A Comparison Between Activated Protein C and Des-1-41-Light Chain-Activated Protein C in Reactions With Type 1 Plasminogen Activator Inhibitor

By Candace L. Gladson, Raymond R. Schleef, Bernd R. Binder, David J. Loskutoff, and John H. Griffin

This study investigates the role of the gamma-carboxyglutamic acid (gla) containing domain of activated protein C in interactions with both platelet-derived and purified type 1 plasminogen activator inhibitor (PAI-1). The activity of human platelet PAI-1 was neutralized to the same extent by bovine activated protein C and bovine des-1-41-light chain-activated protein C. Both forms of activated protein C formed SDS-stable, divalent-cation independent complexes with platelet PAI-1, as demonstrated by immunoblotting using antibodies directed to either protein C or PAI-1. Since activated protein C neutralized PAI-1, the potential inhibition of the enzyme by PAI-1 was studied. Purified PAI-1 inhibited the amidolytic activity of bovine-activated protein C and bovine des-1-41-light chain-activated protein C with a $k_2$ of $2.85 \times 10^4 \text{M}^{-1} \text{sec}^{-1}$ for both proteins. These data suggest that the gla domain of activated protein C is not required for neutralization of PAI-1 activity, for complex formation with PAI-1, or for inhibition of the amidolytic activity of activated protein C by PAI-1.

© 1989 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Materials. S2251 (D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride), S2238 (D-phenylalanyl-L-piperocyl-L-arginine-p-nitroanilide dihydrochloride), and S2366 (L-prolyl-L-arginine-p-nitroanilide hydrochloride) were purchased from Calbiochem-Behring Diagnostics (La Jolla, CA). Hirudin (Grade IV purified from leeches, 1,000 to 1,500 U/mg), human thrombin (1,000 U/mL), SP Sephadex C50-120, ovalbumin, bovine serum albumin, protaglandin E$_1$ (PGE$_1$), theophylline (anhydrous), ethylenediamine tetracetic acid (EDTA), and p-nitrophe-nyl-p-guanidinobenzoate hydrochloride were from Sigma Chemical Co (St Louis), Hepes, (N-2-hydroxyethylpiperazine-N'-2-ethylanesulfonic acid), pka at 20°C = 7.55, was purchased from Gibco (Grand Island, NY). Dibutrioehitol was purchased from Calbiochem-Behring Diagnostics (La Jolla, CA). Prestained protein high molecular weight standards were purchased from Bethesda Research Laboratories Life Technologies Inc (Gaithersburg, MD).

From the Research Institute of Scripps Clinic, Department of Immunology, La Jolla, CA, and the Laboratory for Clinical Experimental Physiology at the Department of Medical Physiology, University of Vienna, Vienna.

Submitted November 3, 1988; accepted February 23, 1989.

Support ed in part by the National Institutes of Health Grants R01-HL-24891, TG-HL-07195, HL-16416, HL-16411 and RR08833, and by the Austrian Fund for Promotion of Scientific Research Grant No. 5654.

Address reprint requests to John H. Griffin, PhD, Department of Immunology, IMM7, The Research Institute of Scripps Clinic, 10666 N. Torrey Pines Rd, La Jolla, CA 92037.

The publications costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1989 by Grune & Stratton, Inc.

0006-4971/89/7401-0007 $3.00/0
All other reagents were of the highest commercial grade available unless otherwise specified.

**Proteins.** Bovine PC (b-PC) was purified according to the method of Stenflo, as previously described. B-APC was prepared by activation of PC with human thrombin, according to the procedure of Sakata et al., using the chromogenic substrate S2238 to monitor the appearance of amidolytic activity. Thrombin was removed from APC by chromatography on a Bio-Rex 70 column (Bio-Rad Laboratories, Richmond, CA). No thrombin activity was detected in the b-APC as measured in a bovine fibrinogen clotting assay. b-APC was approximately 95% pure when analyzed on 10% sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) and migrated as a doublet at 58 kDa.

