Enhancement of Human Protein C Function by Site-directed Mutagenesis of the γ-Carboxyglutamic Acid Domain*

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This study reports properties of site-directed mutants of human protein C that display enhanced calcium and/or membrane binding properties. Mutants containing the S11G modification all showed increased affinity for membranes at saturating calcium concentration. Ser-11 is unique to human protein C, whereas all other vitamin K-dependent proteins contain glycine. This site is located in a compact region of the protein, close to a suggested membrane contact site. Additional changes of H10Q or S12N resulted in proteins with lower calcium requirement for membrane contact but without further increase in membrane affinity at saturating calcium. Mutations Q52E and N33D did not, by themselves, alter membrane affinity to a significant degree. These mutations were included in other mutant proteins and may contribute somewhat to higher function in these mutants. This family of mutants helped discriminate events that are necessary for protein-membrane binding. These include calcium binding to the free protein and subsequent protein-membrane contact. Depending on conditions of the assay used, the mutants displayed increased activity of the corresponding activated protein C (APC) derivatives. The degree of enhanced activity (up to 10-fold) was dependent on the concentration of phospholipid and quality of phospholipid (± phosphatidyethanolamine) used in the assay. This was expected, because APC is active in its membrane-associated form, which can be regulated by changes in either the protein or phospholipid. As expected, the largest impact of the mutants occurred at low phospholipid concentration and in the absence of phosphatidyethanolamine. The anticoagulant activity of all proteins was stimulated by protein S, with the greatest impact on the enhanced mutants. Whereas plasma containing Factor VIIa stimulates activity of APC, protein C variants with enhanced function present new reagents for study of coagulation and may offer improved materials for biomedical applications.

Human protein C, an important inhibitor of blood coagulation (1), requires vitamin K for the formation of nine γ-carboxyglutamic acids (Gla) in its amino-terminal 45 residues (the Gla domain). This domain contains the membrane contact site, which is critical to activated protein C function in proteolysis of factors Va and VIIIa. Activity is expressed upon protein associations on a membrane surface. Despite a high degree of sequence homology, protein C and six other vitamin K-dependent proteins display a large range of membrane affinities (2). Consequently, it should be possible to enhance membrane affinity of low affinity proteins, which include human protein C, by site-directed mutation to approach the structures of high affinity proteins.

At least three events are needed to describe membrane interaction by vitamin K-dependent proteins (as shown in Equation 1).

\[
P + 7Ca \xrightarrow{Ca_{7P}} P^{7Ca}_{\text{Ca}}\]

\[
+ 3 \xrightarrow{PL^{Ca}_{\text{P}}Ca_{\text{Ca}}} (\text{Eq. 1})
\]

\[
PL + nCa \xrightarrow{nCa_{\text{PL}}} \frac{P^{nPL}Ca_{\text{Ca}}}{Ca_{\text{P}}}
\]

Equation 1 is free protein, PL is protein binding sites on the membrane, and \( P^{nPL} \) is the complex. In addition to variations in membrane affinity at saturating calcium (step 3 of Equation 1), members of the vitamin K-dependent protein family have different affinities for calcium (step 1 of Equation 1 (3)), making it possible to enhance protein from this standpoint as well. In fact, it may be possible to find mutants that influence protein-calcium binding (step 1 of Equation 1) or membrane association at saturating calcium levels (step 3 of Equation 1), without influencing the other step. Such mutants would help identify the membrane contact region of the Gla domain and distinguish it from calcium binding to the free protein.

**Experimental Procedures**

Recombinant Proteins—Site-directed mutagenesis was performed using a PCR method as described previously (4). The following oligonucleotides were synthesized: A, 5′-AAA TTA ATA CGA CTC ACT ATA GGG CCC TCT AGA-3′ (corresponding to sense nucleotides 880–895 in the vector pRc/CMV including the HindIII cloning site); B, 5′-GCA TTT AGG TGA CAC TAT AGA ATA GGG CCC TCT AGA-3′ (antisense to nucleotides 984–1019 in the vector pRc/CMV including the XbaI cloning site). The mutagenesis primers were as follows: C, GAG ACC CCC TCT AGA-3′; D, 5′-AAA TTA ATA CGA CTC ACT ATA GGG CCC TCT AGA-3′; E, 5′-GCA TTT AGG TGA CAC TAT AGA ATA GGG CCC TCT AGA-3′.

