Engineering a Disulfide Bond to Stabilize the Calcium-binding Loop of Activated Protein C Eliminates Its Anticoagulant but Not Its Protective Signaling Properties*

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Jong-Sup Bae, Likui Yang, Chandrashekhara Manithody, and Alireza R. Rezaie 1

From the Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, Missouri 63104

In addition to an anticoagulant activity, activated protein C (APC) also exhibits anti-inflammatory and cytoprotective properties. These properties may contribute to the beneficial effect of APC in treating severe sepsis patients. A higher incidence of bleeding because of its anticoagulant function has been found to be a major drawback of APC as an effective anti-inflammatory drug. In this study, we have prepared a protein C variant in which an engineered disulfide bond between two β-sheets stabilized the functionally critical Ca2+-binding 70–80 loop of the molecule. The 70–80 loop of this mutant no longer bound Ca2+, and the activation of the mutant by thrombin was enhanced 60–80-fold independently of thrombomodulin. The anticoagulant activity of the activated protein C mutant was nearly eliminated as determined by a plasma-based clotting assay. However, the endothelial protein C receptor- and protease-activated receptor-1-dependent protective signaling properties of the mutant were minimally altered as determined by stauroporine-induced endothelial cell apoptosis, thrombin-induced endothelial cell permeability, and tumor necrosis-α-mediated neutrophil adhesion and migration assays. These results suggest that the mutant lost its ability to interact with the procoagulant cofactors but not with the protective signaling molecules; thus this mutant provides an important tool for in vivo studies to examine the role of anticoagulant versus anti-inflammatory function of activated protein C.

Protein C is a vitamin K-dependent plasma serine protease zymogen that upon activation by the thrombin-thrombomodulin (TM)2 complex on the endothelial cell surface down-regulates the clotting cascade by a feedback loop mechanism (1, 2). The activation of protein C to activated protein C (APC) by the thrombin-TM complex is markedly stimulated by endothelial protein C receptor (EPCR) (3). APC, upon dissociation from EPCR, forms a complex with the cofactor, protein S, to shut down thrombin generation by limited proteolysis of factors Va (fVa) and VIIIa, the procoagulant cofactors of the prothrombinase and intrinsic Xase complexes, respectively (4). In addition to anticoagulant activity, when APC remains associated with EPCR, it elicits protective signaling responses in endothelial cells (5–9), possibly accounting for the beneficial effect of APC as an anti-inflammatory drug in treating severe sepsis patients (10). The mechanism of the anti-inflammatory and cytoprotective effects of APC is not understood; however, recent results have indicated that APC in complex with EPCR cleaves protease-activated receptor-1 (PAR-1) to initiate protective signaling events in endothelial cells (8, 9).

The two endothelial cell surface receptors EPCR and TM improve the activation of protein C by thrombin by 3–4 orders of magnitude in a Ca2+-dependent reaction (2, 3). The binding of Ca2+ to several low affinity sites in the γ-carboxyglutamic acid (Gla) domain of protein C enables the zymogen to interact with EPCR, thereby markedly improving the K_m of activation by the thrombin-TM complex (3, 11). In addition, the binding of Ca2+ to a high affinity site in the protease domain of protein C is required for the thrombin-TM complex to recognize and activate protein C at a physiologically relevant rate (12). This Ca2+-binding site has been localized to the 70–80 loop of protein C (chymotrypsinogen numbering) (13). The binding of Ca2+ to this loop of APC is also required for the catalytic function of APC in the anticoagulant pathway (12, 14). In addition to Ca2+, the monovalent cation sodium also modulates the catalytic function of APC (14–16). Our recent mutagenesis studies have indicated that the two metal ion-binding sites of APC are linked energetically (14). In a recent study, we engineered a disulfide bond between two anti-parallel β-structures comprising residues 64–69 and 81–91 that join together to form the Ca2+-binding 70–80 loop of protein C/A/PC (17). Studies with this mutant (Cys67–Cys82), which was expressed in the Gla-domainless form, provided new insight into mechanism through which Ca2+ modulates protein C activation by thrombin in the absence and presence of TM.

