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Nonenzymatic anticoagulant activity of the mutant serine protease Ser360Ala-activated protein C mediated by factor Va

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Abstract

The human plasma serine protease, activated protein C (APC), primarily exerts its anticoagulant function by proteolytic inactivation of the blood coagulation cofactors Va and VIIIa. A recombinant active site Ser 360 to Ala mutation of protein C was prepared, and the mutant protein was expressed in human 293 kidney cells and purified. The activation peptide of the mutant protein C zymogen was cleaved by a snake venom activator, Protac C, but the "activated" S360A APC did not have amidolytic activity. However, it did exhibit significant anticoagulant activity both in clotting assays and in a purified protein assay system that measured prothrombinase activity. The S360A APC was compared to plasma-derived and wild-type recombinant APC. The anticoagulant activity of the mutant, but not native APC, was resistant to diisopropyl fluorophosphate, whereas all APCs were inhibited by monoclonal antibodies against APC. In contrast to native APC, S360A APC was not inactivated by serine protease inhibitors in plasma and did not bind to the highly reactive mutant protease inhibitor M358R α_1 antitrypsin. Since plasma serpins provide the major mechanism for inactivating APC in vivo, this suggests that S360A APC would have a long half-life in vivo, with potential therapeutic advantages. S360A APC rapidly inhibited factor Va in a nonenzymatic manner since it apparently did not proteolyze factor Va. These data suggest that native APC may exhibit rapid nonenzymatic anticoagulant activity followed by enzymatic irreversible proteolysis of factor Va. The results of clotting assays and prothrombinase assays showed that S360A APC could not inhibit the variant Gln 506-FVa compared with normal Arg 506-FVa, suggesting that the active site of S360A APC binds to FVa at or near Arg 506.

Keywords: anticoagulant; factor Va; protein C; protein–protein interaction; serine protease

Protein C is a plasma serine protease zymogen (Stenflo, 1976) that, when activated by the thrombin-thrombomodulin complex (Kisiel, 1979; Esmon & Owen, 1981), inhibits blood coagulation by proteolytic inactivation of the blood coagulation cofactors factor Va (FVa) and factor VIIIa (Kisiel et al., 1977; Walker et al., 1979; Marlar et al., 1982). The importance of protein C in the regulation of coagulation is illustrated by the fact that heterozygous protein C deficiency is associated with increased risk of venous thrombosis (Griffin et al., 1981). Furthermore, homozygous protein C deficient infants present with purpura fulminans or venous thrombosis shortly after birth and die within weeks in the absence of aggressive anticoagulant therapy (Branson et al., 1983; Seligsohn et al., 1984).

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Serine proteases are present in virtually all organisms and exist in three families that have independently evolved a similar catalytic mechanism (Liao & Remington, 1990; Perona & Craik, 1995). This mechanism is characterized by the conserved catalytic triad of residues Asp 102, His 57, and Ser 195 in the active site [chymotrypsin numbering system (Hartley, 1970)]. The active site serine is thought to catalyze hydrolysis by nucleophilic attack of the hydroxyl oxygen on the carbonyl carbon of the target amide bond (Kraut, 1977). Thus, mutation of the active site serine of activated protein C to alanine (S360A) would be expected either to ablate or at least to greatly decrease catalytic activity. This mutation can provide a powerful probe for studies of the protein–protein interactions of serine proteases since the mutant protease may bind substrates and cofactors but leave them unproteolyzed. This would allow the study of "unproductive" complexes that presumably resemble productive complexes.

Activated protein C (APC) is normally inactivated by serine protease inhibitors (serpins) present in the blood and has a half life in vivo and in vitro of approximately 20 min (Okajima et al., 1990;

España et al., 1991; Heeb et al., 1991). To prepare a proteolytically inactive "APC" with a structure indistinguishable from native APC for use as a probe of APC protein–protein interactions and to evaluate the importance of the active site serine (Ser360) of APC for its function and metabolism, we made a recombinant Ser360 Ala mutant and studied its anticoagulant activity and its interaction with serpins.

Results

Production and purification of wild-type and S360A recombinant protein C

Wild-type and S360A recombinant protein C (S360A protein C) purified from conditioned cell culture were recovered at a level of about 1 to 1.5 mg/L of media. SDS-PAGE analysis of wild-type and S360A recombinant protein C showed that they have the same mobility as plasma-derived protein C (Fig. 1). In lanes 3 and 3′ Protac C activation of S360A protein C caused a change in the mobility of the non-reduced S360A activated protein C (S360A APC) and the reduced heavy chain of S360A APC. This shift is particularly apparent in the reduced heavy chain doublet [compare lanes 2′ (S360A protein C), 3′ (S360A APC), and 4′ (rwt protein C)]. This suggested that the activation peptide of S360A APC was properly cleaved by Protac C. In the reduced lanes it is also apparent that significant amounts of the recombinant protein Cs are in a single chain form that is not activated. Therefore, the precise amounts of functional APC in these preparations is not known.

Functional activity of S360A APC

The amidolytic activity of recombinant wild-type APC (rwt APC) and S360A APC was tested and compared to that of plasma-derived APC. rwt APC had the same activity towards the chromogenic substrate L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroaniline hydrochloride (S2366) as did the plasma-derived APC (Table 1). However, to obtain measurable levels of substrate cleavage with S360A APC it was necessary to monitor the absorbance of the substrate for four days. The activity of S360A APC was decreased 1300-fold relative to plasma-derived APC. However, since we cannot rule out that there is a small amount of contaminating amidolytic enzyme in the S360A APC preparation, this level of activity must be an upper bound. Therefore, the change of the active site serine to an alanine decreased the amidolytic activity of APC by at least 1300-fold.

