Inhibition and Complexation of Activated Protein C by Two Major Inhibitors in Plasma

By Mary J. Heeb, Francisco España, and John H. Griffin

To determine the major physiologic inhibitors of activated protein C (APC), plasma was incubated with APC or with Protac C and subjected to immunoblotting. APC-inhibitor complexes gave two major bands reacting with antiprotein C antibodies when immunoblotted on nondenaturing gels, and additional minor bands that varied between serum and plasma. Formation of one of the two major bands of APC:inhibitor complex, but not the other, was stimulated by heparin and only this band reacted with antibodies to the previously described APC inhibitor that is here designated PCI-1. Plasma immunodepleted of PCI-1 formed complexes with APC as visualized with antiprotein C but not anti-PCI-1 antibodies, and exhibited heparin-indepent inhibition of APC activity, providing evidence for the existence of a second major physiologic APC inhibitor, PCI-2. Formation of APC:PCI-2 complexes in PCI-1-depleted plasma paralleled inhibition of APC amidolytic activity. PCI-2 was separated from PCI-1 and partially purified using column chromatography. PCI-2 formed inactive complexes of approximately 110,000 molecular weight (mol wt) with APC suggesting that PCI-2 has an approximate mol wt of 50,000. Thus, inhibition of APC in plasma involves two major distinct 50,000 mol wt inhibitors, the heparin-dependent PCI-1 and the heparin-independent PCI-2.

© 1989 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Materials. Reagents were of the best grade available. Electrophoresis reagents and nitrocellulose paper (NCP) were purchased from Bio-Rad, Richmond, CA; the electrophoretic equipment was from Hoefer Scientific, San Francisco; Na137I was from Amersham, Arlington Heights, IL; ovalbumin, benzamidine, chloramine-T, and sodium metabisulfite were from Sigma Chemical Co, St Louis; prestained molecular weight markers were from Bethesda Research Laboratories, Gaithersburg, MD; ethylene diamine tetraacetate (EDTA) was from Baker, Philipsburg, NJ; p-amidino-phenyl-methyl-sulfonyl fluoride (p-APMSF) was from Chemicon, El Segundo, CA; S-2366 and 1-2581 were from Kabi, Stockholm; heparin was from Elkins-Sinn, Inc, Cherry Hill, NJ; polybrene was from Aldrich, Milwaukee; dithiothreitol (DTT), affinity purified goat anti-mouse IgG, swine anti-goat IgG, and goat anti-rabbit IgG were from Boehringer Mannheim Biochemicals, Indianapolis; derivatized Sepharose was from Pharmacia, Uppsala, Sweden; plasma immunodepleted of protein C was obtained from George King Biomedical, Inc, Overland Park, KS; and Protac C (a protein C activator from snake venom) was from American Diagnostica, Greenwhich, CT. Film (X-Omat XRP-1) and enhancing screens were from Eastman Kodak, Rochester, NY. A sample of rabbit anti-PCI-1 antiserum was a generous gift of Dr Koji Suzuki, Mic University School of Medicine, Tsu City, Japan, and a sample of monoclonal antibody to PCI-1 was graciously donated by Dr Johan Stenflo, University of Lund, Malmo, Sweden.

General methods. Electrophoresis was performed in 3-mm thick slab gels, 18 × 15.5 cm, according to Laemmi16 for sodium dodecyl sulfate (SDS) gels, or according to Ornstein and Davis as described by Hames17 for nondenaturing gels. The stacking gel contained 0.5 mmol/L EDTA and the sample contained 20 mmol/L EDTA. Secondary antibodies were 125I-labeled to a specific activity of 0.4 to 0.7 μCi/μg and protein C and PCI were 125I-labeled to a specific activity of 2 to 10 μCi/μg using chloramine T.22 For normal human plasma (NHP), blood was collected from 17 healthy male volunteers in ¼ volume 0.11 mol/L trisodium citrate, and the pooled plasma was snap frozen in individual aliquots. For experiments comparing...
whole blood serum to serum derived from plasma, blood was taken from an individual donor. Five mL of this blood was collected without citrate and allowed to clot overnight at 4°C before separation of the serum, and 10 mL of the blood was used to prepare plasma. Five milliliters of the plasma was treated with 0.5 mL of activated partial thromboplastin time reagent (General Diagnostics, Morris Plains, NY) and 0.5 mL of 0.2 mol/L CaCl₂ and allowed to clot with gentle tilting of the tube. The resultant preparations were frozen in aliquots and designated serum from whole blood, serum from plasma, and plasma.