The abbreviations used are: Gla, γ-carboxyglutamic acid; PCR, polymerase chain reaction; hC-G1E32D33 or APC-G1E32D33, S110Q32EN33D mutant of human protein C or activated protein C; hC-E32 or APC-E32, Q2E2E mutant of human protein C or activated protein C; hC-E32D33 or APC-E32D33, Q32EN33D mutant of human protein C or activated protein C; hC-E32 or APC-E32, Q2E2E mutant of human protein C or activated protein C; hC-E32D33 or APC-E32D33, Q32EN33D mutant of human protein C or activated protein C; hC-E10G11E32D33 or APC-E10G11E32D33, H10E10G11Q32EN33D mutant of human protein C or activated protein C; hC-F10P10H10F mutant of human protein C; TP, tissue plasminogen activator; APC, activated protein C; F, Factor; P, phosphatidylserine; PE, phosphatidylethanolamine; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; nCa, calcium ion.
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5'-CAG TGT GTC ATC AAC ATC TCC GAA AAT TTC CTT GCC C-3' (antisense to amino acids 27–38, with the E32D33 mutation underlined); D, 5'-GCC AAG GAA ATT TTC GAA GTG GAT GAC ACA CTG-3' (complementary to primer C); E, 5'-CAG TGT GTC ATC AAC ATC TCC GAA AAT TTC CTT GCC C-3' (antisense to amino acids 27–38, with the G32D33 mutation underlined); F, 5'-GCC AAG GAA ATT TTC GGA AAT GTG GAT GAC ACA CTG CTC CAG GCT CCC CTC ACG GAG CTC GAG CTC CAG GAA-3' (antisense to amino acids 4–17, with the S11G mutation underlined); H, 5'-GCC CTA CCG CTC GAG CTC GCT CCC CTC ACG GAG CTC GAG CAA-3' (antisense to amino acids 4–17, with the H10Q/S11G mutation underlined); I, 5'-GCC CTA CCG CTC GAG CTC GCT CCC CTC ACG GAG CTC GAG CAA-3' (antisense to amino acids 4–17, with the H10Q/S11G mutation underlined); J, 5'-GCC CTA CCG CTC GAG CTC GCT CCC CTC ACG GAG CTC GAG CAA-3' (antisense to amino acids 4–17, with the S11G/S12N mutation underlined).

Full-length human protein C cDNA was cloned in the pRC/CMV vector using HindIII and XhoI sites. PCR amplification and mutagenesis of the target DNA was performed by the following steps: primers A and C were used to amplify the 5' part of protein C (up to the codon for the amino acid at position 38), whereas primers B and D were used to amplify the 3' part (from the amino acid 27 codon to the end of the protein C cDNA). These two fragments were used as templates to amplify the full-length E32D33 mutant protein C cDNA with primers A and F instead of primers C and D. PCR products were cleaved with HindIII and SalI, and the HindIII-SalI fragment was isolated and ligated together with a SalI-XhoI fragment from protein C cDNA into HindIII and XhoI cleaved pRC/CMV vector.

To prepare the four mutants, G11E32D33, E10G11E32D33, Q10G- 11E2D33 and G11N12E2D33, first human protein C cDNA was used as template for the PCR amplification to prepare mutant G11 (using primers A and I), and G11N12 (using primers A and J). The PCR products were digested by HindIII (at the 5' of human protein C cDNA) and BstBI (located in the primer nucleotide sequence at codons for amino acids Ser-14 and Gly-15). These fragments were ligated to a protein C fragment (BstBI-XhoI, around 1200 base pairs) containing the E32D33 mutation to obtain the full-length hC-G11E32D33 and hC-E10G11E32D33 mutants. All mutations were confirmed by DNA sequencing before transfection. Cell culture in human 293 cells, expression, purification, and quantitation of protein C molecules were performed as described (4).

Gla Analysis—Quantitation of Gla was conducted by amino acid analysis (5). 4-Dimethylaminoazobenzene-4-sulfonic acid and 4-dimethylaminoazobenzene-4-sulfonyl-Apa eluted at 13.5 and 16.5 min, respectively. Gla content was determined from a ratio to Asp in the analysis, assuming 42 Asp or Asn per protein. Gla content (average and range) was determined by high performance liquid chromatography as described (4).