Table 1. *Comparison of specific amidolytic activity of S360A APC with wild-type APCs*[a]

	rwt APC	Pl.-der. APC	S360A APC
mOD_{405}/min	65.8 ± 7.5	60.8 ± 11	0.047 ± 0.004
Relative % specific amidolytic activity[b]	108	100	≤0.08

[a]Protein C samples were adjusted to 100 μg/mL (1.6 μM) and incubated with equal volumes of 1 U/mL Protac C at 37 °C for 30 min. Five microliters of APC and 50 μL of 1 mM S-2366 at pH 8.2 were mixed in each well of an ELISA plate, and OD_{405} was read immediately over 10 min. For S360A APC OD_{405} was followed for four days to obtain a mOD_{405}/min. A negative control of Protac C alone was subtracted from these values.
[b]The specific amidolytic activity of plasma-derived (Pl.-der.) APC was arbitrarily set as 100%.

Clotting assays were performed in protein C-deficient plasma (PCDP) to determine the anticoagulant activity of S360A APC (Fig. 2). In both activated partial thromboplastin time (APTT) assays and FXa-1 stage assays S360A APC exhibited significant levels of anticoagulant activity. Similar results were seen with normal human plasma (data not shown). Under the conditions used S360A APC exhibited 15–25% of the anticoagulant activity of plasma-derived APC and recombinant wild-type APC (data not shown for rwt APC). This level of activity is about 200-fold above the observed amidolytic activity of S360A APC. S360A protein C and Protac C alone had no effect on clotting times (data not shown). S360A APC also showed significant activity in a prothrombinase

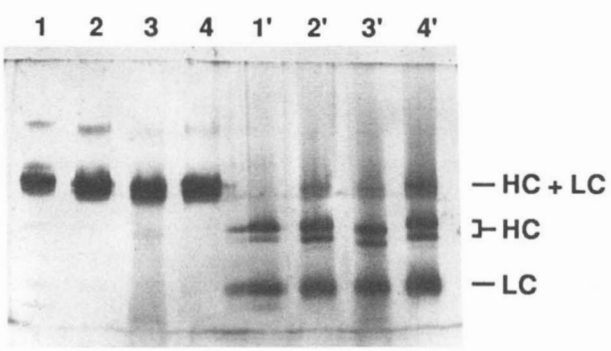

Fig. 1. SDS-PAGE analysis of purified S360A protein C and S360A APC. Protein C samples (50 ng each) were electrophoresed on an 8–25% SDS polyacrylamide Phast Gel (Pharmacia) nonreduced (lanes 1–4) or reduced (lanes 1′–4′), and silver stained. Lanes 1 and 1′ are plasma-derived protein C. Lanes 2 and 2′, recombinant S360A protein C. Lanes 3 and 3′, Protac C-activated S360A protein C (S360A APC). Lanes 4 and 4′, recombinant wild-type protein C. HC, heavy chain; LC, light chain.

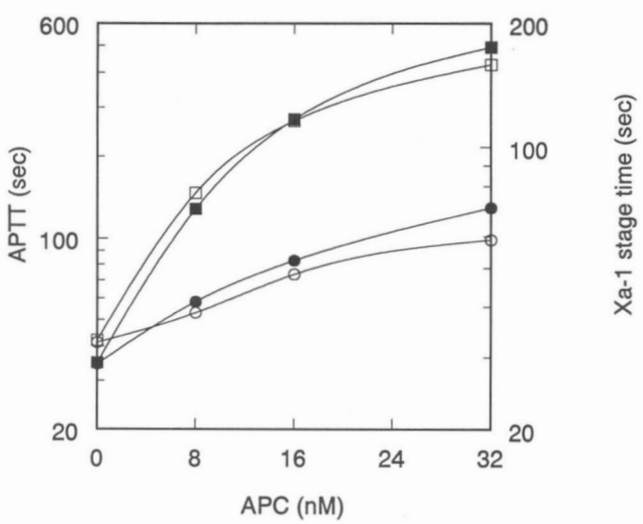

Fig. 2. Anticoagulant activity of S360A APC. Plasma-derived protein C and S360A protein C were activated by Protac C. Clotting assays were performed in PCDP according to the protocols described in Materials and methods. Left axis, APTT assay (open square = plasma-derived APC, open circle = S360A APC); Right axis, FXa-1 stage assay (closed square = plasma-derived APC, closed circle = S360A APC).

assay (Fig. 3). In a prothrombinase assay with 1 nM FXa and 20 pM FVa, S360A APC inhibited prothrombinase activity greater than 90% at 10 μg/mL (160 nM). No significant inhibition was observed in the absence of FVa (data not shown). The zymogen, S360A protein C, did not show this effect. S360A protein C was pretreated with *p*-amidinophenyl methanesulfonyl fluoride (*p*-APMSF) to inactivate any potential traces of APC that might have been present. Controls confirmed that *p*-APMSF and Protac C had no effect on prothrombinase activity.

