoichiometry for PAI-1 inactivation of APC in the presence of vitronectin and APC E192Q in both the absence and presence of vitronectin were determined by titration of 20 nM APC with increasing concentrations of PAI-1 corresponding to PAI-1/ACPC molar ratios of 0–2. In the absence or presence of 600 nM vitronectin in the same TBS buffer system as described previously (20). The residual amidolytic activities of the wild type and mutant enzymes were monitored for up to 12 h at room temperature by the hydrolysis of SpPCa as described above. After completion of the inhibition reactions, the PAI-1/ACPC ratios were plotted versus the residual activities of enzymes, and the inhibition stoichiometry were determined from the x-intercept of the linear regression fit of the inhibition data.
served in previous in vivo and in vitro blood clot lysis assays.

In agreement with this hypothesis, it was found that vitronectin dramatically accelerates the reactivity PAI-1 with APC in a concentration-dependent manner (Fig. 1). The pseudo-first-order rate constant \( k' \) for PAI-1 inactivation of APC at an optimal concentration of vitronectin in large molar excess over PAI-1 was determined at several concentrations of PAI-1 as described under “Experimental Procedures.” Based on reported \( K_d \) values for the PAI-1-vitronectin binary complex interaction, PAI-1 was largely saturated with the cofactor protein under these conditions (27). A saturable dependence of \( k' \) on PAI-1 concentration was observed for APC inhibition in the presence of vitronectin (Fig. 2A). Nonlinear regression analysis of \( k' \) values according to a hyperbolic equation yielded values for the ternary complex dissociation constant, \( K_d \), and the rate constant, \( k \), for stable complex formation as shown in Scheme 1.

\[
\begin{align*}
\text{APC + PAI-1-VN} & \quad \rightarrow \quad \text{APC-PAI-1} + \text{VN} \\
K_d = (22.5 \pm 4.5) \text{ nM} \\
\end{align*}
\]

SCHEME 1

The second-order inactivation rate constant \( k_d \), determined from the ratio of the limiting rate constant, \( k \), to the \( K_d \) value in Scheme 1 was \( 1.8 \pm 0.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \), suggesting that vitronectin enhances the rate of the APC-PAI-1 reaction at least 300-fold (Table I). The observation that in the absence of vitronectin, \( k' \) increases linearly with PAI-1 concentration over the same range (Fig. 2B), indicates that the rate-enhancing effect of vitronectin is due to the promotion of PAI-1-APC encounter complex formation. Similar results were obtained whether the source of APC was from human plasma or from the mammalian cell expression system. Inactivation studies in the presence of excess hirudin further suggested that possible trace contamination of APC with thrombin does not influence the results. Unfractionated heparin maximally accelerated PAI-1 inactivation of APC only 4-fold \( (k_d = 2.4 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}) \) when tested in the presence of 1 \( \mu \text{M} \) heparin. Inhibition kinetic studies with activated Gla-domainless protein C suggested that the rate-accelerating effect of vitronectin requires the Gla-domain of protein C. No effect for vitronectin was detected in PAI-1 inhibition of activated Gla-domainless protein C (data not shown), possibly suggesting that binding of the APC Gla domain to vitronectin was important for the cofactor effect.

The mechanism by which vitronectin may promote the reactivity of PAI-1 with APC was investigated by examining the kinetics of PAI-1 inactivation of two mutants of APC in both the absence and presence of vitronectin. The reactivity of the KKK/PQE mutant of APC with PAI-1 was markedly impaired in both the absence and presence of vitronectin, although the extent of the cofactor function of vitronectin did not appear to be affected with this mutant (Table I). This suggested that the 39-loop of APC is involved in a productive interaction with the reactive site loop of PAI-1 independent of vitronectin. The concentration dependence of PAI-1 inactivation of the mutant in the presence of vitronectin suggested that the \( K_d \) for ternary complex formation was not changed, but the rate constant, \( k \), was impaired with this mutant (data not shown). PAI-1 has two acidic residues at the P4 and P5’ positions (28). Previously, it has been demonstrated that the interactions of the basic residues of the 39-loop of plasminogen activators with these acidic residues is required for an efficient reaction with PAI-1 (29). The same appears to be true for the reaction of the serpin with APC.