To evaluate the anticoagulant and anti-inflammatory properties of Cys67–Cys82 APC, we expressed the full-length form of this mutant in mammalian cells. The activation of this mutant...
Anticoagulant and Protective Signaling Properties of APC

by thrombin was improved 60–80-fold independently of both TM and Ca$^{2+}$. The amydolytic activity of this mutant was comparable with the wild type; however, its anticoagulant activity was dramatically impaired. Further studies revealed that the EPCR- and PAR-1-dependent protective signaling effects of Cys$^{67}$–Cys$^{82}$ APC have remained nearly normal. We conclude that the engineered disulfide bond has stabilized the APC mutant in a conformation that is incapable of normal interaction with procoagulant cofactors but capable of interacting with the target cytoprotective endothelial cell signaling molecules.

EXPERIMENTAL PROCEDURES

Reagents—Staurosporine was purchased from Calbiochem. The following antibodies were used for Western blots: polyclonal human AIF (1:1000), Bax (1:100, Chemicon, Temecula, CA), human p53 (1:1000, Cell Signaling, Beverly, MA), human Bcl-2 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and actin (1:5000, Sigma). Antibodies blocking activation of PAR-1 (H-111, Santa Cruz Biotechnology) or non-blocking (S-19, Santa Cruz Biotechnology) and function-blocking EPCR antibody (clone RCR-252; Cell Sciences, Canton, MA) were used at 25 μg/ml. Tumor necrosis factor-α (TNF-α) was purchased from R&D Systems (Minneapolis, MN). Human plasma factors Va and Xa and antithrombin were purchased from Hemalogic Technologies Inc. (Essex Junction, VT), and Spectrozyme PCa was purchased from American Diagnostica (Greenwich, CT). 5,5’-Dithiobis(2-nitrobenzoic acid) (DTNB) and hirudin were purchased from Sigma.

Cell Culture—Transformed human endothelial cell line EA.hy926 was kindly provided by Dr. C. Edgell (University of North Carolina at Chapel Hill). Cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics (penicillin G and streptomycin). Freshly isolated neutrophils were kindly mented with 10% fetal bovine serum and antibiotics (penicillin G and streptomycin). Freshly isolated neutrophils were kindly provided by Dr. C. Edgell (University of North Carolina at Chapel Hill). Cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics (penicillin G and streptomycin). Freshly isolated neutrophils were kindly provided by Dr. David Ford (Saint Louis University Medical School).

Construction and Expression Methods—Wild-type and Arg$^{67}$/Asp$^{82}$ → 2Cys protein C (Cys$^{67}$–Cys$^{82}$) were expressed in human embryonic kidney cells (HEK-293) as described (17). The Ser$^{195}$ to Ala protein C mutant was prepared using the same expression system. Soluble recombinant TM (18) and protein C inhibitor (19) were expressed and purified as described. Recombinant soluble EPCR (sEPCR), N-terminally tagged with the 12-residue epitope for HPC4, was a generous gift from Dr. Esmon (Oklahoma Medical Research Foundation).

Alkaline Phosphatase–PAR-1–Tissue Factor Assay (AP-PAR-1–TF)

The cDNA encoding secreted human tissue-nonspecific alkaline phosphatase (20) (a generous gift from Dr. William Sly, Saint Louis University Medical School), lacking the last 19 C-terminal residues, was fused to a synthetic DNA fragment encoding the exodomain of PAR-1 (Thr$^{37}$ to Ser$^{99}$) and the membrane-spanning domain of tissue factor (Arg$^{218}$ to Lys$^{244}$). AP-PAR-1–TF cDNA was inserted into the HindIII/XbaI cloning sites of the mammalian expression vector pRc/RSV (Invitrogen).