**Purified proteins and antibodies.** Preparation of polyclonal and monoclonal antibodies and electroimmunoassays for protein C were as previously described. Purification of protein C and preparation of APC were as previously described with the exception that heparin-Sepharose chromatography was substituted for dextran sulfate-Sepharose chromatography. PCI-1 was purified as previously described, except that heparin-Sepharose was substituted for dextran sulfate-Sepharose and the gel filtration step was omitted. Antibodies to PCI-1 were raised by subcutaneous injection into eight rabbits, and electroimmunoassays for protein C were performed by absorbing normal plasma or protein C-depleted plasma with control plasma (Fig 1, lane 1), a new band at 14,000 mol wt was seen that comigrated with protein C antigen. These data suggest that in plasma there was a covalent complex formed between PCI-1 and the heavy chain of APC (lanes 3 through 13) were equally well detected on SDS gels by the monoclonal antibody, and this method was used in the work presented here, except that the buffer used in all steps was 0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, 1% casein, 0.02% NaN₃, pH 7.4. Antilight chain monoclonal antibody to protein C (C3 or C10) was used at 3 μg/mL for all nonreduced immunoblots for protein C, and goat antiserum to protein C diluted 1:400 was used for reduced immunoblots for protein C. Purified rabbit IgG antibody to PCI-1 was used at a dilution equivalent to 1:600 of the original antiserum. Whenever possible, immunoblotting detection was accomplished by the use of radiolabeled purified antigen (ie, [125I]-PCI-1 or [125I]-protein C) rather than with [125I]-secondary antibody, in order to enhance specificity. Dot immunobinding was performed as for immunoblotting, omitting the electrophoresis, and spotting 5 μL of sample directly on the cellulose nitrate paper instead of electrotransferring the sample from electrophoretic gels.

**RESULTS**

**Complexes of activated protein C in plasma.** To identify APC complexes formed in plasma, purified APC was added to plasma immunodepleted of protein C in some experiments and in others normal plasma was incubated with Protac C, a partially purified protein C activator from the venom of *Agkistrodon contortrix contortrix*, to generate APC in plasma. Following incubation of the reaction mixture for five or 60 minutes, one or more new bands containing protein C antigen was detected in each case by immunoblotting with antibodies to protein C under both denaturing (Figs 1 and 2) and nondenaturing (Fig 3) conditions. The new band had an apparent mol wt of 110,000 on a nonreduced SDS gel (Fig 1, lanes 5 and 11) and 84,000 on a reduced SDS gel (Fig 2, lanes 4, 7, 5, 8) and compared with APC at 60,000 on a nonreduced gel or to the heavy chain of APC at 40,000 on a reduced gel. When protein C deficient plasma to which APC had been added (Fig 1, lane 2) was incubated for one hour and then immunoblotted for PCI-1 antigen and compared with control plasma (Fig 1, lane 1), a new band at 110,000 mol wt was seen that comigrated with protein C antigen. These data suggest that in plasma there was a covalent complex formed between PCI-1 and the heavy chain of APC as described by Suzuki et al for the purified molecules.

To see whether the monoclonal anti-protein C antibodies recognized free and complexed APC with the same efficiency, the region of the NCP containing the bands in Fig 1 (lanes 3 through 13) were cut and counted. The radioactivity for APC alone (Fig 1, lane 8) was 11,869 cpm, and the sum of the radioactivities for complexed APC and free APC in lanes 4 through 7 was 11,829, 11,869, 11,415, and 11,415 cpm, respectively. Thus, free APC and complexed APC seem to be equally well detected on SDS gels by the monoclonal anti-protein antibodies under the conditions used. Under this...
Fig 1. Immunoblots of APC:PCI complexes in plasma from a nonreduced SDS gel. A 7% to 11% gradient SDS gel and nonreducing conditions were used. Samples containing 7.5 μL of normal or protein C immunodepleted plasma were incubated at 37°C with 7.5 μL TBS (lanes 1, 3, 9), or with 7.5 μL of 4 μg/mL APC in TBS, 1.25% ovalbumin (lanes 2, 4, 6), or with APC and 1 μL of heparin (160 U/mL) (lanes 5, 7), or with 7.5 μL Protac C (lanes 10, 12), or with Protac C and 1 μL heparin (160 U/mL) (lanes 11, 13). Lane 8 contained 7.5 μL of purified APC alone plus 7.5 μL TBS. Samples were incubated for one hour at 37°C except for samples in lanes 6, 7, 12, and 13, which were incubated for five minutes at 37°C before addition of SDS. Lanes 1 and 2 were analyzed using rabbit anti-PCI-1 followed by 125I-secondary antibody. Lanes 3 through 13 were analyzed using monoclonal antibodies against protein C (C3 and C10), followed by 125I-protein C. The position of mol wt markers is given in the left margin. The complexes of APC and PCI(s) are seen at approximately 110,000 apparent mol wt.