Bovine des-L4-L8 light chain PC or bovine gla-domainless PC (b-GD-PC) was prepared by limited chymotryptic digestion of b-PC, according to the method of Esmon et al. On 10% SDS-PAGE the b-GD-PC appeared at least 95% homogeneous, with the light chain of b-GD-PC migrating 4 kDa faster on reduced gels than the light chain of b-PC, whereas the heavy chains of both species of PC migrated with identical mobility. b-GD-PC was activated by the method used for b-PC with removal of thrombin by immediate Sephadex chromatography. No thrombin activity was detected in the b-GD-APC as measured in a bovine fibrinogen clotting assay over 24 hours. b-GD-APC on 10% SDS-PAGE migrated as a doublet of 54 kDa, and was at least 90% homogeneous.

Human PAI-1 was purified from the conditioned media of the following human cell types: a melanoma cell line (MJJ32) according to the procedure of Wagner and Binder and from a transformed pulmonary fibroblast cell line (SV40 WI-38 VA13 2RA), according to the procedures described by Hekman and Loskutoff. Human melanoma cell PAI-1 demonstrated 1,000 U/mL activity, as measured by inhibition of t-PA and was not treated further. PAI-1 from SV40-transfected WI-38 cells was isolated in a latent form and required activation with denaturants, such as guanidine chloride.

T-PA from a human melanoma cell line was purchased from American Diagnostica Inc (Greenwich, CT) and was >98% single chain with an activity of 450,000 to 550,000 I.U./mg. This t-PA was calibrated to the International t-PA chain with an activity of 450,000 to 550,000 I.U./mg. This t-PA was obtained from Professor Johann Stenflo, University of Lund, Malmo, Sweden, and in immunoblotting experiments did not recognize reduced b-PC or b-GD-PC. Rabbit polyclonal anti-human PAI-1 (anti-PAI-1) was purified from SV40 transformed WI-38 cells was raised by standard techniques and used in immunoblotting studies. Similar results were obtained with antisera to human melanoma cell PAI-1. Monoclonal anti-t-PA (MAB 93) to human melanoma cell t-PA was prepared as described.

Secondary antibodies, goat anti-rabbit IgG and goat anti-mouse IgG (Cappel Laboratories, Malvern, PA.), were 121-labeled to a specific activity of 0.4-0.7 μCi/μg using chloramine T.

**Preparation of platelet releasates.** Human platelet concentrates were purchased from the San Diego Blood Bank. Platelets were isolated from platelet-rich plasma as previously described. The platelet pellet was gently resuspended in 2 mL. Heps buffer (126 mMol/L NaCl, 2.6 mMol/L KCl, 2 mMol/L MgCl2, 20 mMol/L Heps, 5 mMol/L Dextrose, 1 mg/mL ovalbumin, or 1 mg/mL bovine serum albumin, pH 6.5, filtered through a 0.45 μm filter), and was washed by filtration through a Sepharose CL-2B (Pharmacia Fine Chemicals, Uppsala, Sweden) column pre-equilibrated with filtered Heps buffer, pH 7.35, according to the method of Greenberg and Griffin. Three milliliter fractions were collected and counted on a Coulter counter (Coulter Electronics, Inc, Hialeah, FL). One milliliter of each fraction was treated with PGE1, (1 μg/mL final concentration) and theophylline (0.9 mMol/L final concentration) according to the method of Erickson et al. Platelet releasate was obtained after thrombin (0.2 U/mL final concentration) activation of platelets (1.5 minutes, 37°C), followed immediately by addition of hirudin (2.0 U/mL final) and centrifugation at 3,000 rpm (30 minutes, 4°C), as previously described. The supernatant was aliquoted, stored at 70°C and thawed once when used.

