Identification of a Specific Exosite on Activated Protein C for Interaction with Protease-activated Receptor 1*

Likui Yang¹, Jong-Sup Bae¹, Chandrashekhar Manithody, and Alireza R. Rezaie²
From the Edward A. Doisy Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine, St. Louis, Missouri 63104

Activated protein C (APC) is a vitamin K-dependent plasma serine protease which down-regulates the clotting cascade by inactivating procoagulant factors Va and VIIIa by limited proteolysis. In addition to its anticoagulant effect, APC also exhibits cytoprotective and anti-inflammatory activity through the endothelial protein C receptor-dependent cleavage of protease-activated receptor 1 (PAR-1) on endothelial cells. Recent mutagenesis data have indicated that the basic residues of two surface loops including those on 39 and the Ca²⁺-binding 70–80 loops contribute interactive sites for both factors Va and VIIIa, thereby mediating the interaction of APC specifically with these procoagulant cofactors. The basic residues of both loops have been discovered to be dispensable for the interaction of APC with PAR-1. It is not known if a similar exosite-dependent interaction contributes to the specificity of APC recognition of PAR-1 on endothelial cells. In this study, we have identified two acidic residues on helix-162 (Glu-167 and Glu-170) on the protease domain of APC which are required for the protease interaction with PAR-1, but not for its interaction with the procoagulant cofactors. Thus, the substitution of either Glu-167 or Glu-170 with Ala eliminated the cytoprotective signaling properties of APC without affecting its anticoagulant activity. These mutants provide useful tools for initiating in vivo studies to understand the extent to which the anticoagulant versus anti-inflammatory activity of APC contributes to its beneficial effect in treating severe sepsis.

Protein C is a vitamin K-dependent plasma serine protease zymogen that upon activation by the thrombin-thrombomodulin (TM)³ complex at the endothelial cell surface down-regulates the clotting cascade by a feedback loop mechanism (1–3). The catalytic activity of the thrombin-TM complex toward protein C is markedly improved if the substrate is also bound to endothelial protein C receptor (EPCR) at the surface of vascular cells (4). The γ-carboxyglutamic acid (Gla) domain of protein C is responsible for its high affinity (Kₐ ≈ 30 nM) interaction with EPCR, and thus both protein C and APC exhibit identical affinity for the cell surface receptor (5). Recent in vitro and in vivo studies have indicated that in addition to its anticoagulant activity, activated protein C (APC) also exhibits potent cytoprotective and anti-inflammatory activity when the protease remains associated with EPCR, thereby acquiring the capacity to cleave protease-activated receptor 1 (PAR-1) at the surface of endothelial cells (6–9). Because the catalytic efficiency of APC toward PAR-1 is much lower than that of thrombin (10), it is not well understood how APC can signal endothelial cells through the cleavage of the same receptor when thrombin is present. The recent findings that all three receptors TM, EPCR, and PAR-1 are co-localized in the lipid rafts of endothelial cells (11) suggest that the thrombin-TM activation of the EPCR-bound protein C in the same microenvironment of PAR-1 may channel APC into the cytoprotective signaling pathway by enabling the protease to effectively cleave this receptor (12).

The molecular mechanism through which APC interacts specifically with its target substrates factors Va (fVa) and VIIIa (fVIIIa) in the anticoagulant pathway has been extensively studied (13–16). The high specificity of APC interaction with both procoagulant cofactors is determined by several basic residues that are clustered on two exposed surface loops on the protease domain referred to as 39 loop and 70–80 loop (17) in the chymotrypsin numbering system (18). The mutagenesis of the basic residues of either one of these loops in APC (Fig. 1) has resulted in mutant proteases with dramatically impaired anticoagulant activity but normal cytoprotective signaling property (19), suggesting that the determinants of specificity of substrate recognition by APC in the two alternative pathways are different. With the exception of the Gla domain-dependent interaction of APC with EPCR (20), which is essential for the specificity of PAR-1 cleavage by the protease on endothelial cells (6, 11, 19), no other data are available as to how APC recognizes its physiological receptor substrate in the protective signaling pathway.