Fig 2. Immunoblot of APC:PCI complexes in plasma from a reduced SDS gel. A 7% to 11% gradient SDS gel was used and all samples were reduced with 1 mmol/L DTT. The lanes contained the following: lane 1, 7.5 μL of 4 μg/mL protein C in TBS, 1.25% ovalbumin, and 7.5 μL TBS; lane 2, 7.5 μL of 4 μg/mL APC in TBS, 1.25% ovalbumin, and 7.5 μL TBS; lanes 3-5, 7.5 μL of plasma immunodepleted of protein C incubated with 7.5 μL of TBS (lane 3), or with 7.5 μL APC (lane 4), or with 7.5 μL APC and 1 μL 160 U/mL heparin (lane 5); lanes 6-8, 7.5 μL NHP incubated with 7.5 μL TBS (lane 6), or with 7.5 μL Protac C (lane 7), or with 7.5 μL Protac C and 1 μL 160 U/mL heparin (lane 8). All samples were incubated for one hour at 37°C before immunoblotting. Detection was with goat polyclonal anti-protein C antibodies followed by 125I-protein C.

assumption, the proportion of complexed APC was 9% after five minutes of incubation of APC with plasma and 33% after one hour (Fig 1, lanes 6 and 4). Heparin at 10 U/mL promoted an increase in complex formation to 23% at five minutes and 44% at one hour of incubation (Fig 1, lanes 7 and 5). In other experiments not shown, heparin stimulation of complex formation in five minutes of incubation was maximal at 10 U/mL heparin, but even doses in the thera-

Fig 3. Immunoblots of APC:PCI complexes from a nondenaturing gel with two antibodies. Samples were incubated as in Fig 2. Detection in lanes 1 through 3 was with monoclonal anti-protein C (C3) antibodies followed by 125I-protein C, and detection in lanes 4 through 6 was with rabbit anti-PCI-1 followed by 125I-PCI-1.
plasmatic range of 0.1 to 0.5 U/mL caused a twofold stimulation in complex formation. Incubation of NHP with Protac C resulted in the appearance of <2% of APC complexed in five minutes and 35% of APC complexed after one hour (Fig 1, lanes 12 and 10). Heparin was inhibitory of Protac C-dependent APC complex formation during a one-hour incubation with only 13% of the radioactivity in the region of complexed APC (Fig 1, lane 11). Similar results were seen for reaction mixtures analyzed on reduced immunoblots (Fig 2). Heparin appeared to inhibit Protac C action, rather than complex formation between APC and PCI. Following immunoblotting the radioactivity for protein C antigen in NHP (Fig 1, lane 9) was essentially the sum of radioactive signals for protein C and APC complexes (Fig 1, lanes 10 through 13), indicating that protein C and APC are recognized equally well by the monoclonal antibodies used. A proteolytic fragment of APC containing light chain antigenic determinants, ie, determinants recognized by monoclonal antilight chain antibody, was visible on SDS gels at a mobility of approximately 39,000 mol wt, but was not apparent as a distinct species on nondenaturing gels. It should also be noted that the resolution of bands of nonreduced APC in samples containing ovalbumin was greater than that of APC in plasma, as evident by the resolved doublet in Fig 1, lane 8, as compared with lanes 4 through 7. However, the heavy chains of protein C and APC were readily distinguished in reduced gels (Fig 2, lane 6).