Anticoagulant Activity—The anticoagulant activity of wild-type and mutant APC was evaluated in both the purified and plasma-based assay systems as described (21). The time course of fVa inactivation by APC was measured by a three-stage assay in the purified system as described (14). Briefly, in the first stage, fVa (5 nM) was incubated with wild-type or mutant APC (1 nM) on 25 μM phospholipid vesicles composed of 80% phosphatidylcholine and 20% phosphatidylserine in TBS containing 2.5 mM Ca$^{2+}$, 1.0 mg/ml BSA, and 0.1% polyethylene glycol 8000. In the second stage, at different time intervals (0–40 min), the remaining fVa activity was determined in a prothrombinase assay from the fVa-catalyzed prothrombin activation by fXa as described (14). The prothrombinase assay was carried out for 30 s with excess prothrombin (1 μM) and a saturating concentration of fXa (5 nM) at room temperature. The remaining activity of fVa was determined from the decrease in the rate of thrombin generation as monitored by an amidolytic activity assay in the third stage using 200 μM S2238.

The anticoagulant activities in plasma were evaluated in an aPTT assay using a STart 4 fibrinometer (Diagnostica/Stago, VOLUME 282 • NUMBER 12 • MARCH 23, 2007 9252 JOURNAL OF BIOLOGICAL CHEMISTRY
incubated for 1 h at 37 °C in the dark with a TUNEL reaction

citrate for 10 min on ice, washed twice with PBS, pH 7.4, and

temperature, permeabilized with 0.1% Triton X-100, 0.1% sodium

were fixed with 3% paraformaldehyde for 20 min at room tem-

perature. Experiments were performed in triplicate and

repeated multiple times.

Apoptosis Assay—EA.hy926 cells (0.5 × 10⁶) were seeded

onto cover slips coated with gelatin as described (23). After 24 h

at 37 °C, the medium was replaced, and cells were incubated

with protein C or APC for another 24 h. Then, the cells were

incubated with 5 μM staurosporine for 4 h. Thereafter, the cells

were fixed with 3% paraformaldehyde for 20 min at room tem-

perature, permeabilized with 0.1% Triton X-100, 0.1% sodium

citrate for 10 min on ice, washed twice with PBS, pH 7.4, and

incubated for 1 h at 37 °C in the dark with a TUNEL reaction

mixture (Roche Applied Science) for in situ detection of cell
death. After twice washing with PBS, pH 7.4, the cells were

incubated at room temperature with Hoechst 33342 (Sigma) for

15 min. The number of apoptotic cells was expressed as the

percentage of TUNEL-positive cells of the total number of

nuclei determined by Hoechst staining. The cells were counted

in 10–20 random fields (×30 magnification) by two independ-

ent observers blinded to the experimental conditions.

Adhesion Assay—Neutrophil adherence to endothelial cells

was evaluated by fluorescent labeling of neutrophils as
described (24, 25). Briefly, peripheral blood neutrophils were

labeled with 5 μM Vybrant DiD (Molecular Probes) for 20 min

at 37 °C in phenol red-free RPMI 1640 containing 5% fetal

bovine serum. After two washes, the neutrophils (1.5 × 10⁶/ml,

200 μl/well) were resuspended in adhesion medium (RPMI

containing 2% fetal bovine serum and 20 mM HEPES) and

added to confluent monolayers of EA.hy926 cells in 96-well

plates, which were treated with APC derivatives (20 nM for 24 h)

followed by TNF-α (10 ng/ml for 4 h). In blocking experiments,

the monolayers were preincubated for 30 min at 37 °C with the

appropriate antibodies. The fluorescence of labeled cells was

measured (total signal) using a fluorescence microplate reader

(Molecular Devices). After incubation for 60 min at 37 °C, non-

adherent cells were removed by washing four times with pre-

warmed RPMI 1640, and the fluorescent signals of adherent
cells were measured by the same method. The percentage of
adherent leukocytes was calculated by the formula: % adherence =
(adherent signal/total signal) × 100 (as described in Ref. 25).