**Functional assay for platelet PAI-1.** The functional assay of Korninger et al. for PAI-1 activity in plasma was modified to assay PAI-1 activity in the platelet releasate described above. One unit of PAI-1 activity is defined as the amount of platelets that neutralize 1 IU of t-PA. Eppendorf sample tubes were precoated with 5 μL Heps buffer containing 0.5% ovalbumin, pH 7.35. Platelet releasate (35 μL) and b-APC or b-GD-APC (5 μL, 4-200 μmol/L final concentration in the reaction mixture) were incubated together for 20 minutes at 37°C, t-PA (5 μL, 14-49 IU/mL final concentration in reaction mixture) was added and the mixture was incubated another 8 minutes at 22°C. Sodium acetate (0.1 mol/L, pH 3.9, 50 μL) was added and the mixture incubated 10 minutes, 22°C to neutralize other protease inhibitors. The samples were centrifuged (1,000 × g, 15 seconds) and transferred to a 96-well flat bottom microtiter plate (Costar, Cambridge, MA). Substrate buffer (200 μL, 0.15 mol/L Tris-HCL, 0.01% Tween, 0.02% sodium azide, 3% ovalbumin, pH 7.5) containing 0.2 μmol/L plasminogen, 0.8 mMol/L S2251, and 83.3 μg/mL cyanogen bromide fibrinogen fragments, all final concentrations, was immediately added. The concentration of cyanogen bromide fibrinogen fragments was previously determined to be optimum. The absorbance at 405 nm was measured over time (22°C in an ELISA plate reader, EL-309 (Bio-Tek Instruments, Winooski, VT). The rate of plasmin formation (nmol/L/min) and the approximate Km, for plasmin generated in the assay (0.033/sec) were determined from the rate of cleavage of S2251 by human active site titrated plasmin (5-50 nmol/L final concentration). The Km for the substrate plasminogen and the enzyme t-PA was 0.4-1.4 μmol/L in the absence of PAI-1 or platelet releasate. The Km was determined by standard procedures using

nous hydrolysis and deacylation of the enzyme. b-APC and b-GD-APC were 0.92 mol/mol and 0.90 mol/mol active, respectively. Plasminogen was 0.66 mol/mol activatable.

**Antibodies.** Rabbit polyclonal anti-bovine PC (anti-b-PC) was a gift from Professor Johann Stenflo, University of Lund, Malmo, Sweden, and in immunoblotting experiments did not recognize reduced b-PC or b-GD-PC. Rabbit polyclonal anti-human PAI-1 (anti-PAI-1) was purified from SV40 transformed WI-38 cells was raised by standard techniques and used in immunoblotting studies. Similar results were obtained with antisera to human melanoma cell PAI-1. Monoclonal anti-t-PA (MAB 93) to human melanoma cell t-PA was prepared as described. Secondary antibodies, goat anti-rabbit IgG and goat anti-mouse IgG (Cappel Laboratories, Malvern, PA.), were 121-labeled to a specific activity of 0.4-0.7 μCi/μg using chloramine T.

Plasminogen was activated with urokinase (5 smol/L plasminogen activator, 0.002 mol/L calcium chloride, pH 8.3, and nitrophenyl p-nitrophenyl-p-guanidinobenzoate (NPGB) as described by Chase and Shaw. The titration was performed in 0.1 mol/L barbital buffer, 0.002 mol/L calcium chloride, pH 8.3, and nitrophenyl release was monitored at 405 nm in a Cary 210 spectrophotometer. The number of active sites/mol of plasmin from urokinase activation of plasminogen was determined with NPGB, and the titration was performed in 0.1 mol/L barbital buffer, pH 8.3, with nitrophenyl release monitored at 405 nm. The active site-dependent hydrolysis of the titrant was determined by extrapolation to correct for sponta-
three concentrations of both t-PA and plasminogen, followed by construction of a double reciprocal Lineweaver-Burk plot, as previously described.31