The x-ray crystal structure of a Gla-domainless derivative of APC has been resolved (21). The basic residues of the two surface loops that determine the specificity of APC interaction with the procoagulant cofactors are spatially located at the right side of the active site in the structure of the protease in the standard orientation shown in Fig. 1. There are two acidic residues (Glu-167 and Glu-170) at the left side of the active site toward the back of the APC protease domain on a helical struc-
An Exosite for PAR-1 on APC

ture (helix 162–170, hereafter called 162-helix) that are not conserved in the homologous region of other vitamin K-dependent coagulation proteases. By contrast, the 162-helix residues in both factors Ixa (22) and Xa (fXa) (23) carry positive charges (Arg-165 and Arg-169 in factor Ixa and Arg-165 and Lys-169 in fXa) and mutagenesis studies have indicated that the basic regions in both procoagulant proteases provide functionally critical interactive sites for fVIIIa and fVa in intrinsic Xase (24) and prothrombinase (25), respectively. These previous results with the procoagulant proteases provided the rational for investigating the role of the two non-conserved acidic residues of 162-helix in the catalytic activity of APC both in the anticoagulant and antiinflammatory pathways. Hence, in this study, we substituted both Glu-167 and Glu-170 of APC with Ala in separate constructs and evaluated their role in determining the specificity of APC interaction with its physiological substrates in both the anticoagulant and cytoprotective signaling pathways. Our results suggest that both residues play a critical role in the specific APC recognition of PAR-1 in the latter pathway.

EXPERIMENTAL PROCEDURES

Construction, Expression, and Purification of Recombinant Proteins—Construction and expression of wild-type protein C in human embryonic kidney (HEK-293) cells have been described previously (26). The protein C mutants containing Ala substitutions in place of Glu-167 (E167A) and Glu-170 (E170A) (chymotrypsinogen numbering (18)) were constructed by PCR mutagenesis methods in the same expression system as described (26). Both wild-type and mutant zymogens were purified to homogeneity by immunoaffinity chromatography using the Ca$^{2+}$-dependent monoclonal antibody, HPCR4, coupled to Affi-Gel 10 (Bio-Rad) as described (27). The zymogens (0.5–1 mg) were converted to activated protein C by thrombin (25 μg) in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4 (TBS) containing 5 mM EDTA for 2 h at 37 °C as described (27). The APC derivatives were separated from thrombin by an FPLC Mono Q column developed with a 40-mL linear gradient from 0.1 to 1.0 M NaCl. The concentrations of APC derivatives were determined from the absorbance at 280 nm assuming a molecular mass of ~56 kDa and extinction coefficient (E$_{280}^{	ext{cm-1}}$) of 14.5, by an amidolytic activity assay and by stoichiometric titration of enzymes with known concentrations of recombinant protein C inhibitor (PCI) as described (15). PCI was expressed in HEK-293 cells as described (15). The catalytically inactive Ser-195 → Ala (S195A) mutant of APC was expressed and purified as described (28). The homogeneity and quality of all recombinant proteins were verified by SDS-PAGE under non-reducing conditions.

Human plasma proteins, antithrombin (AT), prothrombin, fXa, and fVa were purchased from Hematologic Technologies Inc. (Essex Junction, VT). Human protein S was purchased from Enzyme Research Laboratories (South Bend, IN) phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (29). PT (Thrombomax with Ca$^{2+}$) and aPTT (Alexin) reagents were purchased from Trinity Biotech (St. Louis, MO). Normal pooled plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS). The chromogenic substrate, Spectrozyme PCa (SpPCa) was purchased from American Diagnostica (Greenwich, CT) and S2238 was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH). Tumor necrosis factor-α (TNF-α) was purchased from R&D System (Minneapolis, MN). Antibodies blocking (H-111) and non-blocking (S-19) the activation of PAR-1 were purchased from Santa Cruz Biologics (Santa Cruz, CA), and function-blocking EPCR antibody (clone RCR-252) was purchased from Cell Sciences (Canton, MA).