Characterization of properties of S360A APC

Diisopropyl fluorophosphate (DFP) reacts with the active site serine of serine proteases to form an irreversible diisopropyl phosphate (DIP) derivative, thereby inactivating the protease. Preincubation of plasma-derived APC with DFP totally abolished its activity in an APTT clotting assay. However, preincubation of S360A APC with DFP had no effect on its observed anticoagulant activity in the APTT assay (Fig. 4). With no active site serine DFP cannot derivatize S360A APC. This confirmed that the observed anticoagulant activity was not due to a wild-type revertant of recombinant protein C contaminating the S360A protein C preparation. This experiment also demonstrated that inactivating plasma-derived APC with DFP to block the active site serine did not produce a protein that was equivalent to S360A APC. Though both modifications of APC resulted in a loss of amidolytic activity, DIP-APC had no anticoagulant activity, whereas S360A APC did have anticoagulant activity. This result suggested that the presence of the DIP moiety in the active site of APC was sufficient to prevent binding of APC to FVa at a level sufficient to cause anticoagulant activity.

The effects of two monoclonal antibodies to protein C on S360A APC activity were investigated to further confirm that the anticoagulant effect of S360A APC was not due to a contaminant in the

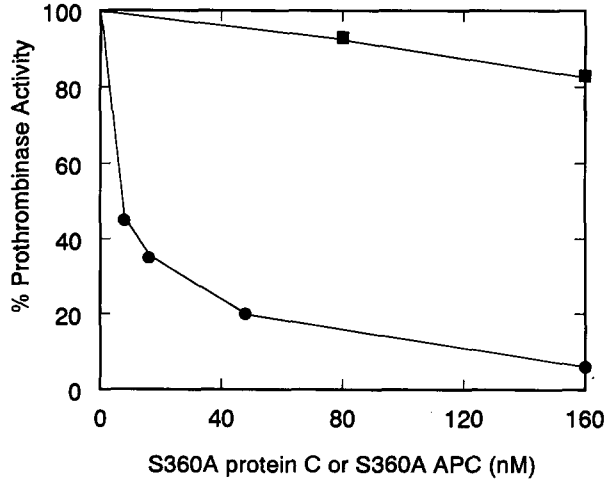

Fig. 3. Inhibition of prothrombinase activity by S360A APC in a purified system. Prothrombinase components (1 nM FXa, 20 pM FVa, 5 mM $CaCl_2$, 50 μM phospholipid vesicles containing 20% phosphatidyl serine, 80% phosphatidyl choline) were preincubated at room temperature for 15 min with or without protein C species. Prothrombin was added to 0.3 μM and 20 μL aliquots were removed at different times and quenched in 80 μL of TBS containing 10 mM EDTA, pH 8.2. S-2238 substrate (50 μL of 0.6 mM) was added and rate of thrombin formation was assessed by measuring OD_{405}/min. Mutant protein C was activated by Protac C (closed circle = S360A APC, closed square = S360A protein C).

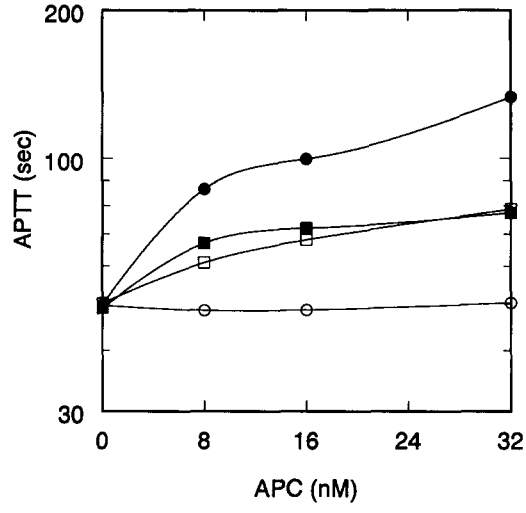

Fig. 4. Effect of diisopropyl fluorophosphate (DFP) treatment on APC and S360A APC. Plasma-derived protein C and S360A protein C were activated by Protac C. Each APC was then treated with 1 mM DFP at 37 °C for one hour. APTT clotting assays were then performed with APC alone (closed circle), APC + DFP (open circle), S360A APC alone (closed square), and S360A APC + DFP (open square).

S360A APC preparation. The monoclonal antibody C1 inhibits amidolytic activity in wild-type APC and binds to the heavy chain of APC on Western blots (Heeb et al., 1988). Therefore, C1 appears to bind close to the active site. This antibody was found to inhibit both plasma-derived APC and S360A APC by >95% in an APTT clotting assay (Table 2). The monoclonal antibody C3 binds to the light chain of APC (Heeb et al., 1988). This antibody had a less striking inhibitory effect on APC (>80% inhibition) (Table 2), but it inhibited S360A APC to the same extent that it inhibited plasma-derived APC.

Gel shift experiments were performed to evaluate the ability of S360A APC to complex with M358R α_1 antitrypsin, a more potent inhibitor of APC than native α_1 antitrypsin (Heeb & Griffin, 1988; Heeb et al., 1990). S360A APC and plasma-derived APC (0.2 μM) with or without M358R α_1 antitrypsin (2 μM) were electrophoresed on a 7.5% acrylamide nondenaturing gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and blotted with the monoclonal antibody C3. Plasma-derived APC and M358R α_1 antitrypsin formed a complex that caused a clear shift in mobility of APC. However, no shift was observed with

Table 2. *Inhibition of APCs by monoclonal antibodies C1 and C3*[a]

	Control	C1 Mab		C3 Mab	
	APTT (s)	APTT (s)	% Inhibition	APTT (s)	% Inhibition
Pl.-der. APC	317	40	>98%	92	>80%
S360A APC	92	50	>95%	56	>80%
Control (no APC)	46	46	None	49	None

[a]APCs (45 μg/mL, 0.73 μM) were incubated with an equal volume of C1 (1.15 mg/mL), C3 (0.46 mg/mL), or buffer at 37 °C for one hour, then added to PCDP and tested in APTT assays.