Immunoblotting studies.20,21 Proteins were electrophoresed on 1.8 x 0.1 x 16 cm 4% SDS polyacrylamide stacking gels, and 12 x 0.1 x 16 cm 7.5% SDS polyacrylamide separating gels according to the method of Laemmli.42 After fractionation by SDS-PAGE, proteins in the gels were electrophoretically transferred to nitrocellulose paper43 and blocked for one hour in "Blotto"44 (3% nonfat dry milk) or 1% casein (0.05 mol/L Tris, 0.15 mol/L NaCl, 1% casein, pH 7.4). The nitrocellulose membranes were then incubated for four hours (22°C) in the indicated first antibody (rabbit anti-PAI-1 diluted 1:250, rabbit anti-b-PC diluted 1:4000, or mouse monoclonal anti-t-PA 6.7 ng/mL, diluted in Blotto or 1% casein). After washing three times with Blotto or 1% Casein, the sheets were incubated (1.0 hour, 22°C) with either 125I-labeled goat anti-rabbit IgG25 or 125I-labeled goat anti-mouse IgG,28 washed extensively, and subjected to autoradiography using Kodak (Rochester, NY) X-Omat film.

Amidolytic assay. b-APC or b-GD-APC (4 μL) was incubated for 5-60 minutes at 37°C with PAI-1 (6 μL) in a reaction mixture containing 0.05 mol/L Tris, 0.15 mol/L NaCl, 2 mmol/L CaCl2, 2 mmol/L MgCl2, 3 mg/mL ovalbumin, pH 7.4. Samples were then briefly centrifuged (10 seconds, 22°C, 1000 x g) and transferred to a 96-well flat-bottom microtiter plate (Costar, Cambridge, MA). Substrate was added (180 μL, 1.0 mmol/L S2238, 0.05 mol/L Tris-Base, 0.15 mol/L NaCl, 2 mmol/L CaCl2, 2 mmol/L MgCl2, 0.05% sodium azide, 3% bovine serum albumin, 0.04% Tween 80, pH 7.4) and the change in absorbance at 405 nm was measured (0-60 minutes, 37°C) in the ELISA plate reader. The percent residual amidolytic activity was calculated as the decrease in absorbance seen in the presence of APC incubated with PAI-1 compared to APC alone. The k2 was determined by linear regression of the slope from a semi-log plot v time for times ranging from 0 to 60 minutes.41 The second order rate constant (k2) was calculated as follows:

\[ k_2 = \frac{[\text{PAI}_1\text{dimer}]}{[\text{PAI}_1\text{dimer}]} \times (2.303)^41 \]

RESULTS

Neutralization of platelet PAI-1 by b-APC or b-GD-APC. To study the PAI-1 activity in platelets, we modified the functional assay previously described by Korninger et al.49 As shown in Fig 1, the releasate obtained from 0.5 x 108 platelets inhibited 14 IU/mL of t-PA. The inhibitory effects related linearly to the concentration of platelet releasate in the mixture, since a twofold increase in the quantity of releasate inhibited 28 IU/mL of t-PA. As previously reported, the PAI-1 activity of a releasate was increased further (twofold) on addition of 1.0% SDS (10 minutes, 37°C) followed by 5.0% Triton X-100,45,46,47 suggesting that PAI-1 in this platelet releasate was partially in a latent form. In contrast, the supernatant from platelets treated with inhibitors of platelet activation (PGE, and theophylline) failed to inhibit t-PA in this assay. Controls in the absence of t-PA (ie, releasate with or without b-APC/b-GD-APC) demonstrated a minimal absorbance change over time.

To examine the effect of APC on platelet PAI-1 activity, b-APC was preincubated with platelet releasate and PAI-1 activity was determined. Figure 2A indicates the dose-dependent neutralization of PAI-1 activity by b-APC. For example, 50% neutralization of PAI-1 in the releasate obtained from 1.1 x 108 platelets was achieved by approximately 35 nmol/L b-APC. We next compared the effects of b-GD-APC and b-APC on PAI-1 activity (Fig 2B). b-GD-APC produced a similar dose-dependent neutralization of the platelet PAI-1 activity in the releasate obtained from 1.1 x 108 platelets.