Cleavage of Chromogenic Substrates—The steady-state kinetics of hydrolysis of SpPCa (15–2000 μM) by APC derivatives (4 nM) was measured in TBS containing 1.0 mg/ml bovine serum albumin and 0.1% polyethylene glycol 8000 (TBS/Ca$^{2+}$) at 405 nm at room temperature in a V$_{max}$ kinetic microplate reader. The $K_m$ and $k_{cat}$ values for the substrate hydrolysis were calculated from the Michaelis-Menten equation as described (15).

Anticoagulant Activity—The anticoagulant activity of APC derivatives was evaluated both in purified and plasma based assay systems as described (15). Briefly, fVa (2.5 nM) was incubated with increasing concentration of wild-type or mutant APC (0–5 nM) on 25 μM PC/PS vesicles in TBS/Ca$^{2+}$. Following 6 min incubation at room temperature, the remaining fVa activity was determined in a prothrombinase assay from the fVa-catalyzed prothrombin activation by fXa as described (15). The prothrombinase assay was carried out for 30 s with excess prothrombin (1 μM) and a saturating fXa (10 nM). The remaining activity of fVa was determined from the decrease of the fVa-dependent rate of thrombin generation as monitored by an amidolytic activity assay using 200 μM S2238. The inactivation of fVa by increasing concentrations of APC was also monitored in the presence of protein S (220 nM) as described above with the exception that the time of incubation was decreased to 1 min. The fVa inactivation assay was also carried out with a fixed concentration of APC (1 nM) in the presence of varying concentrations of protein S (0–220 nM) using the same methods.

The same fVa inactivation assay was employed to evaluate the apparent affinity of the APC derivatives for interaction with PC/PS vesicles. In this case, the rate of fVa (2.5 nM) inactivation by each APC derivative (2.5 nM) was carried out in the presence of increasing concentrations of PC/PS vesicles (0–5 μM) for 6 min using the same methods described above.

The anticoagulant activities in plasma were evaluated in an aPTT assay using a STart 4 fibrinometer (Diagnostica/Stago, Asnieres, France). Briefly, 0.050 ml TBS lacking or containing 0–20 nM final concentrations of APC derivatives were incubated with a mixture of 0.05 ml of normal pooled plasma plus 0.05 ml of aPTT reagent (Alexin) for 5 min before the initiation of clotting by the addition of 0.05 ml of 35 mM CaCl$_2$ at 37 °C as described (15).

Apoptosis Assay—EA.hy926 cells (0.5 × 10$^6$) (kindly provided by Dr. C. Edgell from University of North Carolina at Chapel Hill, NC) were seeded onto coverslips coated with gelatin as described (11, 28). After 24 h at 37 °C, the medium was replaced, and cells were incubated with increasing concentrations of APC (0–50 nM) for another 24 h. Then, the cells were incubated with 10 ng/ml TNF-α for 4 h. Cells were
fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, 0.1% sodium citrate, and incubated for 1 h in the dark with a TUNEL reaction mixture (Roche Applied Sciences) for in situ detection of cell death. After washing with PBS, cells were incubated 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. For the function-blocking antibody treatments of the monolayers, medium was removed, and antibodies were added for 30 min in serum-free medium at a concentration of 25 μg/ml as described (28).

Permeability Assay—EA.hy926 cell permeability in response to TNF-α (10 ng/ml for 18 h) following treatment with increasing concentrations of APC (0–50 nM for 3 h) was quantitated by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional EA.hy926 cell monolayers using a modified 2-compartment chamber model as described (28, 30). For the function-blocking antibody treatments of the monolayers, medium was removed, and antibodies were added for 30 min in serum-free medium followed by analysis of the permeability as described (28).