Another residue, which is known to restrict the specificity of APC with plasma inhibitors, is Glu192 (16). Previous studies have indicated that substitution of Glu192 with Gln, a residue found at the identical site of trypsin and plasminogen activators, results in a mutant (E192Q) that, unlike wild type APC, rapidly reacts with both the serpin and Kunitz-type family of plasma protein inhibitors, including \( \alpha_\text{1}-\text{antitrypsin} \) and tissue factor pathway inhibitor, TFPI (30). Inhibition kinetic studies with the E192Q mutant of APC revealed that this residue also plays a critical role in restricting the reactivity of APC with PAI-1. As shown in Table I, relative to wild type APC, the reactivity of PAI-1 with APC E192Q was markedly improved by ~40-fold. Thus, similar to plasminogen activators, vitronectin was no longer required for efficient reaction of APC E192Q with PAI-1, although it still enhanced the rate of inhibition ~10-fold (Table I). These results suggest that the cofactor function of vitronectin may in large part involve overcoming potentially inhibitory interactions occurring between the acidic residues in...
the reactive site loop of PAI-1 and the extended catalytic pocket of APC.

Vitronectin is also known to markedly accelerate the reactivity of thrombin with PAI-1 (25, 31). The exact mechanism by which vitronectin accelerates the reactivity of PAI-1 with thrombin is not known. Similar to the reaction with APC (Fig. 1), previous studies of the vitronectin concentration dependence of thrombin inactivation by PAI-1 have indicated that the rate of reaction slightly decreases at higher concentrations of vitronectin, possibly suggesting that vitronectin may function by a template mechanism (31). However, other kinetic studies, employing several mutant thrombin derivatives, have not confirmed such a cofactor mechanism for vitronectin function (20). Thrombin also contains a Glu at position 192. Interestingly, the reactivity of thrombin E192Q with PAI-1 was also markedly impaired independent of vitronectin, suggesting that vitronectin may accelerate PAI-1 inhibition of both proteinases by a similar mechanism (data not shown). In the case of thrombin, thrombomodulin occupancy of exosite 1 protects the proteinase from rapid inhibition by PAI-1 in the presence of vitronectin (20). To determine whether complex formation of APC with its cofactor protein S on membrane surfaces protects it from inhibition by the vitronectin-PAI-1 complex, kinetic studies were also carried out in the presence of human protein S (220 nm) on either PC/PS or PC/PS/PE vesicles in the range of 0–50 μg/ml. However, protein S did not influence the reactivity of APC with PAI-1 under either condition (data not shown). Thrombin is also known to cleave PAI-1 in the presence vitronectin (26). In the case of the APC-PAI-1 reaction, however, a 1:1 stoichiometry was observed for both wild type and E192Q APC in the presence of vitronectin (data not shown).

APC is known to circulate in plasma with a long half-life of 27 min (32). This is thought to be due to a poor reactivity of APC with plasma inhibitors. Protein C inhibitor and α2-antitrypsin were believed to be the best inhibitors of APC in circulation, based on reported second-order rate constants of $-0.6 - 7 \times 10^3 \text{m}^{-1} \text{s}^{-1}$ (33–35) and $-10 \text{m}^{-1} \text{s}^{-1}$ (36), respectively, and the known plasma concentrations of these inhibitors. A poor reactivity of APC with plasma inhibitors may allow basal levels of APC (~2 ng/ml) to circulate as an endogenous anticoagulant under normal physiological conditions (32). APC has been demonstrated to function as a potent anticoagulant at such low concentrations (37). However, APC has been reported to exhibit a poor anticoagulant activity on activated platelets (38, 39). Upon activation, platelets release very high concentrations of both PAI-1 and vitronectin (13–15). Furthermore, specific binding sites for vitronectin on platelets following stimulation have been reported (40). The possible physiological significance of these observations is that the vitronectin-PAI-1 complexes on the platelet surface may inactivate the circulating basal APC to allow factor Va to assemble into the prothrombinase complex to generate thrombin in the initial phase of the blood clotting cascade. This can potentially account for the poor anticoagulant activity of APC on activated platelets. In addition, it has been noted previously that APC resistance assays using frozen, but not fresh plasma, containing platelets are associated with frequent false-positive APC sensitivity ratios (41, 42). It has been hypothesized that components released from platelets during freezing and thawing may serve as substrates and/or inhibitors of APC interfering with this assay (41). Results of the current study suggest that this component is most likely vitronectin-PAI-1 complexes that are released and remain attached to the platelet surface, leading to inhibition of the pro-
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