Complex formation between platelet PAI-1 and both b-APC and b-GD-APC. Immunoblotting studies were performed to analyze the ability of b-APC and b-GD-APC to form SDS-stable complexes with platelet PAI-1. Initially, we examined the interactions at concentrations of b-APC or b-GD-APC that neutralized 50% of the PAI-1 activity (Fig 2) in the releasate of 1.1 x 108 platelets. As previously reported,21 b-APC migrated with an M, of 58,000 (panel A, lane 2). An additional immunoreactive band was visible at 107 kDa when b-APC was incubated with platelet releasate and immunoblotted with either anti-b-PC (panel A, lane 3) or anti-PAI-1 (panel B, lane 3). This high M, immunoreactive band was not detected when platelet releasate alone was tested (panels A and B, lane 1). A high M, immunoreactive band (104 kDa) also was detected when b-GD-APC was incubated with platelet releasate and immunoblotted with either anti-b-PC (panel A, lane 3) or anti-PAI-1 (panel B, lane 3). This high M, immunoreactive band was not detected when platelet releasate alone was tested (panels A and B, lane 1). A high M, immunoreactive band (104 kDa) also was detected when b-GD-APC was incubated with platelet releasate and immunoblotted with either anti-b-PC (panel A, lane 5) or anti-PAI-1 (panel B, lane 4). The b-GD-APC alone migrated at 54 kDa (panel A, lane 4), and a small amount of material appeared with a mobility of 43 kDa (Fig 3, panel A, lanes 4 and 5), which presumably represents cleaved b-GD-APC. Furthermore, b-APC or b-GD-APC formed complexes of similar M, with platelet PAI-1 in the presence of 6 mmol/L EDTA (data not shown), suggesting that complex formation was divalent-dependent. The immunoreactive bands at 107 and
104 kDa corresponded to the approximate sum of the molecular weights of b-APC or b-GD-APC (58 and 54 kDa, respectively) and PAI-1 (50 kDa).

Treatment of reaction mixtures containing platelet PAI-1 and either b-APC or b-GD-APC with dithiothreitol, followed by SDS-PAGE and immunoblotting, revealed a high Mr band (89 kDa) using anti-PAI-1 antiserum (Fig 3, panel C, lanes 2 and 3, respectively). This immunoreactive material represents complexes lacking the light chain of APC, since disulfide reduction of b-APC or b-GD-APC is known to dissociate the light chain (residues 1-155 or 42-155, respectively) from the heavy chain (residues 158-419, 39 kDa), and the latter contains the active site region of this protease. Nonactivated b-PC or b-GD-PC failed to complex with platelet PAI-1 (data not shown). In control experiments, when t-PA (30 nmol/L) was incubated with platelet releasate, an immunoreactive band at 107 kDa was formed (panel B, lane 5). Purified PAI-1 (33.4 ng) yielded a single immunoreactive band at 50 kDa (panel B, lane 6), which co-
grated with the 50 kDa band in platelet releasate (panel B, lane 1). The supernatant from PGE, and theophylline-treated nonactivated platelets did not react with anti-PAI-1 (data not shown).

Effect of b-APC on complex formation between t-PA and platelet PAI-1. Competition studies were performed to determine whether b-APC could prevent t-PA from complexing with platelet PAI-1 (Fig 4, panel A). As previously reported,\textsuperscript{24} immunoblotting for t-PA revealed a single band at M, 60,000 using mouse monoclonal anti-t-PA (lane 1). An immunoreactive band at 107 kDa was clearly visible when t-PA (24 nmol/L) was incubated with platelet releasate (8 minutes, 22°C) (lane 2). Complex formation was effectively inhibited when platelet releasate was preincubated with increasing amounts of b-APC for 20 minutes, 37°C, followed by incubation with t-PA (8 minutes, 22°C) (panel A, lanes 3 and 4). Controls consisting of platelet releasate alone, b-APC incubated with platelet releasate, or b-APC alone are shown in lanes 5, 6, and 7, respectively.