Putative APC:PCI complexes of approximately 110,000 mol wt appeared as a closely spaced doublet of bands on some exposures of reduced SDS gels (Fig 1, lanes 6 and 11). When protein C and APC in plasmas were studied by immunoblotting on alkaline nondenaturing gels, ie, without SDS, two well separated and distinct forms of APC:PCI complexes were observed with monoclonal antibody to protein C (Fig 3, bands a and b) as previously described. The percent of the radioactive signal due to added APC that was in complexed forms (bands a and b) was 55% to 65% after one hour of incubation at 37°C of APC with plasma (Fig 3, lanes 2 and 3 compared with lane 1). This percent of APC in complexes was higher than that observed on SDS gels run under denaturing conditions, ie, 33% to 44%, and could indicate that some of the APC:PCI complexes were labile in SDS or noncovalent. The diffuse radioactivity observed between the complexed and free protein C antigen bands in Fig 1 also suggests that the APC:PCI complexes are to some extent labile when heated in SDS. Similar results showing two apparent forms of APC:PCI on alkaline nondenaturing gels were obtained when pooled NHP or individual patient plasmas were incubated with Protac C for one hour (data not shown). The percent of radioactive signal in the complexed region (bands a and b) was 57% for pooled NHP, 46% for a patient with a bleeding disorder of unknown origin, 40% for a patient with hereditary heterozygous protein C deficiency who was on oral anticoagulant therapy, and 50% for a patient with a bleeding disorder known as Passavoy trait. A similar distribution of protein C antigen between bands a and b, representing APC:PCI complexes and free APC or protein C was seen for ten combined factor V/VIII deficient plasmas that were treated with Protac C for one hour and then analyzed using this immunoblotting technique on alkaline nondenaturing gels (data not shown).

Evidence for two distinct protein C inhibitors. In the immunoblots of nondenaturing gels, the APC complex detected by anti-protein C antibodies designated band a in Fig 3, lanes 2 and 3 comigrated with a new band containing PCI-I antigen detected using anti-PCI-I antibodies (band a, Fig 3, lanes 5 and 6). The formation of band a was stimulated by heparin (Fig 3, lanes 3 and 6 compared with lanes 2 and 5, respectively). The new APC complex designated band b (Fig 3, lanes 2 and 3) was detected by the anti-protein C antibodies but not by the anti-PCI-I antibodies (Fig 3, lanes 5 and 6), and formation of band b was diminished in the presence of heparin (Fig 3, lane 3 compared with lane 2). This supports the previous report that band a represents a complex of APC with PCI-I and that another plasma component complexes with APC, in a heparin-independent manner, to give band b. In the absence of heparin, band b accounted for approximately half of the APC complexes formed during one-hour incubation of APC in plasma at 37°C in different experiments (eg, Fig 3, lane 2). In the presence of heparin, band b accounted for less than one fifth of the APC complexes (eg, Fig 3, lane 3). The same pattern of PCI-I antigen distribution seen using our rabbit anti-PCI-I antibodies in Fig 3, lanes 4 through 6, was also seen in immunoblots using rabbit polyclonal antibodies to PCI-I provided by Dr Koji Suzuki or using mouse monoclonal antibodies to PCI-I provided by Dr Johan Stenflo (data not shown). Neither of the APC:PCI bands (ie, bands a or b) appeared to react with antibodies to protein S or to β2-glycoprotein I in immunoblotting studies (data not shown), suggesting that complexation of APC with either of these proteins cannot account for band b.

When bands a and b of complexes were eluted from a nondenaturing gel and electrophoresed on a nonreduced SDS gel and immunoblotted for protein C, each complex had a mol wt of approximately 110,000 (data not shown). The complex formation with inhibition of APC. Studies were performed to correlate directly the extent of inhibition of APC activity in plasma with the extent of complex formation as determined on nondenatured immunoblots. As seen in Fig 4, the inhibition of APC correlated well with the appearance of APC:PCI complexes, ie, with the sum of bands a and b. In the immunoblot for this experiment, band a and band b of APC:PCI complexes each appeared to increase over time at approximately the same rate, demonstrating that each type of complex arose independently and not by proteolytic processing of one into the other. NHP or plasma immunodepleted of protein C each inhibited the amidolytic activity of APC by 47% to 73% in different experiments in one hour of incubation at 37°C. The APC amidolytic activity generated in Protac C-treated NHP was followed as a function of time; it peaked at 15 minutes at 37°C, and fell to 48% to 54% of that value after one hour of incubation, with half maximal inactivation occurring at 30 minutes, ie, 15 minutes after the peak activity was measured. Control studies showed that the effect of Protac C alone on the substrate, S-2366, was negligible. The amidolytic activity generated after 15 minutes by Protac C treatment of plasma
The percent inhibition of APC amidolytic activity was compared with APC controls as described in Materials and Methods. For the corresponding reaction mixture remaining in the wells, 6 μL of sample buffer containing 20 mmol/L benzamidine at 4°C for immunoblotting analysis. The reaction mixture remaining in the wells was tested for amidolytic activity compared with APC controls as described in Materials and Methods. The percent inhibition of APC amidolytic activity was plotted as a function of time (closed circles). For the corresponding samples subjected to immunoblotting analysis, the percent of complex formation (open circles) was calculated from the percent of total radioactivity in the region of APC:PCI complexes, i.e., band a plus band b.