Adhesion Assay—Neutrophil adherence to confluent monolayers of TNF-α (10 ng/ml for 4 h) treated EA.hy926 cells in 96-well plates, which were treated with APC derivatives (10 nM for 24 h) was evaluated by fluorescent labeling of neutrophils with 5 μM Vybrant DiD (Molecular Probes) for 20 min at 37 °C in phenol red-free RPMI containing 5% fetal bovine serum as previously described (28, 31, 32). The fluorescence of labeled cells was measured using a fluorescence microplate reader (Molecular Device). The percentage of adherent neutrophils was calculated by the formula: % adherence = (adherent signal/total signal) × 100.

Interaction with EPCR—The affinity of APC derivatives for interaction with EPCR was evaluated by an ELISA-based binding assay using HPC4-tagged recombinant soluble EPCR (sEPCR) (kindly provided by Dr. Esmon from Oklahoma Medical Research Foundation) as described (28). The affinity of APC derivatives for interaction with EPCR was also evaluated at the endothelial cell surface employing the permeability assay described above. In this case, the competitive effect of APC derivatives (E167A, E170A, and S195A) and wild-type protein structure in human APC (Fig. 1). Further support for the lack of a mutagenesis-mediated global effect on the conformation of APC derivatives was provided by the observation that both mutants reacted with the target serpin, PCI, with similar second-order association rate constants (Table 1).

### TABLE 1

| APC         | kcat | kcat/Km | kcat/Km/Km | k2 (PCI) |
|-------------|------|---------|------------|----------|
| APC-WT      | 234 ± 25 | 33.1 ± 1.7 | 0.14 ± 0.02 | (1.3 ± 0.1) × 10^2 |
| APC-E167A   | 249 ± 35 | 34.0 ± 2.7 | 0.14 ± 0.03 | (1.2 ± 0.2) × 10^3 |
| APC-E170A   | 258 ± 45 | 35.0 ± 4.1 | 0.14 ± 0.04 | (1.1 ± 0.2) × 10^3 |

### FIGURE 1

The space-filling model of the crystal structure of the catalytic domain of active site-inhibited APC. The side chains of basic residues of 39-loop (Lys-37–39) and 70–80-loop (Arg-74 and Arg-75) are shown in blue. The side chains of acidic residues of 162-helix (Glu-167 and Glu-170) are shown in red. The catalytic residue Ser-195 is shown in green in the center of the molecule. The coordinates (Protein Data Bank accession code 1AUT) were used to prepare the figure (21).

### Statistical Analysis

Results are expressed as mean ± S.E., and Student’s 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

**Amidolytic Activity**

The steady state kinetics of hydrolysis of the tripeptidyl chromogenic substrate, SpPca, by wild-type and mutant APCs is presented in Table 1. All APC derivatives cleaved the chromogenic substrate with a similar catalytic efficiency, suggesting that the mutagenesis of either Glu-167 or Glu-170 did not adversely affect the folding and/or the reactivity of either the catalytic residues or the extended P1-P3 binding sites of the active site groove. These results were expected, because both Glu-167 and Glu-170 are located on a solvent-exposed helical structure in human APC (Fig. 1). Further support for the lack of a mutagenesis-mediated global effect on the conformation of APC derivatives was provided by the observation that both mutants reacted with the target serpin, PCI, with similar second-order association rate constants (Table 1).
Anticoagulant Activity

The anticoagulant activity of APC derivatives was evaluated both in the absence and presence of protein S. As presented in Fig. 2, the APC concentration dependence of fVa inactivation both in the absence (panel A) and presence of protein S (panel B) indicated that both E167A and E170A mutants have a normal anticoagulant activity. Further fVa inactivation studies in the presence of increasing concentrations of protein S suggested that both mutants interact with protein S with a similar apparent affinity as evidenced by essentially identical specific activity for both wild-type and mutants at all concentrations of protein S (220 nM). The plots are representative experiments out of 2–3 independent and reproducible measurements.