The reciprocal experiment was also performed to determine whether t-PA could prevent b-APC from complexing with platelet PAI-1. Figure 4, panel B (anti-b-PC) shows the immunoreactive band at 107 kDa (lane 2) resulting when b-APC (30 nmol/L) was preincubated with similar quantities of platelet releasate (20 minutes, 37°C). However, no complexes were detected when platelet releasate was preincubated with t-PA (24 nmol/L) (8 minutes, 22°C), followed by incubation with b-APC (30 nmol/L) (20 minutes, 37°C) (panel B, lane 3). Furthermore, when t-PA (24 nmol/L) and b-APC (30 nmol/L) were simultaneously incubated with platelet releasate (20 minutes, 37°C), no visible complexes of b-APC and PAI-1 formed (panel B, lane 4).

Inhibition of the amidolytic activity of b-APC or b-GD-APC by PAI-1. The above experiments have shown that b-APC and b-GD-APC neutralize and form complexes with platelet PAI-1 in a similar manner. To quantitate in more detail the interaction between APC and PAI-1, we have studied whether purified PAI-1 directly inhibits the amidolytic activity of APC. When b-APC or b-GD-APC was incubated with PAI-1, a time-dependent decrease in the residual APC amidolytic activity was observed for both forms of APC (Fig 5). At three different concentrations of PAI-1 (3.4, 7.5, and 12 nmol/L), the observed rate constants for the inhibition of the amidolytic activity of b-APC (3.32 nmol/L) or b-GD-APC (3.84 nmol/L) were co-linear (Fig 5, inset). Calculation of an approximate second-order rate constant (k\textsubscript{2}) for b-APC or b-GD-APC revealed a k\textsubscript{2} of 2.85 x 10\textsuperscript{-5} M\textsuperscript{-1} s\textsuperscript{-1}. In the presence of 8 mmol/L EDTA the k\textsubscript{2} decreased by 30% for b-APC and by 10% for b-GD-APC based on a 60 minute incubation at 37°C (data not shown). The addition of phospholipid (10 µg/mL, 80% phosphatidyl choline, 20% phosphatidyl serine) and bovine protein S (15-fold molar excess over b-APC) in the presence of CaCl\textsubscript{2} (2 mmol/L) did not enhance the inhibition of b-APC (8.0 and 12 nmol/L) amidolytic activity by PAI-1 (6.0 nmol/L). Also, the inhibition of human APC (h-APC) (4.0, 8.0, and 16 nmol/L) amidolytic activity by PAI-1 (6.0 nmol/L) was not enhanced by the addition of phospholipid (10 µg/mL) and human protein S (15-fold molar excess over
**DISCUSSION**

The balance between blood coagulation and fibrinolysis is crucial for vascular hemostasis and blood flow. APC is a pivotal molecule in this balance since it can modulate both the coagulation and fibrinolytic pathways as demonstrated in several animal model systems of thrombosis. Neutralization of endothelial cell PAI-1 activity by complex formation with APC provides at least one explanation for the recently shown profibrinolytic effect of APC. PAI-1 is a key regulatory protein in the fibrinolytic cascade and functions by inhibiting both tissue-type and urokinase-like plasminogen activators. This study was undertaken to delineate the role of the gla domain of APC in its interaction with PAI-1. In addition to investigating APC-neutralization of PAI-1 activity, functional assays were also employed to assess the ability of PAI-1 to inhibit directly the amidolytic activity of APC. b-APC was used for gla domain structure-function studies since it demonstrates a more marked neutralization of human platelet PAI-1 compared to h-APC. Since bovine and human PC are highly homologous, the reactions between PAI-1 and b-APC or h-APC are most likely extremely similar.