Analysis of Expression of Adhesion Molecules—The expres-

sion of vascular cell adhesion molecule 1 (VCAM-1), intercel-

lular adhesion molecule 1 (ICAM-1), and E-selectin on

EA.hy926 cells was determined by a whole-cell ELISA as
described (26). Briefly, confluent monolayers of EA.hy926 cells

were treated with APC derivatives (20 nM) for 24 h followed by

TNF-α for 4 h. The medium was removed, and cells were

washed with PBS and fixed by adding 50 μl of 1% paraformal-

dehyde for 15 min at room temperature. After washing, 100 μl

of mouse anti-human monoclonal antibodies (VCAM-1,

ICAM-1, E-selectin, 1:50 each) were added. After 1 h (37 °C, 5%

CO₂), the cells were washed three times, and then 100 μl of

1:2000 peroxidase-conjugated anti-mouse IgG antibodies

(Sigma) was added for 1 h. The cells were washed again three
times and developed using o-phenylenediamine substrate
(Sigma). Colorimetric analysis was performed by measuring

absorbance at 490 nm. All measurements were performed in

triplicate wells.

Transendothelial Migration Assay—Migration assays were

performed in Transwell plates (6.5-mm diameter, 8-μm pore

size filters). EA.hy926 cells (6 × 10⁴) were cultured for 3 days to

obtain confluent endothelial monolayers. The cell monolayers

were washed three times with PBS before the addition of freshly

isolated neutrophils (1.5 × 10⁶/0.2 ml) to the upper compart-

ment. In blocking experiments, the EA.hy926 cells were prein-

cubated for 30 min at 37 °C with indicated antibodies. Trans-

well plates were incubated at 37 °C, 5% CO₂ for 2 h. Cells in the

upper chamber of the filter were aspirated, and nonmigrating

cells on top of the filter were removed with a cotton swab.

Neutrophils on the lower side of the filter were fixed with 8% glut-

araldehyde and stained with 0.25% crystal violet (Sigma) in 20%

methanol (w/v). Each experiment was repeated in duplicate

wells, and within each well counting was done in nine randomly

selected microscopic high power fields.

Enzyme-linked Immunosorben
t Assays—To evaluate the interaction of the HPC4-tagged EPCR with APC, 96-well flat

microtiter plates were coated with the HPC4 monoclonal anti-

body in ELISA buffer (TBS containing 1 mM CaCl₂) overnight at

4 °C. After the plates were washed three times in the same TBS

buffer containing 0.05% Tween 20, they were incubated with 1%

BSA in TBS/ Ca²⁺ for 2 h at room temperature. After the plates

were washed as described above, they were incubated with sol-

uble EPCR (0.5 μM in TBS/ Ca²⁺ containing 0.1% BSA) for 1 h.

The plates were rinsed and incubated with wild-type or mutant

APC (7–1000 nM) diluted in the same TBS/ Ca²⁺ buffer for 1 h.

After the plates were rinsed again, they were incubated with a

goat anti-protein C polyclonal antibody (1:1000) for 1 h. Then,

the plates were washed and incubated with rabbit anti-goat IgG

(KPL, MD, 1:1000) for 1 h. After washing, the plates were incu-

bated with 2,2’-azino-di(3-ethylbenzthiazoline-6-sulfonate)

(ABTS; KPL, Gaithersburg, MD). Colorimetric analysis was

performed by measuring absorbance values at 405 nm as
described above. All treatments were performed in duplicate

and repeated at least three times.
Anticoagulant and Protective Signaling Properties of APC

Western Blot Analysis—Equal amounts of total cellular protein were subjected to 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Bio-Rad). Blots were blocked overnight at 4 °C with 5% skim milk and incubated with the indicated primary antibodies. Blots were further incubated with diluted (1:2000) horseradish peroxidase-conjugated secondary antibodies (Sigma). Immunoreactive protein bands were visualized by SuperSignal West Pico (Pierce). Signal intensities were quantitated by densitometry (Gel-Pro Analyzer).

Statistical Analysis—Results are expressed as mean ± S.E., and t tests (paired or independent) were used to assess data. Differences were considered statistically significant at p values of <0.05. Statistics were performed using the software package SPSS, version 14.0 (SPSS, Chicago, IL). All experiments were repeated at least three times.