Cytoprotective Activity of APC Derivatives

Cytoprotective and antiinflammatory activity of APC derivatives were evaluated in three established cellular assays: Anti-apoptotic Activity—Thrombin and proinflammatory cytokines can induce apoptosis in endothelial cells (33). As presented in Fig. 5, the treatment of EA.hy926 cells with TNF-α-induced apoptosis and wild-type APC inhibited cell death by a concentration-dependent manner, which reached saturation at 5 nM protease (Fig. 5C). In agreement with previous results (6, 7, 28), the anti-apoptotic activity of APC required interaction with EPCR and its subsequent cleavage of PAR-1 because func-
ties of wild-type (E) E167A (s) and E170A (d) were antibodies, as a function of different concentration of APC derivatives at 37 °C as described under “Experimental Procedures.” The plots are representative experiments out of three independent and reproducible measurements.

Figure 5. Anti-apoptotic activity of APC derivatives in TNF-α-induced apoptosis assay. A, EA.hy926 cells were treated with APC derivatives (10 nM) for 24 h followed by induction of apoptosis with TNF-α (10 ng/ml) for 4 h. The cells were fixed with paraformaldehyde and incubated with the TUNEL reaction mixture followed by Hoechst 33342 to stain the apoptotic cells (green) and the total number of nuclei (blue), respectively. B, the same as above except that the number of apoptotic cells is expressed as the percentage of TUNEL-positive cells of the total number of nuclei. B-αPAR-1 and NB-αPAR-1 represent blocking and non-blocking anti-PAR-1 antibodies, respectively, and B-αEPCR represents blocking anti-EPCR antibody. C, the same as B except that the percentage of TUNEL-positive cells are presented as a function of varying concentrations of wild-type APC (E), E167A (s), and E170A (d). The number of TUNEL-positive cells in the absence of TNF-α was 10–15%. *p < 0.001 in both panels B and C.

Inhibition of Neutrophil Adhesion—APC is known to inhibit the interaction of the cytokine-activated neutrophils with endothelial cells by down-regulation of the expression of adhesion molecules including vascular cell adhesion molecule 1, intracellular adhesion molecule 1 and E-selectin (28, 33, 34). In agreement with previous results, APC effectively inhibited the binding of neutrophils to TNF-α-stimulated endothelial cells (Fig. 6C). As with other two cellular assays described above, neither E167A nor E170A influenced the enhanced interaction of neutrophils with the cytokine-stimulated EA.hy926 cells (Fig. 6C). No effect on neutrophil binding to endothelial cells was observed for either mutant if their concentrations were raised from 10 nM (Fig. 6C) to 50 nM (data not shown). Both function-blocking anti-EPCR and anti-PAR-1 antibodies effectively abrogated the protective effect of APC in this assay (Fig. 6C).

Interaction with EPCR

APC exerts its cytoprotective activity through EPCR-dependent cleavage of PAR-1 on endothelial cells (6, 11, 19). To study the effect of the mutagenesis on the affinity of mutant proteases for binding to EPCR, their ability to interact with soluble EPCR (sEPCR) was evaluated by an ELISA-based binding assay as described (28). As shown in Fig. 7A, all APC derivatives bound to EPCR with similar affinities, exhibiting K_d(app) values of ~35.5 ± 2.0 nM for wild-type APC, 47.8 ± 4.2 nM for E167A and 44.3 ± 2.2 nM for E170A, suggesting that the loss of the protective activity of mutant proteases in the assays presented above may not be due to a defect in their interaction with EPCR on endothelial cells. To provide further support for this hypothesis, the competitive effect of increasing concentrations of the APC mutants on the inhibition of the barrier protective effect of wild-type APC was evaluated in the permeability assay. As shown in Fig. 7B, both E167A and E170A exhibited essentially identical inhibitory effect as did either the S195A mutant of the protease or the wild-type protein C zymogen. These results clearly suggest that the affinity of the APC mutants for interaction with EPCR at the endothelial cell surface is normal. The results further support the previous observation that the protective activity of APC in endothelial cells requires an intact active site pocket (7, 28). It should be noted that both APC and protein C exhibit similar high affinities for interaction with EPCR (5).