Since proteolytic neutralization of a serine protease inhibitor can occur without reciprocal inhibition of the enzyme, as in the case of elastase inactivation of antithrombin III, studies of the inhibition of APC amidolytic activity by PAI-1 were performed. The enzyme-activity of both b-APC and b-GD-APC was inhibited by PAI-1 with a $k_2$ of $2.85 \times 10^6$ M$^{-1}$sec$^{-1}$. This inhibition of APC appears to be associated with complex formation.

The equally efficient inhibition of b-APC and b-GD-APC amidolytic activity by PAI-1 suggests that the gla domain of b-APC may not be required for the APC neutralization of PAI-1. Indeed, our results show that 35 nM b-APC or b-GD-APC produces a 50% neutralization of PAI-1 activity in the releasate from $1.1 \times 10^9$ platelets. The report of de Fouw et al., which found no enhancement of the h-APC-mediated inactivation of PAI-1 in the presence of phospholipid and protein S, implied but did not demonstrate that the gla domain is not required, based on the assumption that APC binds to phospholipid through the gla domain.
Protein S is a vitamin K-dependent protein that enhances the anticoagulant action of APC. In contrast, D’Angelo et al. described the ability of h-APC to neutralize an inhibitory activity directed against urokinase, which was not neutralized by GD-APC. The differences in these observations may be explained by the presence of phospholipid vesicles in the experiments of D’Angelo et al. Available data indicate that APC binds to negatively charged phospholipids in a divalent-cation dependent manner with a Kd of 150 nmol/L, whereas GD-APC presumably exhibits greatly reduced affinity for phospholipids under these conditions. Since PAI-1 has been recently reported to interact with phospholipids, it is possible that the conformation of PAI-1 may be altered such that this inhibitor is not inactivated by solution-phase GD-APC. This may be analogous to the situation observed for phospholipid-bound Factors Va and VIIIa, which are not inactivated by solution-phase GD-APC, but readily inactivated by phospholipid-bound APC. In the absence of phospholipids, the ability of both APC and GD-APC to inactivate Factor Va similarly would be consistent with our observation that the gla-domain is not an essential component for solution-phase APC:PAI-1 interactions. It should be noted that APC from warfarin-treated individuals, i.e., molecules that are only partially carboxylated, might not reciprocally inhibit PAI-1 in the same manner as GD-APC. In addition, it should be noted that the ability of phospholipid vesicles to stimulate the activity of latent PAI-1 by as much as 50-fold may complicate the interpretation of studies focusing on the direct proteolytic inactivation of PAI-1. The ability of the local calcium ion concentration to also modulate the phospholipid-activation of PAI-1 is further evidence for the complex interactions that may occur in an
Fig 5. Effect of PAI-1 on the amidolytic activity of b-APC and b-GD-APC. PAI-1 (12 nmol/L) was incubated with b-APC (3.22 nmol/L; ○) or b-GD-APC (3.84 nmol/L; ●) for the indicated periods at 37°C, substrate S2366 was added and the A405 nm was measured over time. The change in absorbance over time was proportional to the amidolytic activity of APC. Inset, three different concentrations of PAI-1 (3.4, 7.5, and 12 nmol/L) were incubated (0-60 minutes, 37°C) with b-APC (3.32 nmol/L; ○) or b-GD-APC (3.84 nmol/L; ●), followed by the addition of S2366 and measurement of the A405 nm over time. The kobs was calculated as described in Materials and Methods.

assay system using phospholipid vesicles, calcium ions, and PAI-1.