RESULTS

Protein C Activation by Thrombin—The activation of protein C by thrombin requires the cofactor functions of both TM and Ca\(^{2+}\) (2). In the absence of TM, Ca\(^{2+}\) plays an inhibitory role, and thus thrombin is a very poor activator of protein C in the absence of the cofactor (2). The initial rate of wild-type and mutant protein C activation by thrombin in the absence of TM and in the presence of physiological concentrations of Ca\(^{2+}\) is presented in Fig. 1A. Comparisons of the activation rates suggest that relative to wild-type, the activation of Cys\(^{67}–\)Cys\(^{82}\) protein C by thrombin has been improved 60–80-fold independently of TM. Nevertheless, TM did not accelerate the activation of the protein C mutant by thrombin because of the inability of the mutant to interact with TM, as demonstrated previously for the corresponding mutant in the Gla-domainless form (17). The Ca\(^{2+}\) dependence of protein C activation by thrombin revealed that the 70–80 loop of the mutant no longer binds Ca\(^{2+}\) (data not shown).

Biochemical Characterization of Recombinant APC Derivatives—SDS-PAGE analysis of wild-type and Cys\(^{67}–\)Cys\(^{82}\) APC under nonreducing conditions indicated that both proteins had been purified to homogeneity and exclusively migrated in monomeric form as two subforms with the expected apparent molecular masses that correspond to α- and β-APC (not shown), which are glycosylation variants observed with this protein (27, 28). Spectrophotometric titrations with DTNB indicated that neither wild-type nor Cys\(^{67}–\)Cys\(^{82}\) APC (up to 10 \(\mu\)M) increased the absorbance of DTNB at 412 nm, suggesting that both proteins lack a free sulfhydryl group (not shown). These results suggested that a correct disulfide bond has been formed between the two engineered Cys residues in the mutant protein. The active site concentrations were determined by an amidolytic activity assay and active site titrations with known concentrations of protein C inhibitor as described (19). The concentrations of enzymes were within 95–100% of those expected based on their absorbance at 280 nm. The homogeneity of Cys\(^{67}–\)Cys\(^{82}\) APC was further confirmed in a sensitive colorimetric assay measuring the APC cleavage of Arg-41 of PAR-1 in a construct in which alkaline phosphatase cDNA was coupled to the exodomain of PAR-1 and anchored to the cell surface by the membrane-spanning domain of tissue factor. In this assay, both wild-type and mutant APC, for which concentrations were determined based on their absorbance at 280, exhibited essentially identical activity (Fig. 1B), suggesting that the mutant has no heterogeneity and that both APC derivatives have identical catalytic activity toward PAR-1. The activity of 0.1 nM thrombin was similar to that of 75 nM APC, suggesting that thrombin is ~750-fold more potent than APC. This is consistent with results of a previous study which compared the activities of thrombin and APC toward PAR-1 in a similar assay system (29). APC S195A did not exhibit any activity in this assay (not shown).

Binding of Ca\(^{2+}\) to the 70–80 loop and Na\(^{+}\) to 225 loop of APC is required for both the amidolytic and proteolytic activities of APC (12, 14, 16). Calcium enhances the amidolytic activity of APC by <2-fold; however, the amidolytic activity of APC is strictly dependent on Na\(^{+}\) (14, 15). We demonstrated previously that the two metal ion-binding sites are linked energetically (14). In the absence of Ca\(^{2+}\) (100 \(\mu\)M EDTA), APC exhibited a \(K_{d(app)}\) of 45 mM for Na\(^{+}\). In agreement with previous
results, the same value in the presence of Ca\(^{2+}\) was reduced to \(~3—5\, \text{mm} \, \text{Na}^+\) (14). By contrast, the 70—80 loop of Cys\(^{67}\)–Cys\(^{82}\) APC did not interact with Ca\(^{2+}\), and the Na\(^+\)-binding loop was stabilized in a high affinity state as evidenced by the mutant exhibiting a \(K_{d(app)}\) of 5 mm for Na\(^+\) in EDTA. The amidolytic activity of the mutant APC toward Spectrozyme PCa was nearly normal in EDTA, but the 2-fold stimulatory effect of Ca\(^{2+}\) was abolished. These results suggested that the APC mutant had been trapped in a high affinity Na\(^+\) conformation independent of Ca\(^{2+}\).