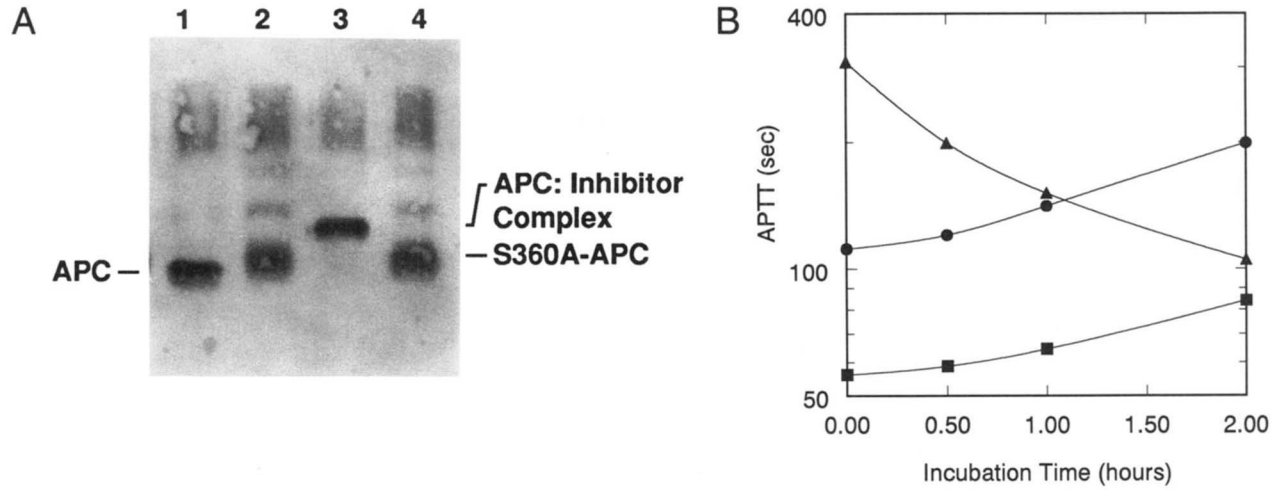

Fig. 5. Effect of serpins on S360A APC. **A:** Detection of complex formation with M358R α_1 antitrypsin on a nondenaturing gel. Aliquots of S360A APC or plasma-derived APC (0.2 μM) with or without M358R α_1 antitrypsin (2 μM) were loaded on a 7.5% acrylamide nondenaturing minigel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and blotted with the anti-protein C monoclonal antibody C3. Lane 1 contained plasma-derived APC; lane 2 contained S360A APC; lane 3 contained APC + M358R α_1 antitrypsin; lane 4 contained S360A APC + M358R α_1 antitrypsin. **B:** Inactivation of APC anticoagulant activity by human plasma. S360A APC and plasma-derived APC were added to human PCDP to a final concentration of 32 nM and incubated at 37 °C. At each time point 50 μL of plasma was removed and the APTT was determined (closed triangle = APC, closed circle = S360A APC, closed square = control with no APC added).

S360A APC and M358R α_1 antitrypsin under these conditions (Fig. 5A).

In vivo plasma serpins provide the major mechanism for inactivating and clearing APC (Okajima et al., 1990; España et al., 1991; Heeb et al., 1991). When plasma-derived APC (2 μg/mL) was incubated with PCDP at 37 °C it lost >90% of its activity over the course of two hours (Fig. 5B). However, under the same conditions S360A APC retained all of its anticoagulant activity. The shift in the baseline control (PCDP without added APC) was probably due to the instability of FV and FVIII in plasma. These gel shift binding experiments and activity experiments in plasma demonstrated that S360A APC does not form complexes with the serpins that commonly inactivate APC. Therefore, S360A APC would probably have a long half-life in vivo.

Characterization of FVa inhibition by S360A APC

S360A APC, rwt APC, and plasma-derived APC were incubated with FVa under conditions described in Materials and methods from 1.5 to 30 min and then electrophoresed on a 4–15% SDS-PAGE gel, transferred to PVDF membrane, and Western blotted with anti-FVa heavy chain antibodies (Fig. 6). The plasma-derived APC and rwt APC reactions both showed a decrease in the intensity of the FVa heavy chain band over time. Furthermore, fragments with the apparent molecular weights of 76 kDa, 44 kDa, and 28 kDa attributable to known APC cleavage products of the FVa heavy chain (Kalafatis et al., 1994; Heeb et al., 1995) were visible. In contrast, S360A APC at five times the concentration (16 nM) of plasma-derived APC (3.2 nM) did not appear to cleave the FVa heavy chain appreciably under these conditions. The faint band visible at 46 kDa is present also in the negative control. A very faint band is visible at 28 kDa only in the 30-min digestion. This band is attributable to the fragment from 506 to 709 of the heavy chain and is known to contain the epitope for the monoclonal antibody used in the Western blots. Thus, it is generally disproportionately bright.