Cleavage of PAR-1

To determine whether the APC mutants can recognize and cleave PAR-1, their catalytic activity toward the receptor was evaluated by a sensitive colorimetric assay measuring the APC

tion-blocking anti-EPCR and anti-PAR-1 antibodies both effect-

Inhibition of the Permeability Barrier—Previous studies measuring the flux of albumin in a dual chamber system have indicated that both thrombin (30) and TNF-α (28) disrupt the permeability barrier of EA.hy926 cells and that APC exerts a potent protective effect. In agreement with previous results, treatment of EA.hy926 cells with TNF-α resulted in enhanced permeability, which was effectively reversed by wild-type APC in a concentration-dependent manner that reached saturation at 10 nM APC (Fig. 6A). As with the anti-apoptotic activity assay, neither E167A nor E170A exhibited a protective effect in this assay at up to 50 nM APC. Function-blocking antibodies to either EPCR or PAR-1 eliminated the barrier protective effect of APC, confirming that the EPCR-dependent cleavage of PAR-1 by APC mediates the cellular effect of the protease (Fig. 6B).

An Exosite for PAR-1 on APC

The clotting activity of APC derivatives was determined as a function of different concentration of APC derivatives at 37 °C as described under "Experimental Procedures." The plots are representative experiments out of three independent and reproducible measurements.

Figure 4. Plasma clotting activity of APC derivatives. The clotting activities of wild-type (E) E167A (s) and E170A (d) were antibodies, as a function of different concentration of APC derivatives at 37 °C as described under "Experimental Procedures.” The plots are representative experiments out of three independent and reproducible measurements.

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Cleavage of PAR-1

To determine whether the APC mutants can recognize and cleave PAR-1, their catalytic activity toward the receptor was evaluated by a sensitive colorimetric assay measuring the APC
An Exosite for PAR-1 on APC

![Graph showing the barrier protective and antiinflammatory activity of APC derivatives in TNF-α-induced permeability and neutrophil adhesion assays.](image)

**FIGURE 6.** The barrier protective and antiinflammatory activity of APC derivatives in TNF-α-induced permeability and neutrophil adhesion assays. A, the APC (○ wild-type, ● E167A, □ E170A) concentration dependence of inhibition of TNF-α-induced permeability and neutrophil adhesion assays. B, the permeability was quantitated in the absence and presence of function-blocking antibodies to either PAR-1 or EPCR. C, TNF-α-mediated adherence of neutrophils to EA.hy926 monolayers was analyzed after treating monolayers with APC derivatives as described under “Experimental Procedures.” B-αEPCR and N8-αPAR-1 represent blocking and non-blocking anti-PAR-1 antibodies, respectively, and B-αEPCR represents blocking anti-EPCR antibody in both panels B and C. *, p < 0.005 in all three panels.

cleavage of Arg-41 of PAR-1 in a reporter construct in which alkaline phosphatase cDNA was coupled to the exodomain of PAR-1 and the membrane spanning domain of tissue factor, thereby anchoring the cleavage reporter construct to the cell surface upon its transfection to endothelial cells (28). In this assay, wild-type APC cleaved the Arg-41 scissile bond of the PAR-1 cleavage reporter with ~750-fold lower catalytic efficiency than thrombin (Fig. 8A). In contrast to wild-type APC, the PAR-1 cleavage efficiency of both E167A and E170A mutants were dramatically impaired so that no significant alkaline phosphatase activity was detected at concentrations of up to 200 nM APC for either one of the mutants (Fig. 8B). The catalytically inactive S195A mutant of APC did not exhibit any activity in this assay (not shown). These results suggest that the loss of the cytotoxicity of both APC mutants is due to their inability to interact and cleave PAR-1 in endothelial cells. Thus, both Glu-167 and Glu-170 of APC are specific recognition sites for interaction with PAR-1.