Anticoagulant Activity—The anticoagulant activity of the APC mutant was evaluated in both purified and plasma-based clotting assays. The APC mutant exhibited dramatically impaired activity in a fVa degradation assay monitoring the inhibition of thrombin generation by prothrombinase (Fig. 2A). In the aPTT-initiated clotting assay, the APC mutant did not exhibit any anticoagulant activity for up to 100 nm (Fig. 2B).

Effect of APC on Thrombin-induced Permeability—Previous studies measuring the flux of albumin in a dual chamber system have indicated that thrombin disrupts the permeability barrier of EA.hy926 cells and APC has a potent protective effect (22). In a similar assay, treatment of EA.hy926 cells with thrombin resulted in an enhanced permeability that was effectively reversed by both wild-type and mutant APC (Fig. 3), although the APC concentration dependence of the protective effects indicated that the mutant requires a 2-fold higher concentration to yield an equal effect (Fig. 3A). In agreement with the literature (22), the protective effect required interactions with both EPCR and PAR-1, because the function-blocking antibodies to both receptors eliminated the effect with both APC derivatives (Fig. 3B), suggesting that the mutant functions through the same signaling pathways.

To determine the basis for the 2-fold lower efficacy of the APC mutant, the ability of the mutant to interact with sEPCR was evaluated by an ELISA-based binding assay. As shown in Fig. 4, APC bound to sEPCR with a \(K_{d(app)}\) of \(~31 \pm 2\, \text{nm} (n = 3)\), which is similar to that previously reported for APC interaction with sEPCR (29 nm) in solution (30). A \(K_{d(app)}\) of \(~132 \pm 3\, \text{nm} (n = 3)\) for Cys\(^{67}\)–Cys\(^{82}\) APC interaction with sEPCR was obtained, suggesting that the 2-fold lower activity of the mutant is likely because of its lower affinity for EPCR. Because the interactive sites for EPCR are localized to the Gla domain of APC, the basis for a lower affinity of the mutant for sEPCR is unknown. It is possible that the stabilization of the Ca\(^{2+}\) loop alters the conformation of the Gla domain, thereby decreasing the affinity of the mutant for EPCR. In support of this hypothesis a Ca\(^{2+}\)-dependent conformational linkage between the Gla and protease domains of factor Xa has been reported (31). Alternatively, a fraction of the APC mutant may be not fully \(\gamma\)-carboxylated. Both wild-type and Cys\(^{67}\)–Cys\(^{82}\) APC cleaved Arg\(^{41}\) of PAR-1 in AP-PAR-1-TF-transfected cells with identical efficiency (Fig. 1B), most likely suggesting that this cleavage occurs independently of EPCR in this system. This could happen if AP-PAR-1-TF were not co-localized with
EPCR on the membrane surface. In support of this hypothesis both APC derivatives exhibited identical activities in the absence and presence of the anti-EPCR function-blocking antibody (not shown).

**Anti-apoptotic Activity of APC**—APC exhibits cytoprotective activity in the staurosporine-induced apoptosis assay (23). The APC mutant exhibited normal anti-apoptotic activity in EA.hy926 cells treated with staurosporine (Fig. 5), although the concentration dependence of the cytoprotective activities suggested that, instead of 5 nM APC, 10 nM APC mutant was required to obtain a maximal effect (Fig. 5C). Similar to previous results (23), the cytoprotective activity of APC was mediated, at least partially, through inhibition of caspase-3 activity, and the function-blocking antibodies to either EPCR or PAR-1 abrogated the anti-apoptotic activity (not shown). The cytoprotective activity required an intact active site and was mediated through the up-regulation of Bcl-2 and the down-regulation of p53, Bax, and AIF genes (Fig. 5D) as demonstrated previously (32, 33).