The inactivation of FVa was also tested in a clotting assay with FV-deficient plasma (FVDP). FVa was incubated with plasma-derived APC or S360A APC at 37 °C under the conditions described in Materials and methods. Aliquots were removed at the indicated times and added to FVDP in a prothrombin time clotting assay (Fig. 7). In this assay the activity of FVa incubated with plasma-derived APC decreased with time. This is characteristic of inactivation of FVa due to enzymatic cleavage. However, the activity of FVa incubated with S360A APC at two different concentrations was independent of the time of incubation over the time range of 10–240 s. The clotting times in this experiment (Fig. 7) increased in proportion to the S360A APC concentration, reflecting the concentration dependence of S360A APC activity seen above

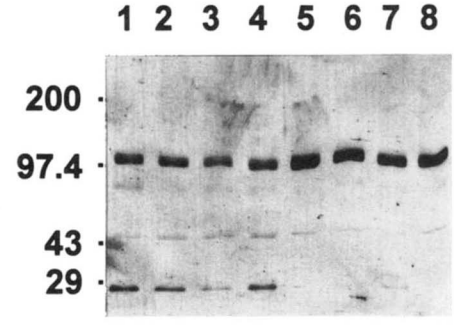

Fig. 6. Western blot of FVa inactivated by plasma-derived APC or S360A APC. FVa (150 ng/lane, 45 nM) was incubated with plasma-derived APC, rwt APC, or S360A APC for varying times and the samples were electrophoresed on an SDS-PAGE gradient gel (4–15%) and subjected to Western blotting as described in Materials and methods. Lanes: 1, 2, and 3 = FVa with 3.2 nM plasma-derived APC incubated for 1.5, 15, and 30 min, respectively; 4 = FVa with 3.2 nM rwt APC incubated for 30 min; 5, 6, and 7 = FVa with 16 nM S360A APC incubated for 1.5, 15, and 30 min, respectively; 8 = FVa with 0.01 U/mL Protac C alone incubated for 30 min.

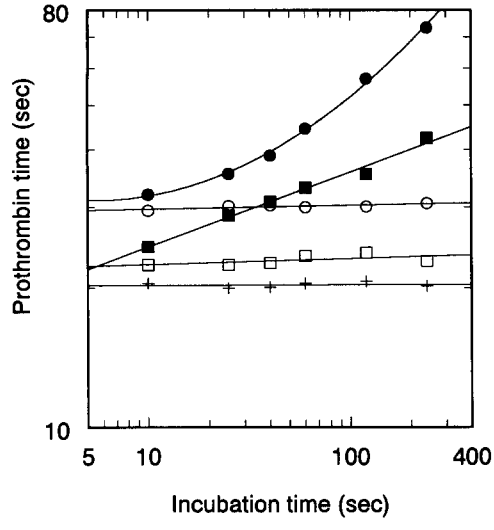

Fig. 7. Time course of the inactivation of FVa by plasma-derived APC or S360A APC. FVa at a concentration of 3.0 nM was preincubated with either plasma-derived or S360A APC. Aliquots of 5 μL were removed at the indicated times and added to 45 μL of FVDP after which 100 μL of Innovin was added to initiate clotting (+ = FVa alone; open square = 0.80 nM S360A APC; open circle = 4.0 nM S360A APC; closed square = 0.80 nM plasma-derived APC; closed circle = 4.0 nM plasma-derived APC). Each experiment was done several times and a representative single experiment is shown.

in the APTT and FXa-1 stage clotting assays (Fig. 2) and in the prothrombinase assays (Fig. 3). This indicated that the inhibitory effect of S360A APC was not due to enzymatic activity but was more likely due to a binding interaction with FVa.

At the highest plasma-derived APC concentration (4.0 nM), the activity of FVa did not approach the baseline value at time = 0. It appeared to extrapolate to the level of FVa activity present in the S360A APC incubation at the same concentration. The form of the FVa activity curve versus time was also non-linear in a log-log plot. This suggests that the initial anticoagulant activity of plasma-derived APC is nonenzymatic due to a binding reaction with a fast equilibrium relative to the enzymatic activity, followed by enzymatic inactivation of FVa and substrate turnover.

APTT assays were performed in normal plasma and Q^{506}-FV plasma (homozygous) with plasma-derived APC or S360A APC added with the recalcification reagent in order to determine the effect of the Q^{506}-FV mutant on the properties of S360A APC (Table 3). For normal plasma, plasma-derived APC at a concentration of 4.8 nM increased the APTT by a factor of 2.55, while in Q^{506}-FV plasma there was a slight increase in the APTT to give an APC resistance ratio of 1.16. S360A APC at a concentration of 11 nM also significantly increased the clotting time of normal plasma but, remarkably, it had no effect at all on Q^{506}-FV plasma. The ability of S360A APC to inhibit purified normal FV and FVa compared with purified Q^{506}-FV and FVa was also studied. Factor V or factor Va (20 pM) was incubated for 10 min at room temperature with 1 to 40 nM of S360A APC in HBS with 0.5% BSA, 5 mM $CaCl_2$, and 25 μM phospholipid vesicles. Then residual factor Va activity was measured in a prothrombinase assay by the addition of 1 nM factor Xa and 0.3 μM prothrombin to each reaction mixture, followed by measuring the rate of thrombin formation with the chromogenic substrate S-2238 under conditions where this rate was linearly correlated with the FV/Va concentration. The results showed that under these conditions, S360A APC gave dose-dependent inhibition of normal (R^{506}) FVa and FV, with 70 and 93% inhibition, respectively, at 40 nM S360A APC. In contrast, S360A APC from 1 to 40 nM in the reaction mixtures gave no significant inhibition (±10%) of Q^{506}-FV or Q^{506}-FVa (data not shown). These observations, consistent with the data in Table 3, show that replacement of Arg 506 by Gln in FV or FVa ablates the susceptibility of FV/Va to inhibition by S360A APC.