Fig 4. Time course for inhibition and complexation of APC by PCls in heparinized plasma. Protein C-depleted plasma containing 10 U/mL heparin was incubated at 37°C in the wells of microtiter plates (15 μL/well) with 15 μL of 4 μg/mL APC in TBS, 1.25% ovalbumin for various times. At the end of each incubation time, 8 μL of the mixture was put in 50 μL of sample buffer containing 20 mmol/L benzamidine at 4°C for immunoblotting analysis. The reaction mixture remaining in the wells was tested for amidolytic activity compared with APC controls as described in Materials and Methods. The percent inhibition of APC amidolytic activity was plotted as a function of time (closed circles). For the corresponding samples subjected to immunoblotting analysis, the percent of complex formation (open circles) was calculated from the percent of total radioactivity in the region of APC:PCI complexes, i.e., band a plus band b.

Containing 4 μg/mL of protein C was the same (A4 = 0.076/10 min/μL plasma at 405 nm) as that measured for 5 μL of 4 μg/mL purified APC incubated under the same conditions, suggesting that all of the protein C in plasma was activated after 15 minutes of Protac C treatment.

APC inhibition by plasma depleted of PCI-1 and by partially purified PCI-2. In order to study whether formation of band b on nondenaturing immunoblots of mixtures containing APC in plasma was associated with inhibition of APC, plasma depleted of PCI-1 and protein C antigen was prepared as described in Materials and Methods. The immunodepleted plasma contained <5% of plasma PCI-1 as measured by Laurell rocket immunoelectrophoresis and by quantitative dot immunobinding. The immunodepleted plasma retained <10% of the ability of normal plasma to inhibit APC amidolytic activity in 20 minutes in the presence of heparin; however, it still retained approximately 50% of the ability of normal plasma to inhibit APC amidolytic activity in 150 minutes in the absence of heparin (Fig 5). APC was incubated with plasma depleted of PCI-1 and protein C and aliquots were analyzed at various times in order to measure simultaneously inhibition of APC amidolytic activity and APC complex formation (Fig 5). The appearance of APC complexes as band b paralleled inhibition of APC amidolytic activity, suggesting that the complexes seen primarily as band b represent APC in an inactive form complexed with an inhibitor other than PCI-1. This inhibitor responsible for band b formation is here designated PCI-2. Control experiments showed no influence of heparin on the inhibition of APC by PCI-2 in the plasma depleted of PCI-1 and protein C.

Other immunoblotting studies used PCI-1 depleted plasma, designated Id. Complexes with APC were formed in this Id plasma, as detected by antibodies to protein C (Fig 6, lanes 3 and 4), and most of these complexes had the mobility of band b. Controls showed no formation of band a complexes when APC was added as seen with antibodies to PCI-1 (data not shown). Controls on these blots also showed that the bands detected by antibodies to PCI-1 in normal plasma incubated with APC had the mobility of band a.