Previously, the anti-apoptotic activity of APC was examined in staurosporine-treated endothelial cells (23). TNF-α treatment of endothelial cells also induced an apoptotic pathway, and the pretreatment of endothelial cells with either 10 nM wild-type or mutant APC potently inhibited cell death induced by TNF-α (data not shown).

**Effect of APC on TNF-α-mediated Leukocyte Adhesion and Migration**—TNF-α treatment of human umbilical vein endothelial cells is associated with up-regulation of several cell surface adhesion molecules like VCAM-1, ICAM-1, and E-selectin, and APC has been demonstrated to inhibit the expression of these molecules (7). The results presented in Fig. 6A support these findings in EA.hy926 cells and further demonstrate that APC suppression of the TNF-α-mediated expression of adhesion molecules is EPCR and PAR-1-dependent, as evidenced by the ability of function-blocking antibodies to either of the receptors to neutralize the modulatory effect. Similar to wild-type, the APC mutant down-regulated the TNF-α-mediated expression of all three adhesion molecules in EA.hy926 cells (Fig. 6A). Further studies were initiated to determine whether the expression of these adhesion molecules correlates with enhanced binding of neutrophils and whether APC can block the adhesion of neutrophils to TNF-α-activated EA.hy926 cells. The results presented in Fig. 6, B and C, demonstrate that both APC derivatives effectively inhibited the binding of neutrophils to the TNF-α-activated endothelial cells by EPCR and PAR-1-dependent pathways. Similar to other cellular effects, an ~2-fold higher concentration of mutant was required to obtain a maximal inhibitory effect (Fig. 6C). Further studies revealed that the adhesion of neutrophils to endothelial cells is associated with their sub-
sequent transendothelial migration and that both APC derivatives inhibit this step by a similar enzyme concentration-dependent as well as EPCR- and PAR-1-dependent mechanisms (Fig. 6, D and E). Taken together, these results demonstrate that, unlike the nearly complete loss of the anticoagulant activity, the in vitro indices of the anti-inflammatory and cytoprotective activities of the APC mutant have remained nearly normal.

DISCUSSION

Recently, recombinant APC has been approved as a drug for treating severe sepsis patients (10). There is growing evidence suggesting that the beneficial effect of APC in severe sepsis is separate from its anticoagulant effect and is, at least partially, attributable to its direct protective signaling effect in endothelium (5, 9, 22, 32, 33). Nevertheless, similar to heparin therapy, a major drawback of APC is the increased incidence of bleeding in ~2% of treated patients (10). Thus, there is interest in developing APC variants with decreased anticoagulant but normal cytoprotective activity (9). Here, we have demonstrated that stabilization of the 70–80 loop by an engineered disulfide bond nearly eliminates the anticoagulant activity of APC but minimally affects its anti-inflammatory and cytoprotective properties.

The mechanism through which APC functions in the anticoagulant pathway has been studied extensively and is relatively well understood. Briefly, upon activation of EPCR-bound protein C on endothelial cells by the thrombin-TM complex, APC can dissociate from EPCR and bind to protein S to function in the anticoagulant pathway by degrading factors Va and VIIIa by limited proteolysis (4). The specificity of the APC recognition of both procoagulant cofactors is determined by basic residues of an exosite that is known to be a heparin-binding site in APC (21, 34, 35). Basic residues of this exosite are clustered on three exposed surface loops referred to as 37–39 loop, 60–68 loop and 70–80 loop (chymotrypsin numbering (13)). With the exception of basic residues of 60 loop, these residues are involved in recognition and subsequent degradation of factors.
Anticoagulant and Protective Signaling Properties of APC