**DISCUSSION**

In this study, we have demonstrated that Glu-167 and Glu-170, two residues unique for 162-helix of APC which are not conserved on the homologous helix of other vitamin K-dependent coagulation proteases, constitute a specific PAR-1-binding exosite on APC that facilitates the recognition and cleavage of the receptor by the protease at the endothelial cell surface. The specific interaction of APC with an unknown site of the PAR-1 extracellular domain through this exosite appears to be essential for the EPCR-dependent cytoprotective signaling function of APC, as evidenced by the inability of either E167A or E170A to exhibit significant protective activity in response to TNF-α-induced inflammatory events in endothelial cells. Both APC mutants showed normal anticoagulant activity in both fVa-inactivating and plasma-based clotting assays, suggesting that neither one of the acidic residues of 162-helix are involved in interaction of APC with the procoagulant cofactors. Previous results have demonstrated that several basic residues located on either 39-loop (Lys-37, Lys-38, and Lys-39) or the Ca²⁺-binding 70–80 loop (Arg-74 and Arg-75) of APC specifically interact with the procoagulant cofactors (13–16). The mutagenesis of either one of these basic residues has resulted in mutant proteases with markedly impaired anticoagulant activity (13–16), however, the APC mutants have retained their normal protective cell signaling activity (19). Thus, the specificity of the APC protease domain interaction with its natural substrates in the alternative anticoagulant and antiinflammatory pathways are determined by two distinct exosites with different polarity, with acidic residues of 162-helix, on the left side of the active site toward the back of the molecule, determining the specificity of the protease interaction with PAR-1 in the latter pathway, and the basic residues of 39 and 70–80 loops on the right side of the active site, determining the specificity of the protease interaction with the procoagulant cofactors in the former pathway (Fig. 1).

The residues of 162-helix are also known to play critical roles in determining the specificity of other vitamin K-dependent coagulation proteases by providing interactive sites for binding to their respective cofactors on activation complexes. In factor VIIa, this loop interacts with tissue factor, thereby facilitating the rapid activation of factor X by the extrinsic Xase complex (35, 36). In factor IXa, two residues Arg-165 and Arg-169 provide critical binding sites for interaction with IVIla, thereby leading to the rapid activation of factor X by the intrinsic Xase complex (24). In the case of fXa, two residues Arg-165 and Lys-169 are interactive-sites for fVa in the prothrombinase complex, which is responsible for the activation of prothrombin to thrombin in the final step of the coagulation cascade (25). Both basic residues are also conserved on the same helix of thrombin and form part of the heparin binding exosite of the molecule (18). The results of this study now suggest that the reversed polarity of these residues on 162-helix endows specificity for APC, enabling the protease to interact with PAR-1 in
the antiinflammatory pathway. Thus, the interaction of all coagulation proteases with their natural cofactors and/or substrates through residues of this helix plays a critical role in modulation of the physiological functions of these proteases in different pathways.

APC has been approved as a drug for treating severely septic patients (37). It is believed that the beneficial effect of APC in severe sepsis, at least partially, is derived from its direct protective signaling activity in the endothelium (6–9). However, APC therapy increases the incidence of bleeding in ~2% of the treated patients (37). An understanding of the mechanism by which APC specifically interacts with its procoagulant cofactor substrates has allowed investigators to design mutants that have lost their anticoagulant activity but retained their normal cytoprotective properties (19, 28). These mutants may provide excellent alternative approaches with no risk of bleeding for treating septic patients. The evaluation of the potential therapeutic utility of some of these mutants is ongoing in animal models of sepsis. The findings that the substitution of acidic residues of 162-helix specifically eliminates the cytoprotective signaling activity of APC without influencing the anticoagulant activity of the protease, for the first time, open up new strategies for developing APC variants with potential therapeutic values in treating thrombotic patients (i.e., cancer patients) for whom the cytoprotective signaling activity of APC may not be desirable. Furthermore, in addition to a direct antiinflammatory activity, APC also exerts indirect antiinflammatory activity through down-regulation of thrombin generation. The extent to which the direct and indirect antiinflammatory effects of APC contribute to its beneficial effect in septic patients is not known. The APC mutants described in this study may provide a plausible approach to investigate this important question.

Acknowledgment—We thank Audrey Rezaie for proofreading the manuscript.

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An Exosite for PAR-1 on APC

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