Discussion

Analysis of an alanine mutant of the active site serine of *Bacillus amyloliquefaciens* subtilisin showed a greater than 10^6-fold reduction of k_{cat} at pH 8.6 (Carter & Wells, 1988). In a similar study with rat anionic trypsin Corey and Craik demonstrated that mutation of the active site serine to alanine decreased k_{cat} by 4×10^4 at pH 8.0 (Corey & Craik, 1992). In APC the mutation, S360A, caused a ≥1300-fold decrease in the rate of cleavage of the chromogenic substrate S-2366 at pH 8.2 (Table 1). However, in spite of this striking decrease in hydrolytic activity, S360A APC exhibited significant anticoagulant activity. Characterization of this anticoagulant property of S360A APC suggests that it requires an intact active site, except for the hydroxyl group of residue 360, since neither the S360A PC zymogen nor DIP-APC exhibited any anticoagulant activity. The binding of S360A APC to FVa may involve the catalytically inactive active site of S360A APC and/or other exosites whose conformation depends on a "native" conformation of APC. This binding appears to prevent or modulate the proper interaction between FVa and the other components of the prothrombinase complex.

The crystal structures of the serine proteases trypsin and chymotrypsin, as well as the structures of their respective zymogens, have been solved at high resolution (Freer et al., 1970; Birktoft & Blow, 1972; Stroud et al., 1974; Fehlhammer et al., 1977; Kossiakoff et al., 1977). Comparisons between the structures of the active protease and its respective zymogen show that very little change takes place in the structure and orientation of the catalytic triad

Table 3. *Anticoagulant activities of APC and S360A APC in normal plasma compared with plasma containing Q^{506}-FV*[a]

	Normal plasma		Q^{506}-FV plasma	
	Clotting time (s)	APC resistance ratio[b]	Clotting time (s)	APC resistance ratio
+ APC	96.0	2.55	38.5	1.16
− APC	37.6		33.1	
+ S360A-APC	64.8	1.74	33.1	1.03
− S360A-APC	37.3		32.2	
+ S360A-PC	37.0	0.98	31.8	0.98
− S360A-PC	37.6		32.4	

[a]The clotting assay was a standard activated partial thromboplastin time (APTT). The presence (+) or absence (−) of APC at 4.8 nM, S360A PC at 11 nM, or S360A PC at 110 nM in the recalcification reagent is indicated.

[b]The APC resistance ratio is the ratio of clotting times for the presence versus the absence of APC. Clotting times are the means for two different experiments, each performed in duplicate.

residues with respect to each other. The main conformational changes that take place upon activation are in the binding sites for the substrate side chains (Freer et al., 1970; Fehlhammer et al., 1977; Kossiakoff et al., 1977). Activation of chymotrypsin also causes the formation of the oxyanion hole characteristic of serine proteases (Wang et al., 1985). The conclusion for both serine proteases is that formation of the proper conformation of the substrate binding pocket is a major contributing factor in the activation of the protease. By analogy, activation of protein C likely causes some conformational change in the substrate binding pocket for FVa that is responsible, at least in part, for expression of the nonenzymatic anticoagulant activity. The active site of DIP-APC is blocked by the presence of the DIP moiety and is also possibly conformationally altered. This is enough to prevent the binding interaction between APC and FVa that is responsible for the inhibition of FVa cofactor activity. This lack of binding may be due to steric hindrance of FVa binding or to structural changes in APC.

The lack of inhibition of the Q^{506}-FVa by S360A APC suggests that the residue Arg 506 in FVa is critical for interactions between APC and FVa, which are responsible for non-enzymatic inhibition of FVa. A structural model of the A domains of factor VIII has been constructed based on homology to the known structure of nitrite reductase (Pan et al., 1995). By homology to this model Arg 506 in factor V and factor Va is suggested to be in a polypeptide loop that is solvent exposed and located between two β-barrel domains in the A2 domain of factor V. Therefore, this site should be accessible for APC binding and then subsequent cleavage by APC at Arg 506. Apparently, cleavage at Arg 506 facilitates subsequent cleavage at Arg 306 (Kalafatis & Mann, 1993; Kalafatis et al., 1994). The results showing that S360A APC does not inhibit Gln 506-FV or Gln 506-FVa are consistent with the concept that appreciable recognition of the Arg 306 cleavage site by APC follows cleavage at Arg 506. This region of FVa has also been shown to be involved in APC, FXa and protein S binding (Heeb et al., 1994, 1996). Furthermore, this region is homologous to a FIXa binding site on FVIIIa (Fay et al., 1994; O'Brien et al., 1995). Therefore, binding of APC to FVa at the Arg 506 site would be expected to interfere with the FVa–FXa interaction.