Preparation of APC—Approximately 0.1 mg of protein C (1 mg/liter) was mixed with human thrombin (3 μg/liter) and incubated at 37 °C for 3 h in standard buffer (0.05 M Tris, pH 7.5, 0.1 mM NaCl). The reaction product was diluted with an equal volume of water and applied to a 1-ml column of SP-Sephadex C-50. The column was washed with standard buffer, and the flow-through was pooled as activated protein C (APC). APC was purified by hydrolysis of 0.1 mM S2366 (l-prolyl-t-lysyl-t-arginyl-p-nitroanilino hydrochloride) and gave a constant activity per mg of protein for all preparations (±10%). Concentrations were normalized to S2366 activity.

Coagulation Assays—Coagulation was measured with APTT reagent (Sigma) (0.05 ml of APTT reagent that was diluted 1:10 or 1:100 with standard buffer containing 0.1 mM ethylacrylate) or diluted TP (Sigma) (0.1 ml of TP-high sensitivity that had been diluted 1:100 with standard buffer containing 0.01 mM CaCl2). The total volume of assays was 0.2 ml. For APTT assays, 0.05 ml of plasma, 0.05 ml of APC and/or protein S solution, and APTT reagent were incubated at 37 °C for 5 min. Coagulation was initiated with 0.05 ml of standard buffer containing 0.025 mM CaCl2. For TP assays, TP was diluted to 1:1000 in standard buffer, and then 0.05 ml of plasma, 0.05 ml of APC and/or protein S solution, and TP were incubated at 37 °C for 5 min, and clotting was started with diluted TP. Time to form a clot was determined by a manual method. Clotting times were reported as the average and range of two determinations. Plasma samples used were human plasma (Sigma), protein S-deficient plasma (American Diagnostica, Greenwich, CT), and heterozygous and homozygous factor V Leiden plasma (kindly provided by Dr. Nigel Key, Department of Medicine, University of Minnesota Medical School).

A one-stage coagulation assay was carried out using bovine factor Xa. Buffer (total volume of 112.5 μl, containing 6.7 mM CaCl2) was mixed with factor Xa, sufficient to give a clotting time of 30 ± 2 s under the conditions of the assay, APC, and phospholipid. The mixture was incubated for 1 min at 37 °C, and coagulation was initiated by addition of 37.5 μl of normal human plasma (at 37 °C).

Vesicle Preparation—Small unilamellar vesicles (SUVs) were prepared by methods described (5, 16). Highly pure phospholipids with natural fatty acid distributions included phosphatidylethanolamine (PS, from bovine brain, purchased from Avanti Polar Lipids) and phosphatidylcholine (PC, from egg, purchased from the Sigma) and phosphatidylethanolamine (PE, from egg, purchased from Avanti). These were reconstituted in degassed solvent, dried by a stream of argon, and suspended in standard buffer (20 mg in 3 ml). SUVs were formed by sonication and gel filtration. Large unilamellar vesicles (LUVs) were prepared by extrusion as described by Hope et al. (17). Phospholipids included PS/PC/PE (natural abundance fatty acids, described above). This PE preparation contained 60% plasmalogens. Alternatively, LUVs were made from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, and with and without 1,2-dilinoleoyl-sn-glycero-3-phosphoethanolamine. All synthetic lipids were from Avanti. Vesicles containing dilinoleoyl-PE were stored under argon and were used within 24 h of preparation. Others were used within 5 days.

Sonication of LUVs was carried out essentially as described for preparation of SUVs. Vesicle size was measured by quasi-elastic light scattering with an LSA2 laser light scattering apparatus coupled with a Langley Ford 1096 autocorrelator. Average diameters for SUVs were 35–40 nm, and average diameters for LUVs were 110–120 nm. Phospholipid concentrations were determined by organic phosphate assay assuming a phosphorous:phospholipid weight ratio of 25 (18).