Va and VIIIa by APC in the anticoagulant pathway (21, 34, 35). These structural-functional data have recently allowed rational design of several recombinant APC variants containing substitutions on basic residues of 37–39 loop and 70–80 loop which exhibited greatly reduced anticoagulant activity but normal protective signaling properties (9). The results presented above suggest that our novel approach of stabilizing the Ca^{2+}-binding loop of APC by an engineered disulfide bond also yields an APC variant that has dramatically lost its anticoagulant activity, but has retained its cytoprotective signaling properties. In light of a minimal role for 60 loop of APC in recognition of FVa, it is not known how an engineered disulfide bond between residues 67 and 82 nearly abolishes the anticoagulant activity of the APC mutant without significantly impacting its direct effect on cell signaling pathways. Nevertheless, the anticoagulant function of APC is known to require the cofactor functions of the metal ions Ca^{2+} and Na^+ both of which allosterically modulate the structure and catalytic function of APC (14). Such a coordinated metal ion modulation of the APC structure and function has been disrupted in the mutant protease because the engineered disulfide bond abolishes the requirement for Ca^{2+} and stabilizes the Na^+-binding site of the mutant protease in the high affinity state. These structural changes appear to be important for the anticoagulant function but not for the protective signaling effects of APC. It is also possible that the binding of Ca^{2+} to the 70–80 loop of APC is required for the exposure of the basic residues of this loop for interaction with the procoagulant cofactors and the stabilization of the loop by a disulfide bond stabilizes these residues in internally oriented conformations.

For APC to elicit protective signaling responses, it must remain associated with EPCR on endothelial cells. Unlike the anticoagulant effect, the exact mechanism through which APC exerts its cell signaling effects is not known. Recent studies have demonstrated that, in addition to EPCR, the protective signaling effect of APC also requires the presence of PAR-1 on the surface of endothelial cells (22, 32). Subsequent studies have indicated that the EPCR-dependent cleavage of PAR-1 at Arg-41 is responsible for the direct cell signaling effect of APC (9, 23). Other recent studies have indicated that APC down-regulates expression of several key pro-inflammatory cytokines (i.e. TNF-α and IL-1), adhesion molecules (i.e. ICAM-1 and VCAM-1), and transcription factors (i.e. nuclear factor-κB related molecules) in endothelial and non-endothelial cells (7). In agreement with these observations, we demonstrated that both wild-type and mutant APC inhibited the TNF-α-mediated up-regulation of ICAM-1, VCAM-1, and E-selectin. During inflammation the expression of these adhesion molecules is up-regulated and leukocytes, by binding to these molecules on the surface of endothelial cells, undergo extravasation (25). In the present study, we demonstrated that the enhanced TNF-α-mediated expression of the adhesion molecules on the transformed endothelial cells is indeed associated with enhanced binding of neutrophils and their subsequent transendothelial migration and that both APC derivatives effectively inhibited these processes by EPCR and PAR-1-dependent mechanisms. These in vitro anti-inflammatory activities of APC required a functionally active site because neither protein C nor S195A APC exhibited activity. The results suggest that, unlike the nearly complete loss of the anticoagulant function, the anti-inflammatory function of the APC mutant has relatively intact and is mediated through the same EPCR- and PAR-1-dependent pathways.

Increased vascular permeability is another hallmark of inflammatory disorders such as sepsis. Several recent in vitro studies have indicated that APC reverses thrombin-induced endothelial permeability, possibly contributing to the anti-inflammatory properties of APC in improving survival in severely septic patients (10, 22). Similar to the results presented above, the APC mutant exhibited normal barrier protective activity by EPCR- and PAR-1-dependent mechanisms. Moreover, similar to wild-type, the cytoprotective activity of the mutant in a stauroporine-induced apoptosis assay was nearly normal and mediated (at least partially) through the inhibition of caspase-3, the up-regulation of Bel-2 and down-regulation of the p53, Bax, and AIF genes as demonstrated previously (23, 32, 33).

In conclusion, we have demonstrated that although the anticoagulant activity of the APC mutant was dramatically impaired, all in vitro indices of its anti-inflammatory and cytoprotective signaling properties were minimally impacted. Thus this novel APC mutant potentially may be a safer drug for treating severe sepsis patients who experience a higher incidence of bleeding due to the anticoagulant function of APC.

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Anticoagulant and Protective Signaling Properties of APC

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