It is known that, in the presence of phospholipid vesicles, cleavage of Arg 506 in FVa by APC initially is preferred over cleavages at Arg 306 and 679 and that, furthermore, cleavage at Arg 506 facilitates subsequent cleavages at Arg 306 and 679, presumably by exposing these sites to APC (Kalafatis & Mann, 1993; Kalafatis et al., 1994). This supports the view that Arg 506 is critical for an initial fast binding interaction of APC with FVa followed by much slower cleavage at Arg 506. Mutation of Ser 360 to Ala in APC to eliminate the catalytic activity of APC has revealed this binding interaction by preventing the subsequent cleavages of FVa.

Though the complete structural details of the general form of an inhibitory complex between a serpin and a serine protease are not understood, it is known that only catalytically active serine proteases react with serpins. The serpin then appears to be in a covalent complex with the serine protease and undergoes an extensive conformational change that stabilizes the serpin and the serpin:protease complex (Gettins et al., 1992). Since S360A APC does not have catalytic activity, it appears that it is not inactivated by the serpins in plasma that are responsible for inactivation and turnover of APC in vivo (Fig. 5) (Okajima et al., 1990; España et al., 1991; Heeb et al., 1991). Thus, it could potentially be a more effective anticoagulant in vivo than wild-type APC.

The long-lived anticoagulant activity of S360A APC may be an advantage for the potential use of APC as a therapeutic agent. Purified protein C concentrates have been used successfully to treat homozygous protein C deficiency that causes life-threatening neonatal purpura fulminans (Dreyfus et al., 1991, 1995). Warfarin-induced skin necrosis is associated with protein C deficiency (heterozygous or acquired deficiencies) and is believed to be due to a transient imbalance of the vitamin K-dependent procoagulant factors and the vitamin K-dependent anticoagulant protein C at the initiation of oral anticoagulant therapy (Gladson et al., 1987). Purified protein C concentrate has been used during the initiation of oral anticoagulation therapy to prevent skin necrosis (Schramm et al., 1993). Protein C concentrate has also been used to treat purpura fulminans associated with meningococcemia (Rivard et al., 1995).

APC is implicated to play roles in many other processes, raising the possibility that it may be therapeutically useful for treatment of other conditions. Coronary artery occlusion in pigs induces protein C activation and blocking this activation impairs recovery (Snow et al., 1991), and APC has been shown to inhibit thrombosis in dogs and baboons (Emerick et al., 1985; Gruber et al., 1989). APC also appears to have anti-inflammatory properties (Esmon et al., 1991). Tumor necrosis factor production during human renal allograft rejection is inversely correlated with levels of plasma protein C antigen (Tsuchida et al., 1992). APC prevents the lethal effects of *E. coli* infusion in baboons (Taylor et al., 1987), and APC attenuates LPS induced pulmonary vascular injury in rats, an effect mediated by the inhibition of activated leukocytes (Murakami et al., 1996). In tissue culture, APC appears to inhibit selective responses of human mononuclear macrophages to LPS, IFN-γ, or phorbol ester (Grey et al., 1994).

Protein C has a half-life in vivo of six to eight hours (Viganò et al., 1993) and APC has a half-life in vivo and in vitro of 20–30 min (Okajima et al., 1990; Heeb et al., 1991). Infusion of protein C concentrate results in the conversion of 1–2% of the protein C to activated protein C in vivo (Gruber & Griffin, 1992; Conard et al., 1993; Dreyfus et al., 1995; Pichler et al., 1995). The longer half-life of S360A APC might allow the use of significantly smaller amounts of protein C or the direct infusion of APC at appropriate concentrations. A variant of protein C has been engineered that can be activated by free α-thrombin independently of thrombomodulin (Richardson et al., 1992). This opens up the possibility of the use of protein C for the treatment of macrovascular thrombosis where thrombomodulin concentration is low. Both of these studies demonstrate the power of molecular biology for altering highly specific serine proteases to enhance their potential therapeutic benefits.

Materials and methods

Proteins and reagents

Plasma-derived protein C was purified and activated according to (Gruber et al., 1989; Mesters et al., 1991). FVa was prepared as described (Heeb et al., 1993). FXa was purchased from Enzyme Research Laboratories (South Bend, IN). Phospholipid vesicles (80% phosphatidyl choline, 20% phosphatidyl serine) were prepared as described (Mesters et al., 1991). The chromogenic substrates L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide hydrochloride (S-2366) and H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2238) were purchased from

Chromogenix (Franklin, OH). Normal human citrate-anticoagulated plasma, protein C-deficient plasma, and FV-deficient plasma were purchased from George King Bio-Medical, Inc. (Overland Park, KS). Biotinylated goat-anti-rabbit-IgG, streptavidin alkaline phosphatase conjugate (SAAP), nitro-blue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3′-indolylphosphate-*p*-toluidine salt (BCIP) were purchased from Pierce (Rockford, IL). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Bedford, MA). Protac C, a snake venom enzyme activator of protein C, was obtained from American Diagnostica (Greenwich, CT).

Expression and purification of recombinant protein C

The cDNA coding for wild-type recombinant protein C (rwt protein C) in pUC119 and an expression system including kidney 293 cells, and the expression vector pCIS2M were generous gifts from Drs. Francis J. Castellino and Li Zhang of Notre Dame University. Site-directed mutagenesis as described (Kunkel et al., 1987) was used to construct the S360A mutant using the mutagenic oligonucleotide primer 5′-AGGGCGACGCTGGGGGGC-3′ (the underlined letters indicate the mutated bases). The S360A protein C gene was then inserted into the expression vector pCIS2M, transfected into human kidney 293 cells, and expressed as described (Zhang & Castellino, 1990).