Measurement of Protein-Membrane Interaction—Light scattering at 90° was measured to quantify protein-membrane binding (19). Briefly, the intensity of phospholipid vesicles alone (I1) and after addition of protein (I2) were measured. Background from buffer and unbound protein was subtracted. The ratio of the molecular weight of the protein-vesicle complex (M1) to that of the vesicles alone (M2) was estimated from the following relationship: I1/I2 = [M1/M2]P*PL/(1 + [M1/M2]P*PL), where [M1/M2]P*PL is the refractive index of the respective species. Oxysterol-containing vesicles were labeled with 3H in the hydroxyl group of 3-keto-sterols; these vesicles were analyzed by thin-layer chromatography to assure complete labeling. The calculations were performed in triplicate, and the binding of bound P*PL and free protein P can be estimated. These values, together with the maximum protein binding capacity ([P*PL]max) of the vesicles (was assumed to be 1.0 g/g for all proteins) was used to obtain the dissociation constant for protein-membrane interaction from the following relationship: KD = ([P*PL]max − P*PL)/([P*PL]), where all values are in molar concentrations of protein or protein binding sites. Equilibrium binding constants are reported as KD values and are primarily valuable for comparisons. Values at 5 mM calcium were calculated at a total protein:phospholipid ratio of 2:0.1:0 (w/w). Values at 2 mM calcium were determined at a total protein:phospholipid ratio of 0.7:1.0 (w/w).

Protein binding to LUVs is reported as the light scattering intensity ratio (I2/I1). Vesicle size is a significant fraction of the wavelength of light so that nondideal light scattering may occur. However, when all solution parameters, vesicle sizes, and distributions were the same for titrations of two similar proteins, the light scattering intensity ratio provided an indication of relative binding by the two proteins. For several reasons, direct comparison of two proteins by this method is more accurate than absolute binding constants obtained (19). The variations in membrane binding constants reported are (reviewed in Ref. 7) may partially reflect these factors. Direct comparisons between proteins in a single study is an important aspect of this study.

Other Proteins—The bovine proteins factor X (20) and prothrombin (21) were purified by published procedures. Activation of factor X was performed as described (22). Human protein S was generously provided.
 jubboine factor X (G11E32D33 (f), hC-Q10G11E32D33 (G), hC-E32D33 (GA)) thromboplastin assay (Fig. 3). Activity of APC-E32 was similar to that of wild type APC in a diluted situation used in the assay. The impact of calcium concentration resulted in lowered calcium requirement. Thus, the relative efficacy of these proteins should differ with the calcium concentration used in the assay. The impact of calcium concentration is illustrated in Table I. For example, the difference between hC-G11E32D33 and hC-Q10G11E32D33 is greater at 2 mM calcium than at 5 mM calcium.

Activity of APC in Standard Coagulation Assays—The activity of APC-E32 was similar to that of wild type APC in a diluted thromboplastin assay (Fig. 3A, also see Ref. 13). This assay showed that APC-G11E32D33 was about 10-fold superior to wild type protein and that the APC-Q10G11E32D33 and APC-G11N12E32D33 mutants had approximately 12-fold higher activity.

The relative activity was dependent on the commercial batch of thromboplastin used. Fig. 3B shows approximately 5.5-fold difference between the APC-G11E32D33 and APC-E32 mutants. In keeping with the slight differences of membrane binding by other mutants, APC-E32D33 was approximately equal to APC-G11E32D33 (Fig. 3B, inset). Nevertheless, APC-G11E32D33 was more effective than APC-E32 in these plasmas.

Activity in a diluted APTT was also determined (data not shown). This assay relies on the intrinsic coagulation pathway and therefore utilizes factor VIIIa. APC inhibits both Va and VIIIa, and it was of interest to determine whether a difference was observed for the mutants in these situations. As for the thromboplastin assay, larger differences between APC-G11E32D33 and APC-E32 occurred in diluted APTT reagent and/or at longer coagulation times. There was minimal impact of the mutations when full strength APTT reagent was used.

Greatest impact of enhanced membrane affinity at low coagulation stimulus was common to other proteins with improved membrane affinity, including bovine protein C (4) and...
Factor VII (5).

**Sensitivity to Protein S**—Both coagulation assays were sensitive to protein S but showed somewhat different requirements (Fig. 4). The diluted APTT test showed a midpoint of about 7 nM protein S, whereas the diluted TP assay showed a midpoint at 30–40 nM protein S. In both assays and in the protein S titrations (Fig. 4), APC-G11E32D33 showed continuous increase of inhibition at higher protein S concentrations. A difference in APC concentration requirement was also apparent; inhibition in the diluted APTT assay occurred at 0–2 nM APC but required 0–15 nM APC in the diluted TP reaction (Fig. 3).