Fractionated plasma was chromatographed on DEAE-Sephadex (see Materials and Methods) and assayed for PCI-1 as band a and PCI-2 as band b using nondenaturing immunoblots of APC incubated with column fractions. PCI-2 eluted after PCI-1 from DEAE-Sephadex, and a pool of partially purified PCI-2 displayed no detectable PCI-1 antigen or complexes with APC as seen on immunoblots with antibodies to PCI-1 (data not shown). When APC was incubated with partially purified PCI-2 in the absence of heparin, complexes were formed that comigrated with band b, as seen in immunoblots using antibodies to protein C (Fig 6, lane 1). When APC was incubated with partially purified PCI-2 in the presence of heparin, most of the APC complex appeared as band b (Fig 6, lane 2) although a small amount of band a appeared possibly due to some slight PCI-1 contamination or due to the presence of another heparin-dependent minor inhibitor. The immunoblotting pattern for the partially purified PCI-2 is nearly identical to that of the APC in plasma depleted of PCI-1 and protein C. The experiment was conducted as in Fig 4, except that no heparin was added, and plasma depleted of PCI-1 and protein C was substituted for protein C-depleted plasma.
incubation mixture, as shown in Fig 7B, in which the Nondenaturing immunoblot analysis was performed on each plexes plasma and treated with Protac C to allow APC:PCI complexes from protein C-immunodepleted plasma. The reduced SDS-PAGE analysis of the fractions was performed as in Fig 3. The presence or absence of APC, 10 U/mL heparin, normal human plasma (NHP), PCI-1 depleted protein C deficient plasma (Id), or partially purified PCI-2 is indicated by + or −.

**Fig 6.** Immunoblots of APC complexes formed in normal plasma, in plasma depleted of PCI-1 and protein C, and in a PCI-2 preparation, using two antibodies. Incubations were as in Fig 2 except that volumes of plasma or APC were 8 μL. Immunoblotting was performed as in Fig 3. The presence or absence of APC, 10 U/mL heparin, normal human plasma (NHP), PCI-1 depleted protein C deficient plasma (Id), or partially purified PCI-2 is indicated by + or −.

Id plasma (lanes 1 and 2 compared with lanes 3 and 4). When APC was added to the Id plasma reconstituted with PCI-1 and immunoblotted using protein C antibodies, both band a and band b were observed in normal plasma in Fig 6, lanes 5 and 6 (data not shown). Partially purified PCI-2 formed complexes with APC that had a mol wt of approximately 110,000 on an immunoblot from an SDS gel, suggesting that PCI-2 has a mol wt of approximately 50,000. In other preliminary immunoblotting experiments not shown, the complex formed on incubation of partially purified PCI-2 with APC did not appear to react with antibodies to β2-glycoprotein I, α2-macroglobulin, α1-antitrypsin, α1-antichymotrypsin, protein S, or antithrombin III. Also, hereditary deficient plasmas from a patient with <5% of normal levels of α2-antiplasmin and a patient with <5% of normal levels of C1-inhibitor each formed normal amounts of the two major forms of APC complexes seen as bands a and b on nondenaturing immunoblots, suggesting that PCI-2 is not related to any of these known proteins and inhibitors.

**Complexes of activated protein Cα and protein Cβ.** Protein Cα and protein Cβ were studied to see whether each can be activated by Protac C and form complexes with the PCIs in plasma, and whether this might represent another basis for two major types of APC complexes. During preparative electrophoresis for purification of protein C,4 fractions were isolated that contained predominantly protein Cα or protein Cβ (Fig 7A, arrows) as determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE) of the fractions. Protein Cα and protein Cβ differ in the mobility of their heavy chains on reduced SDS gels (Fig 7).2 Fractions of protein Cα and protein Cβ were mixed with protein C-immunodepleted plasma and treated with Protac C to allow APC:PCI complexes to form for comparison to Protac C-treated NHP. Nondenaturing immunoblot analysis was performed on each incubation mixture, as shown in Fig 7B, in which the electrophoresis was allowed to run 10% longer than was routine. The added resolution achieved revealed that the band of APC complexes designated band a was a doublet. The same general pattern of complexes was seen for Protac C-treated normal plasma as for Protac C-treated protein C immunodepleted plasma supplemented with either protein Cα or protein Cβ. It is evident that both protein Cα and protein Cβ can be activated with Protac C and can form complexes with PCI(s) to yield the same general pattern of APC complexes as found for Protac C-treated NHP, but with slightly altered mobilities, with APCα migrating slightly faster than APCα. Therefore, the two major types of APC complexes in plasma, ie, bands a and b, do not originate from the two forms, APCα and APCβ, although the existence of APCα and APCβ may account for some microheterogeneity observed on nondenaturing immunoblots showing the two major forms.