Purification of recombinant wild-type and S360A protein C from conditioned cell culture media was accomplished using a two-step chromatography procedure. Conditioned media was dialyzed against TBS (50 mM Tris, pH 7.4, 150 mM NaCl) containing 2 mM EDTA and 2 mM benzamidine. The media was then loaded onto a 5 mL Fast Flow Q column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Protein C was eluted with a 0–30 mM $CaCl_2$ gradient in TBS. Fractions containing protein C were identified by SDS-PAGE and pooled. This pool was dialyzed against TBS + 4 mM EDTA and loaded onto a second Fast Flow Q column. Protein C was then eluted with a 0.15–0.5 M gradient of NaCl. The protein C containing fractions were pooled and dialyzed against TBS for storage at −80 °C.

Functional assays

Each purified protein C was activated by Protac C (American Diagnostica, Inc., Greenwich, CT). Aliquots of protein C in TBS at a concentration of 100 μg/mL (1.6 μM) were incubated with an equal volume of 1 U/mL Protac C in H_2O at 37 °C for 15–30 min. Amidolytic activity of each APC was measured by adding an aliquot of APC to the chromogenic substrate S-2366 (1 mM) in TBS, 1 mg/mL BSA, 0.02% NaN_3, 3 mM $CaCl_2$, pH 8.2. Hydrolysis of the substrate was monitored by the change in absorbance at 405 nm over 10 min using an EL312 Microplate Bio-Kinetics reader (Bio-Tek Instruments, Inc., Winooski, VT) (Mesters et al., 1991).

The APTT clotting assays were performed as follows. APC (0.5–2 μL of 50 μg/mL, 8–32 nM final concentration in the plasma) was added to 50 μL of PCDP, which was then mixed with 50 μL of APTT reagent (Platelin LS, Organon Technika Corp., Durham, NC) and preincubated at 37 °C for five minutes. Clotting time was recorded on an ST4 coagulometer (Stago, Asnieres, France) after the addition of 50 μL of 25 mM $CaCl_2$. The FXa-1 stage assays were performed as follows. Fifty microliters of PCDP with APC added as in the APTT assays was mixed with 50 μL of rabbit brain cephalin (100 μg/mL) (Sigma, St. Louis, MO) and incubated at 37 °C for three minutes. Clotting was initiated by the addition of 50 μL of FXa (39 ng/mL, 0.5 nM) (Enzyme Research Laboratory, Inc., South Bend, IN) and 50 μL of 25 mM $CaCl_2$.

For some APTT assays, plasma-derived APC and S360A APC were treated with DFP as follows. After activation with Protac C as described earlier, DFP in isopropanol was added to a final concentration of 1 mM. These mixtures were incubated for one hour at 37 °C. Amidolytic activity was tested as described earlier to verify inactivation of APC to greater than 99%.

For APTT assays testing the inhibition of the APCs by monoclonal antibodies each APC was incubated at 45 μg/mL (0.73 μM) with an equal volume of monoclonal antibody C1 (1.15 mg/mL) or monoclonal antibody C3 (0.46 mg/mL) at 37 °C for one hour. APTT assays were then performed with these samples as described earlier.

Inhibition of prothrombinase activity was measured in a prothrombinase assay as follows. Prothrombinase components (1 nM FXa, 20 pM FVa, 5 mM $CaCl_2$, and 50 μM phospholipid vesicles containing 20% phosphatidyl serine and 80% phosphatidyl choline) were preincubated at room temperature for 15 min with or without protein C species in 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% BSA, and 0.1 mM $MnCl_2$. Prothrombin was added to a final concentration of 0.3 μM and 20 μL aliquots were removed at different time points and quenched in 80 μL of TBS containing 10 mM EDTA, 0.1% BSA, pH 8.2. Chromogenic substrate S-2238 (50 μL of 0.6 mM in TBS, 0.1% BSA, 0.02% NaN_3, 3 mM $CaCl_2$, pH 8.2) was added and the rate of thrombin formation was assessed by measuring the change in absorbance at 405 nm as described earlier. The zymogen protein C was pretreated with 400 μM p-APMSF to inactivate any traces of activated protein C.

FVa inactivation was measured in a prothrombin time clotting assay as follows. FVa was incubated with APC in a volume of 40 μL at the defined concentrations in 50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 0.5% BSA, 0.1 mM $MnCl_2$, and 32 μM phospholipid vesicles (20% phosphatidyl serine, 80% phosphatidyl choline) at 37 °C, and 5 μL aliquots were removed at the indicated time points and added to 45 μL FVDP. Innovin (100 μL) (Baxter Diagnostics, Inc., Deerfield, IL) was added immediately to initiate clotting.

For Western blots of FVa inactivated by APC, 45 nM FVa was incubated with the indicated concentrations of plasma-derived APC, rwt APC, or S360A APC in the buffer described above. Aliquots were removed at the indicated time points and boiled in SDS sample buffer, then electrophoresed in a 4–15% gradient gel and transferred to PVDF membrane. FVa and FVa cleavage products were monitored by Western blotting with a polyclonal antibody against the human FVa heavy chain (generous gift from Drs. Jan Rosing and Guido Tans, University of Limburg, Maastricht, The Netherlands) and a monoclonal antibody against the human FVa heavy chain (Enzyme Research Laboratory, Inc., South Bend, IN) (Heeb et al., 1995).

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