**Impact of Phospholipid Type**—Phospholipid concentration and composition have an impact on coagulation. Although attention is normally paid to the need for anionic lipids, an interesting effect was observed for neutral phospholipids, PC versus PE. The presence of PE in the membrane has a large synergistic impact on function of PS for membrane binding by vitamin K-dependent proteins (24). Similar effects were seen for diverse proteins such as protein kinase C, annexins (25), pentraxins (26), myristoylated alanine-rich C kinase substrate (27), and blood clotting factor VIII (28).

Enhanced protein binding to membranes containing PE was evident from the comparison shown in Fig. 5A. Phospholipids containing natural fatty acid distributions gave similar results for both wild type and the hC-E32 mutant (not shown). The impact of PE was apparent for hC-G11E32D33 (Fig. 5A).

PE also enhances coagulation reactions, such as those involving factor VIIa (29). The importance of PE was shown in this study by the fact that membranes of PS/PC (5/95) were not competent to support coagulation in the assay shown in Fig. 5B, whereas PS/PE/PC (5/50/45) was an excellent membrane source (data not shown).

Phosphatidylethanolamine also impacts on anticoagulation by APC. It supports higher activity of APC against factor Va in a purified system (30). More interesting is a selective promotion of anticoagulation. That is, APC prolongs coagulation to a higher extent in membranes containing PE, under conditions where procoagulation (clotting time) in the absence of APC is constant (30). This study supported that finding, although with a smaller impact of PE. Synthetic phospholipids (Fig. 5, B and C), as well as purified phospholipids (data not shown), showed higher anticoagulant activity for APC in membranes containing PE than in those containing only PC (Fig. 5, B and C). The difference varied somewhat with phospholipid preparation and storage time. The largest difference (about 4-fold) was observed in preparations that had been stored at 4 °C for 2 months.

An important implication of these comparisons was that the enhanced activity of the site-directed mutants differed with the phospholipid concentration and composition used in an assay. The effect of PE on wild type APC was highest at low phospholipid concentration (Fig. 5C), whereas the APC-G11E32D33 mutant showed small differences at the different phospholipid concentrations (compare clotting time versus APC-G11E32D33 concentrations in Fig. 5, B and C). APC-G11E32D33 was also relatively insensitive to the presence of PE (Fig. 5, B and C). As a result, the enhanced function of mutants of higher activity will differ with the conditions selected for the assay. High membranes concentrations or the presence of PE reduced the apparent effect of the mutations. This outcome was qualitatively similar to the impact of a chimera protein C that contained residues 1–44 of human prothrombin (30), except that PE virtually eliminated the difference between wild type and chimera APC proteins.

**DISCUSSION**

Earlier predictions (2) and the outcome for other proteins (4, 5) suggest that enhanced vitamin K-dependent plasma proteins can be created by site-directed mutation of the Gla domain to enhance membrane binding affinity. This study showed success in generation of human protein C with enhanced membrane binding. Several results indicated that human protein C has unique features among this family of proteins. For example, bovine protein C responded to incorpo-
and human factor X (32). Thus, identification of residues that can be altered to improve membrane interaction by human protein C is an important step to understanding the unique properties of this protein. It also provides methods to potentiate this important regulator of blood coagulation for research and biomedical applications.

The S11G mutation generated protein C with higher membrane affinity at saturating calcium levels. This mutation therefore appeared to alter the actual membrane contact site on the protein. This could occur by a direct impact of glycine-11 on membrane interaction or by indirect improvement of other positions in the protein. Other residues could include placement of E32D33 to a more optimum location that allowed them to enhance membrane contact. Although the actual site of membrane contact is still unknown, it can be noted that this site is adjacent to position 10, one of the three residues that appeared to regulate most of the diversity in membrane affinity among the naturally occurring proteins (sites 10, 32, and 33 (2)). Position 11 is in a compact region of the protein that is closely placed to Ca-2 in the x-ray crystal structure (33). The Ser-11 hydroxyl may function as a ligand to the calcium ion, thereby modifying structure in this region. Alternatively, the serine side chain may not allow optimum protein folding due to steric factors. An importance of position 11 is also suggested by low activity of a Gly (position 12 of factor IX) to Arg mutation of factor IX (34).