Minor forms of APC complexes in plasma and serum. Besides the two major forms of APC complexes formed on incubation of APC with plasma, some minor forms of complexes were observed on immunoblots after long film exposures (Fig 8). The immunoblot patterns varied reproducibly between plasma, serum derived from plasma, and serum derived from whole blood (Fig 8). The minor
forms of APC antigen were of slower mobility than bands a and b, the two major forms of complexes (Fig 8). No APC complexes or amidolytic activity were observed in serum treated with Protac C, as compared with plasma (Fig 8, lanes 7 and 11, compared with lane 3), although PCI(s) were evidently present in the serum because complexes were formed when exogenous APC was added (Fig 8, lanes 6 and 10). This was found to be largely due to inhibition of Protac C by calcium ions in the serum, since when calcium ions were added to plasma (Fig 8, lane 4), no complexes and very little amidolytic activity were observed on Protac C treatment. Addition of EDTA to the serum to a final concentration of 20 mmol/L increased the yield of APC complexes on Protac C treatment, but not to the same level as seen in plasma without EDTA (Fig 8, lanes 8 and 12, compared with lane 3). In experiments not shown, addition of 20 mmol/L EDTA to plasma inhibited Protac C-dependent activation, since it resulted in 62% less amidolytic activity at the peak of activation (15 to 20 minutes) and a lower yield of APC:PCI complexes at 60 minutes of incubation. When serum or plasma containing varying concentrations of EDTA was incubated with Protac C, peak APC amidolytic activity and yield of complexes were equivalent from 6 to 12 mmol/L EDTA in serum and 0 to 12 mmol/L EDTA in plasma, but higher concentrations of EDTA were increasingly inhibitory in each case. In other experiments not shown, addition of 2.5 mmol/L CaCl₂ and 1.6 mmol/L MgCl₂ to a mixture of NHP and APC, followed by incubation at 37°C for one hour, resulted in a pattern similar to that of serum in Fig 8, lane 6 (ie, a minor band was formed near the top of the gel and band a was slightly diminished compared with NHP incubated with APC without added metal ions, as in lane 2).

**DISCUSSION**

In normal plasma, APC associates with two protease inhibitors, PCI-1 and PCI-2, to give stable, presumably covalent complexes of approximately 110,000 mol wt (Figs 1 through 3). One of these two major forms of APC:PCI complexes reacts with antibodies to PCI-1 and its formation is stimulated by heparin, but the other does not (Fig 3), indicating that another major inhibitor of APC, here designated PCI-2, exists in plasma, as our group and van der Meer et al have recently suggested. This inhibitor, designated PCI-2, is separable from PCI-1 and has been partially purified. PCI-2 is present in PCI-1-depleted plasma (Fig 7). PCI-2 inhibits APC in a heparin-independent reaction and forms inactive complexes with APC of approximately 110,000 mol wt.

At levels of APC equivalent to that of protein C in plasma, approximately 60% of APC was complexed with PCI-1 and PCI-2 in one hour of incubation at 37°C (Figs 3 and 4), and this complexation was correlated with a proportional loss in amidolytic activity of APC. The time course of APC inhibition by plasma seen during incubations with heparin as in Fig 4 may represent a faster heparin-dependent inhibition by PCI-1 superimposed on a slower, heparin-independent inhibition by PCI-2. In the absence of heparin and PCI-1, the inhibition of APC by plasma is slower and linear for at least 140 minutes, as in Fig 5. The comparison of immunoblot results from non-denaturing gels v denaturing SDS gels suggests that complex formation is extensively covalent, as previously reported for purified protein studies, although as much as one third of the APC:PCI complexes appears labile or noncovalently linked. Since a nearly equivalent degree of complex formation was obtained with Protac C-treated normal plasma as with protein C immunodepleted plasma supplemented with purified APC, the activation of protein C in plasma by Protac C is efficient, and the APC generated in plasma in this way reacts with PCI-1 and PCI-2 in essentially the same way as that activated in vitro by thrombin cleavage. The slightly smaller proportion of protein C antigen in complexed form in Protac C-treated plasma v plasma incubated with APC may reflect the lag time required for protein C activation and the absence of heparin in the former. Slight degradation of the complexes by Protac C cannot be excluded; however, no new fragments were observed on immunoblots with up to 90 minutes of incubation in the reaction mixture. The specificity of Protac C has not been completely defined and another article in this issue describes cleavage of protein S by Protac C.