SDS-stable complexes between platelet PAI-1 and either b-APC or b-GD-APC can be detected by immunoblotting at enzyme concentrations (~35 nmol/L) that neutralize approximately 50% of PAI-1 in the releasate of 1.1 x 10^8 platelets. Consistent with this observation is the report of de Fouw et al[23] describing complex formation between b-APC and human endothelial cell PAI-1, and our previous finding of complexes between b-APC and bovine endothelial cell PAI-1.[24] Our present data extends these observations and demonstrates that the heavy chain of b-APC or b-GD-APC can be detected in a complex with platelet PAI-1 following disulfide reduction and SDS-PAGE. This interaction may represent a covalent bond between the active site region of APC and PAI-1. Although a high percentage of platelet PAI-1 was neutralized by both forms of APC, only a small amount of SDS-stable complexes was detected. Dissociation of complexes between t-PA and PAI-1 is routinely observed during SDS-PAGE[48] and is believed to occur by nucleophilic attack on the acyl-enzyme complex by the Tris molecule at high pH. On the other hand, it is also known that acylation of a protease is not required for an inhibitor to neutralize a protease, thus a stable but nonacylated inhibitory complex can exist.[49] In addition, an equilibrium between the nonacylated enzyme-inhibitor complex and the acylated enzyme-inhibitor complex has been observed for several plasma proteases and their inhibitors.[50] Thus, the possibilities exist that either minimal SDS-stable covalent complex formation occurs under the conditions described, or the majority of the APC:PAI-1 complexes are SDS-labile and dissociation occurs during electrophoresis.

The potential importance of interactions between APC and platelet PAI-1 is strengthened by the data obtained from competition experiments followed by immunoblotting analysis (Fig 4). For example, b-APC preincubated with platelet releasate prevents complex formation between t-PA and platelet PAI-1. Our data indicate that APC-mediated inhibition of t-PA and platelet PAI-1 complex formation occurs when b-APC is in a 2.5-fold molar excess over t-PA. This effect is probably not a result of t-PA interacting with b-APC since this protease has previously been demonstrated not to alter the activity of t-PA.[25] Thus, this situation may be physiologically relevant since plasma concentrations of PC activation peptide are 0.48 nmol/L in acute deep venous thrombosis,[17] and normal plasma concentrations of t-PA antigen, including t-PA complexed to PAI-1, are on the order of 0.04-0.1 nmol/L.[35] To confirm that b-APC can compete with t-PA for binding to PAI-1, we have studied the inhibition of b-APC or b-GD-APC by purified PAI-1, and find a 100-1,000-fold smaller kD (Fig 5) than reported for PAI-1 inhibition of t-PA.[24,25] Exogenous APC has been shown to prevent thrombosis in animal model studies at a plasma concentration of approximately 10-20 nmol/L.[54] In this situation, APC would be expected to be present at approximately a 100-1,000-fold greater concentration than t-PA.[55]

In summary, the data presented here demonstrate that b-APC, independent of the gla domain, and PAI-1 neutralize each other in a reciprocal manner and form SDS-stable complexes that may have physiologic significance under certain circumstances.

ACKNOWLEDGMENT

The authors thank Professor Johann Stenflo for kindly providing the polyclonal anti-bovine PC, Drs Jan Rosing and Guido Tans for the gift of bovine protein C in crude preparations, and Leslie Sherry, Peggy Tayman, and Lisa Johnson for secretarial assistance.
REFERENCES


27. de Fouw NJ, de Jong YF, Haverkate F, Bertina RM: Activated protein C increases fibrin clot lysis (purified system) by inactivating plasminogen activator inhibitor: No evidence for a cofactor role of protein S. Fibrinolysis 2:129, 1988 (suppl 1) (abstr)


34. van Mourik JA, Lawrence DA, Loskutoff DJ: Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. J Biol Chem 259:14914, 1984


41. Hekman CM, Loskutoff DJ: Kinetic analysis of the interac-
tions between plasminogen activator inhibitor 1 and both urokinase and tissue plasminogen activator. Arch Biochem Biophys 262:199, 1988


44. Levin EG: Quantitation and properties of the active and latent plasminogen activator inhibitors in cultures of human endothelial cells. Blood 67:1309, 1986


A comparison between activated protein C and des-1-41-light chain-activated protein C in reactions with type 1 plasminogen activator inhibitor

CL Gladson, RR Schleef, BR Binder, DJ Loskutoff and JH Griffin