Other site-directed mutations produced proteins that displayed a different type of enhanced function. For example, an additional change of H10Q or S12N resulted in proteins with a lower calcium requirement for membrane association, but with nearly identical membrane binding at saturating calcium levels. This suggested that Gln-10 and Asn-12 modify calcium binding to the protein without participation in actual membrane contact. These mutants should show higher activity at low calcium concentrations, but identical activity at saturating calcium. In the assays used, these mutants were similar to APC-G11E32D33, indicating that calcium levels were saturating in these assays. The calcium concentration under in vivo conditions is not known, and whole animal experiments may be needed to determine whether improvements in calcium binding to the protein can aid function.

In all cases and with all membranes used, improved enzyme activity of APC correlated with increased membrane affinity. In fact, the behavior appeared to fit to a general equilibrium expression for assembly of the active APC complex. Although the correct binding expression for APC function as an anticoagulant may be quite complex, it should contain protein and phospholipid components such as those given in boldface in Equation 2.

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\[
K_0 = \frac{[P_{\text{free}}][P_{\text{free}}][\text{Va}^+][\text{Xa}^+]\left[\text{Va}^+\text{PL}\text{Xa}^+\text{Xa}\right]}{[\text{Va}^+\text{S}][\text{Xa}^+]\left[\text{Va}^+\text{PL}\text{Va}^+\text{S}^+\text{Xa}\right]} \quad (\text{Eq} \ 2)
\]

where \( [P_{\text{free}}] \) and \([PL_{\text{free}}]\) are the concentrations of free APC and unfilled APC binding sites on the membrane, Va,S, and Xa may also contribute to the complex. Assuming that bound protein \([P^*\text{PL}]\) is active and free protein \( [P_{\text{free}}] \) is inactive, any change that lowers the ratio of \([P_{\text{free}}][P^*\text{PL}]\) in Equation 2 will enhance the activity of APC. This is a common proposal for protein S function; by enhancing membrane bound APC, activity of APC is increased. Other ways in which the ratio of \([P_{\text{free}}]/[P^*\text{PL}]\) can be decreased include the addition of more PL to the reaction. Indeed, higher phospholipid levels in the assay gave higher APC activity (compare APC-wild type activity in Fig. 5A versus Fig. 5B). An alternative way to lower \([P_{\text{free}}]/[P^*\text{PL}]\) is with membrane content that results in higher affinity. This can include an increase in PS or use of PE as a portion of the neutral phospholipid. Again, improvements in the phos-
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pholipid, by inclusion of PE enhanced the activity of APC (Fig. 5). A third way of reducing $[P_{\text{free}}]/[P^{\text{PL}}]$ is by improvements of the membrane binding site on the protein. Higher activity by APC-G11E32D33 illustrated this property. Finally, these three approaches to higher activity of APC impact on the same phenomenon, the ratio of $[P_{\text{free}}]/[P^{\text{PL}}]$, and a maximum limit for activity is reached when $[P_{\text{free}}]$ becomes zero. Lowered $[P_{\text{free}}]/[P^{\text{PL}}]$ by one mechanism will decrease the potential impact of other mechanisms. As expected from this interrelationship, the highest impact of the enhanced mutants was observed at low concentrations of membranes that did not contain PE (Fig. 5C).

The largest impact of PE was observed with low affinity proteins (Fig. 5, B and C) and was almost undetected for high affinity proteins. The impact of phospholipid concentration was greatest for low affinity proteins and was minimal for the APC mutants with high membrane affinity (compare Fig. 5B with Fig. 5C). Due to these properties, it is possible that neither the impact of PE nor the increased activity of the mutants will be detected in assays that use high phospholipid concentrations. Standard clotting assays often use an optimum phospholipid concentration, and that may mask the benefit of both PE and the mutant proteins.

The pattern of protein S impact may suggest a function more complex than simple enhancement of membrane-bound APC. Rather than reducing the importance of protein S, APC mutants with increased membrane affinity showed a higher response to protein S that did wild type protein. In addition, the two coagulation assays showed different requirements for protein S. The thromboplastin assay is sensitive to components of the prothrombinase complex and the major site of APC function is factor Va. This assay required a higher protein S concentration for maximum activity than did the APTT assay. The latter relies on the intrinsic pathway, including factor VIIIa/Xa. Thus, the result of protein S titrations with APTT versus thromboplastin assays (Fig. 4) suggested that protein S interacted somewhat differently for APC action on factor VIIIa versus factor Va.

Acknowledgments—We acknowledge the excellent technical assistance of Astra Anderson in mutant production and protein purification and the contributions of Dr. Stephen Harvey to activity analysis.