Immunoblotting studies of protein C antigen in plasmas activated by Protac C provide a convenient method to assay

---

**Figure 8.** Immunoblot of APC complexes in plasma and serum. Plasma and serum from the same donor were prepared as in Materials and Methods, and incubated with Protac C or APC as in Fig 2. The final concentrations of reagents, when added as indicated, were: CaCl₂, 15 mmol/L; EDTA, 20 mmol/L. Immunoblotting was performed as in Fig 3.
the APC formation and reactivity with PICIs in various patients' plasma. A deficiency of PCI has been hypothesized to be associated with a bleeding diathesis. Since Passavoy trait plasma, combined factor V/VIII deficient plasmas, and another plasma of a patient with an unexplained bleeding diathesis treated with Protac C each contained a normal proportion of APC in its complexed forms, the basis of these defects does not seem to involve an abnormality of the formation of APC:PCI complexes, at least not in the cases studied here.

Since the two major forms of APC complexes in plasma arise at approximately the same rate in the absence of heparin and remain in the same proportion over a long time, it is unlikely that one form arises from proteolytic processing of the initially formed APC:PCI complex involving only one PCI. Since multiple forms of PCI-1 exist and a form of PCI-1 has been found in human urine by Geiger et al,33 multiple forms of PCI-1 may account for microheterogeneity in one of the two major forms of complex observed for band a. We recently demonstrated35 that the plasma and urinary PICIs previously described16,33 are immunologically and functionally very similar if not identical to one another and to a heparin-dependent urokinase inhibitor also known as plasminogen activator inhibitor-3.34

Both protein C, and protein C, in plasma can be activated by Protac C and form complexes with the two major plasma APC inhibitors, and differences in these forms of APC may account for some microheterogeneity with the two major forms of APC:PCI complexes, APC:PCI-1 and APC:PCI-2 (Fig 7). Thus, in principle the following is the minimum number of complexes formed in plasma: APC:PCI-1, APC,PCI-2, APC,PCI-1, and APC,PCI-2.

In addition to the two major forms of APC complexes, some additional minor forms of APC antigen that vary between plasma, serum derived from plasma, and serum derived from whole blood were observed (Fig 8). These minor molecular species containing APC antigen have not yet been identified nor shown to involve APC inhibitors. However, addition of calcium and magnesium ions to a mixture of APC and plasma results in a pattern similar to that of serum incubated with APC. Protac C treatment of serum results in formation of less APC amidolytic activity and less APC:PCI complexes than seen in plasma, unless EDTA is added to a final concentration of 6 to 12 mmol/L. Higher concentrations of EDTA are inhibitory to Protac C activation.

That APC may play a physiologically important role as a regulator of thrombosis is suggested by the demonstration that infants with a hereditary homozygous deficiency of protein C develop shortly after birth intravascular coagulation that is fatal when untreated. Moreover, APC inhibits venous thrombosis in a dog model19 and platelet-dependent arterial thrombosis in a baboon model,24 and APC prevents the coagulopathy as well as the fatal outcome associated with Escherichia coli-induced shock in a baboon model of sepsis.37 Therefore, the regulation of APC in plasma by two major inhibitors, PCI-1 and PCI-2 is of considerable interest. The studies here suggest that the previously described15-18 heparin-dependent PCI-1 plays an important role in plasma, but that a second heparin-independent inhibitor, PCI-2, may also play an important role. In another article in this issue we report that for the majority of patients with disseminated intravascular coagulation, APC:PCI-2 complexes are found in plasma while a few patients have both APC:PCI-1 and APC:PCI-2 complexes.38 It is not yet known what other enzymes may compete for PCI-1 and PCI-2 in various disease states. Identification and characterization of PCI-2 and definitive proof that PCI-2 is not related to PCI-1 will require its isolation and study. PCI-2 was later isolated and identified as α1-antitrypsin.39

ACKNOWLEDGMENT

We are grateful for the excellent and cheerful assistance of Christian Nova and Anthony Potente in technical matters and of Cheryl McLean and Leslie Sherry in clerical matters. We thank Terry White, Dr Andras Gruber, and Richard Cavicke for assistance in preparing antibodies and APC and Drs Mauro Berretti, Peter Schwarz, and Judith Greengard for helpful discussions. We acknowledge Drs K. Suzuki, J. Stenflo, and M. Laurell for kindly providing antibodies against PCI-1.

REFERENCES

12. Taylor FB, Lockhart MS: A new function for activated protein C: Activated protein C prevents inhibition of plasminogen
Inhibition and complexation of activated protein C by two major inhibitors in plasma

MJ Heeb, F Espana and JH Griffin

Updated information and services can be found at:
http://www.bloodjournal.org/content/73/2/446.full.html

Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml