

Multifunctional Specificity of the Protein C/Activated Protein C Gla Domain*

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Activated protein C (APC) has potent anticoagulant and anti-inflammatory properties that are mediated in part by its interactions with its cofactor protein S and the endothelial cell protein C receptor (EPCR). The protein C/APC Gla domain is implicated in both interactions. We sought to identify how the protein C Gla domain enables specific protein-protein interactions in addition to its conserved role in phospholipid binding. The human prothrombin Gla domain, which cannot bind EPCR or support protein S cofactor activity, has 22/45 residues that are not shared with the human protein C Gla domain. We hypothesized that the unique protein C/APC Gla domain residues were responsible for mediating the specific interactions. To assess this, we generated 13 recombinant protein C/APC variants incorporating the prothrombin residue substitutions. Despite anticoagulant activity similar to wild-type APC in the absence of protein S, APC variants *APC(PT33–39)* (N33S/V34S/D35T/D36A/L38D/A39V) and *APC(PT36/38/39)* (D36A/L38D/A39V) were not stimulated by protein S, whereas *APC(PT35/36)* (D35T/D36A) exhibited reduced protein S sensitivity. Moreover, *PC(PT8/10)* (L8V/H10K) displayed negligible EPCR affinity, despite normal binding to anionic phospholipid vesicles and factor Va proteolysis in the presence and absence of protein S. A single residue variant, *PC(PT8)*, also failed to bind EPCR. Factor VIIa, which also possesses Leu-8, bound soluble EPCR with similar affinity to wild-type protein C, collectively confirming Leu-8 as the critical residue for EPCR recognition. These results reveal the specific Gla domain residues responsible for mediating protein C/APC molecular recognition with both its cofactor and receptor and further illustrate the multifunctional potential of Gla domains.

Protein C is the key component of the anticoagulant protein C pathway (1, 2). Homozygous protein C deficiency is associated with severe neonatal thrombosis (3), whereas individuals with heterozygous deficiency are thrombophilic (4). Protein C

is activated by the thrombin-thrombomodulin complex on endothelial cells (5). Protein C activation is enhanced ~5-fold by protein C binding to the endothelial cell protein C receptor (EPCR)² (5). Activated protein C (APC) binds to anionic phospholipid surfaces, where it inactivates procoagulant cofactors factor Va (FVa) and factor VIIIa (FVIIIa), thereby attenuating thrombin generation (6, 7). Proteolysis of FVa and FVIIIa by APC is enhanced by the presence of the APC cofactor, protein S (8). APC also has potent anti-inflammatory (9–11) and anti-apoptotic properties (12, 13), which may be mediated in part by EPCR-bound APC activation of protease-activated receptor 1 (14).

Protein C is a vitamin K-dependent protein with a multidomain structure comprising a serine protease domain, two epidermal growth factor (EGF) domains, and a Gla domain (15). Situated at the protein C N terminus, the Gla domain consists of 45 amino acid residues. Of these, 9 glutamic acid (Glu) residues are post-translationally modified to γ -carboxyglutamic acid (Gla) residues. Gla residues facilitate Ca^{2+} ion coordination, which causes the domain to undergo a profound structural transition (16). In this conformation, the protein C Ca^{2+} -bound Gla domain binds to anionic phospholipids exposed on the surface of activated endothelial cells and platelets, albeit with lower affinity than other vitamin K-dependent proteins (17–19).

The mechanism by which protein S exerts its cofactor activity on APC is not fully understood. Protein S enhances APC affinity for phospholipids and is required for optimal alignment of the APC active site for substrate cleavage (8, 20). Protein S has also been reported to remove factor Xa (FXa) protection of FVa in the prothrombinase complex (21). Several protein S domains have been shown to contribute to APC cofactor activity, including the thrombin-sensitive region, EGF1, EGF2, and the Gla domain (21–28). A direct role for the APC Gla domain in mediating protein S cofactor activity was suggested by a study in which a recombinant protein C chimera was generated and the protein C Gla domain (residues 1–45) was replaced by that of prothrombin (18). This chimera was resistant to protein S cofactor stimulation. Furthermore, the anticoagulant activity

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² The abbreviations used are: EPCR, endothelial cell protein C receptor; sEPCR, soluble EPCR; APC, activated protein C; RU, resonance unit; ETP, estimated thrombin potential; PE, phosphatidylethanolamine; FVIIa, factor VIIa; Gla, γ -carboxyglutamic acid.

of an APC chimera in which the APC Gla domain residues 1–22 were substituted with the corresponding prothrombin residues was enhanced by protein S, suggesting a role for APC residues 22–45. However, the precise residues responsible, present in the protein C/APC Gla domain but not in prothrombin, were not determined.

The protein C Gla domain is also required for EPCR binding (29). A crystal structure of part of the protein C Gla domain (residues 1–33) bound to soluble EPCR (sEPCR) indicated that hydrophobic residues Phe-4 and Leu-8 contained within the ω -loop of the protein C Gla domain and Gla residues 7, 25 and 29, bind EPCR (30). Despite the significant amino acid sequence conservation between the EPCR binding residues of the protein C/APC Gla domain and that of other vitamin K-dependent coagulation proteins, surprisingly only protein C/APC has been reported to bind EPCR. Binding competition experiments using FX, FXa, and protein S failed to prevent high affinity protein C-EPCR binding (31). Consequently, the residues that enable the EPCR to recognize and bind specifically to the protein C/APC Gla domain are currently unknown.

In this study, we evaluated 13 protein C/APC recombinant variants where protein C residues were substituted for those of prothrombin at non-identical positions (22/45 Gla domain residues). We assessed the binding of each variant for both phospholipids and sEPCR and evaluated the APC variants in anticoagulant activity assays in the presence and absence of protein S in both purified protein and plasma systems. We describe the critical residues in the APC Gla domain that mediate protein S cofactor activity. Moreover, we have identified the protein C Gla domain residue responsible for the specific “recognition” of EPCR and how its presence in factor VII (FVII) also enables it to bind EPCR.

EXPERIMENTAL PROCEDURES

Generation of Protein C Variants—The pRc/CMV vector (Invitrogen) containing full-length protein C cDNA was used to generate recombinant wild-type protein C and as a template for PCR site-directed mutagenesis (QuikChange mutagenesis kit; Stratagene) using mutagenic oligonucleotide primers (available on request) as before (32). Expression vectors for each protein C variant were used to transfect human embryonic kidney 293 cells (European Collection of Cell Cultures, Wiltshire, UK), and G418-selected colonies expressing protein C at high levels were picked for further expansion. Serum-free conditioned medium containing each recombinant protein C was buffer exchanged against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. Protein C was purified by ion exchange chromatography using a Q-Sepharose Fast Flow column (Amersham Biosciences) with a gradient elution using 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 30 mM CaCl₂ as previously described (19, 33, 34). Protein C concentrations were determined either by absorbance at 280 nm or by enzyme-linked immunosorbent assay (32). The purity of all recombinant proteins was confirmed by SDS-PAGE and Coomassie staining, and Western blotting was performed using a horseradish peroxidase-conjugated polyclonal anti-protein C antibody (Dako, Ely, UK). To generate APC, wild-type protein C and protein C variants were activated with Protac as previously

described (32). Kinetic values were used to active site titrate each recombinant APC variant against wild-type APC.

Phospholipid Binding of Protein C Variants—A BIAcore X system (BIAcore, Uppsala, Sweden) was used to evaluate protein C binding to anionic phospholipids. 10 μ g/ml of a Ca²⁺-dependent anti-protein C monoclonal antibody (Hematologic Technologies Inc.) was immobilized onto both flow cells of a CM5 sensor chip (BIAcore), corresponding to \sim 12,000 RU on each flow cell. 50 μ l of wild-type or variant protein C was then passed over the surface of one flow cell at a flow rate of 30 μ l/min until a response of \sim 2,000 RU was achieved for each protein C species tested. The flow cell with no protein C bound was used to detect nonspecific binding. Phospholipids phosphatidylcholine/phosphatidylserine (PC/PS, 80%:20%) were prepared as described previously (32) in HBS-N buffer (100 mM HEPES, pH 7.4, 150 mM NaCl) (BIAcore) containing 3 mM CaCl₂ and 0.6 mM MgCl₂ and then passed over the surface of the sensor chip at 30 μ l/min for 1 min. Phospholipids were dissociated from the surface using 5 μ l of 40 mM octyl glucoside (Sigma) at the same flow rate. The maximum binding response at each phospholipid concentration was assessed using BIA-evaluation software (3.0) (BIAcore). The sensor chip surface was regenerated using HBS-EP buffer (100 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) (BIAcore), for 5 min at 30 μ l/min.

EPCR Binding Affinity of Protein C Variants—Protein C binding to sEPCR was quantitatively assessed as previously described (32). Briefly, 300 ng of RCR-2 (a kind gift from K. Fukudome, Saga Medical School, Japan; characterized in Ref. 32) was injected for 6 min across both flow cells of a CM5 sensor chip, generating a response of 8,000–9,000 RU. sEPCR was expressed as described previously (32). sEPCR in HBS-P buffer (100 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% v/v surfactant p20) containing 3 mM CaCl₂ and 0.6 mM MgCl₂ was injected and equilibrated. Protein C concentrations (12.5–200 nM) were sequentially injected over both flow cells at a flow rate of 30 μ l/min for 80 s. The flow cell immobilized with RCR-2 without sEPCR bound was used to detect nonspecific binding. Any influence of mass transport effects was discounted from results of binding and dissociation at different flow rates. HBS-EP buffer (BIAcore) was used to dissociate the protein C-sEPCR complex. The RCR-2 surface was regenerated with 10 mM glycine-HCl, pH 2.5, after each set of experiments.

Determination of APC-mediated Factor Va Proteolysis—To determine FVa degradation by APC, 0.32 nM APC was incubated at 37 °C with phospholipid vesicles (PC/PS, 80%:20%) and 4 nM FVa (Hematologic Technologies Inc.) in 40 mM Tris-HCl, 140 mM NaCl, 3 mM CaCl₂, and 0.3% w/v bovine serum albumin (0.08 nM APC, 19 μ M phospholipids, and 1 nM FVa, final concentration). At specified time points over 20 min, 2- μ l aliquots were removed and added to a prothrombinase mixture consisting of 75 μ M phospholipids, 3 nM factor Xa, and 1.5 μ M prothrombin (25 μ M phospholipids (PC/PS, 80%:20%), 1 nM factor Xa, and 0.5 μ M prothrombin, final concentrations) (Hematologic Technologies Inc.) for 3 min. Each reaction was then stopped using 5 μ l of ice-cold 0.5 M EDTA. 100 μ l of the reaction mixture was removed and incubated with 50 μ l of chromogenic substrate S-2238 (Chromogenix, Milan, Italy) to assess

	1	45
Protein C (1–45)	ANSFLEELRHSSLERECIEEICDFEAAKEIFQNVDDTLAFWSKHV	
PC (PT3)	AN TFLEELRHSSLERECIEEICDFEAAKEIFQNVDDTLAFWSKHV	
PC (PT8)	ANSFLEEL V RHSSLERECIEEICDFEAAKEIFQNVDDTLAFWSKHV	
PC (PT10)	ANSFLEEL RK SSLERECIEEICDFEAAKEIFQNVDDTLAFWSKHV	
PC (PT8/10)	ANSFLEEL V RKSSLERECIEEICDFEAAKEIFQNVDDTLAFWSKHV	
PC (PT11/12)	ANSFLEELRH GN LERECIEEICDFEAAKEIFQNVDDTLAFWSKHV	
PC (PT18)	ANSFLEELRHSSLEREC V EEICDFEAAKEIFQNVDDTLAFWSKHV	
PC (PT21/23/24)	ANSFLEELRHSSLERECIEE TC SYEEAKEIFQNVDDTLAFWSKHV	
PC (PT28/30)	ANSFLEELRHSSLERECIEEICDFEAA FEA FQNVDDTLAFWSKHV	
PC (PT31–35)	ANSFLEELRHSSLERECIEEICDFEAAKEIF LESST DLAFWSKHV	
PC (PT33–39)	ANSFLEELRHSSLERECIEEICDFEAAKEIF QSSTATD VFWSKHV	
PC (PT35/36)	ANSFLEELRHSSLERECIEEICDFEAAKEIFQNV TAT LAFWSKHV	
PC (PT36/38/39)	ANSFLEELRHSSLERECIEEICDFEAAKEIFQNV AD DVFWSKHV	
PC (PT42/44/45)	ANSFLEELRHSSLERECIEEICDFEAAKEIFQNVDDTLAF WAKYT	
Prothrombin (1–45)	AN TFLEEL V RK GN LEREC V EE TC SYEE FAE LES STATD VFW AKYT	

FIGURE 1. Amino acid sequence alignment of recombinant protein C Gla domain variants. The human protein C Gla domain (residues 1–45) shares 33% sequence identity with the human prothrombin Gla domain. 13 protein C Gla domain variants were generated in which residues were substituted with the corresponding human prothrombin Gla domain residues.

thrombin generation. The rate of S-2238 cleavage was measured at 405 nm using an iEMS plate reader MF (Labsystem, Basingstoke, UK).

Protein S-enhanced Proteolysis of FVa by APC—Human protein S (2.5–25 nM) was incubated with 0.8 nM APC, 8 nM FVa, and 75 μM phospholipid vesicles (PC/PS/phosphatidylethanolamine (PE), 60%:20%:20%) in 40 mM Tris-HCl, pH 7.4, 140 mM NaCl, 3 mM CaCl₂, 0.3% (w/v) bovine serum albumin (0.2 nM APC, 2 nM FVa, and 19 μM phospholipid vesicles, final concentrations) for 2 min at 37 °C. A 2-μl aliquot was added to 0.3 nM FXa, 1.5 μM prothrombin, and 75 μM phospholipid vesicles (0.1 nM FXa, 0.5 μM prothrombin, and 25 μM phospholipid vesicles, final concentrations) at 37 °C for 3 min. 5 μl of ice-cold 250 mM EDTA stopped the reaction. 50 μl of S-2238 was diluted 1:2 with 50 mM Tris-HCl, 150 mM NaCl, pH 8.3, and added to 50 μl of the reaction mixture. The rate of S-2238 cleavage was then measured as before.

Protein S-dependent APC Anticoagulant Activity in Plasma—Thrombin generation was assessed using a Fluoroskan Ascent Plate Reader (Thermo Lab System, Helsinki, Finland) in combination with Thrombinoscope software (SYNAPSE BV) as previously described (35, 36). 80 μl/well of protein C-deficient plasma (Affinity Biologicals Inc., Ontario, Canada) was incubated with 240 μg/ml of anti-protein S polyclonal antibody or the equivalent volume of water for 15 min at room temperature. Each plasma pool was then incubated with 16 pM recombinant tissue factor (Dade Innovin, Dade Behring, Marburg, Germany) and 17.5 μM phospholipid vesicles (PC/PS/PE, 60%:20%:20%). 42 μg/ml corn trypsin inhibitor (Hematologic Technologies Inc.) was added to prevent contact activation. APC (3.5–7 nM) was added to microtiter plate wells and the plasma mix with or without anti-protein S polyclonal antibody subsequently added, making a final volume of 100 μl/well. Thrombin generation was initiated by automatic dispensation of 2.5 mM Z-Gly-Gly-Arg-AMC-HCl, 2.5% Me₂SO, 18 mM HEPES, pH 7.4, 54 mg/ml bovine serum albumin, and 100 mM CaCl₂ into each well. Thrombin generation was quantitated using a thrombin calibration standard (Synapse, Maastricht, Netherlands). Measurements were taken at 20-s intervals for 40 min at wavelengths 390 nm (excitation) and 460 nm (emission).

RESULTS

Generation and Characterization of Protein C Variants—To identify protein C Gla domain residues that enable protein S cofactor activity and EPCR recognition, a series of protein C variants with prothrombin residue substitutions at non-identical positions were generated (Fig. 1). These variants were divided into two groups, those situated at the N-terminal end of the protein C Gla domain (residues 1–25) and those located at the C-terminal end incorporating the hydrophobic stack (residues 26–45). The purity of each recombinant pro-

tein C variant was confirmed by Coomassie staining and exhibited identical characteristic double bands under non-reducing conditions upon Western blotting with an anti-protein C polyclonal antibody (data not shown).

Phospholipid Binding of Protein C Variants—To characterize the Gla domain integrity of the recombinant protein C variants, the ability of each variant to bind to phospholipids was assessed. Protein C was bound to the surface of a CM5 sensor chip by a Ca²⁺-dependent anti-protein C monoclonal antibody directed against the heavy chain, and not the Gla domain-containing light chain, of protein C. A protein C variant (E16D), possessing a residue substitution that causes aberrant Ca²⁺ binding and Gla domain misfolding (32), was found to bind to the immobilized antibody with equal affinity to human protein C, indicating that the antibody epitope was not on the Gla domain (data not shown). Increasing concentrations of anionic phospholipid vesicles were passed over the protein C surface and specific binding was detected (Fig. 2A). Due to the slow dissociation rate of the phospholipid-protein C interaction, thorough analysis of the binding kinetics was not possible. The maximum binding response at each phospholipid concentration was therefore measured and used to qualitatively assess phospholipid binding. The binding response of both human protein C and recombinant wild-type protein C at each phospholipid concentration tested was essentially identical (Fig. 2B). When the E16D protein C variant (which does not bind phospholipids) (32) was immobilized, a minimal response to phospholipids was detected (Fig. 2B, top panel), demonstrating the specificity of this approach. The phospholipid binding of the remaining variants was found to be very similar to that of wild-type protein C (Fig. 2B, middle and lower panels), with the exception of PC(PT21/23/24) and PC(PT31–35). These variants exhibited markedly reduced binding to phospholipids (Fig. 2B, middle and lower panel, respectively), suggesting that these variants most likely possess a misfolded Gla domain due to suboptimal γ-carboxylation. The defective phospholipid binding of both variants also resulted in severely impaired anticoagulant activity in protein C-deficient plasma (data not shown). Consequently, these variants were not investigated further.

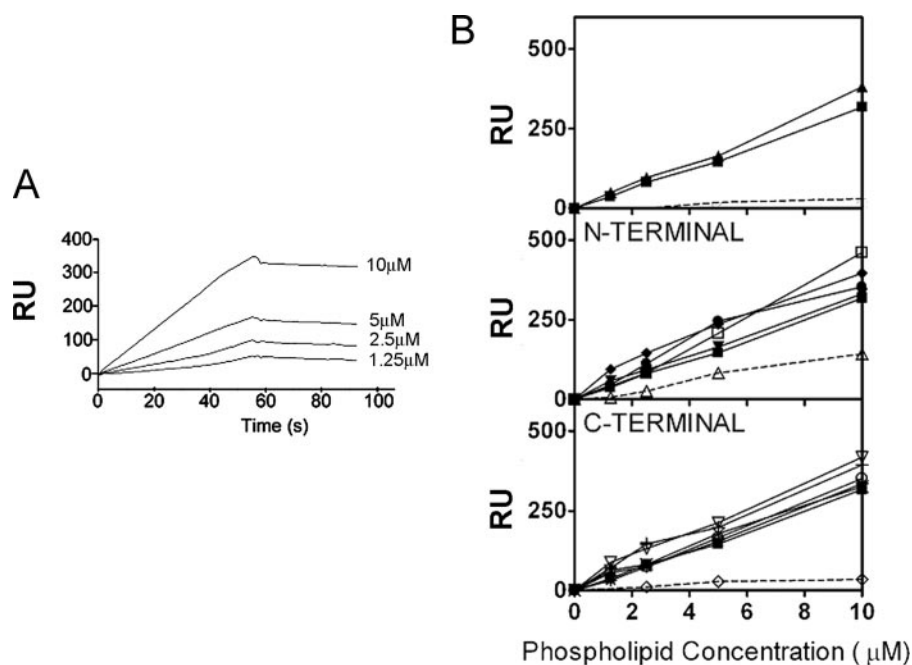


FIGURE 2. **Phospholipid binding to protein C variants.** Recombinant protein C affinity for negatively charged phospholipids (PC/PS, 80%:20%) was assessed by surface plasmon resonance (see "Experimental Procedures"). A Ca^{2+} -dependent anti-protein C monoclonal antibody was immobilized onto both flow cells of a CM5 sensor chip. Each protein C species was then bound to the second flow cell (2,000 RU), the other flow cell acting as a "no protein C" control for nonspecific binding. *A*, increasing concentrations of phospholipid vesicles (1.25–10 μM) were passed over a wild-type protein C surface, and the maximum binding response was measured. *B*, each of the protein C variants was tested in an identical manner to that of wild-type protein C. The maximum binding response (in RU) at each phospholipid concentration tested was determined using BIAEvaluation software and plotted against phospholipid concentration. *Top*, human plasma purified protein C, (\blacktriangle); wild-type protein C (\blacksquare); protein C Gla domain variant E16D (dashed line). *Middle*, wild-type protein C, (\blacksquare); PC(PT3), (\blacktriangledown); PC(PT8/10), (\blacklozenge); PC(PT11/12), (\bullet); PC(PT18), (\square); PC(PT21/23/24), (dashed line, \triangle). *Lower*, wild-type protein C, (\blacksquare); PC(PT28/30), (\blacktriangledown); PC(PT31–35), (dashed line, \diamond); PC(PT33–39), (*); PC(PT35/36), (+); PC(PT36/38/39), (\circ); and PC(PT42/44/45), (\times).

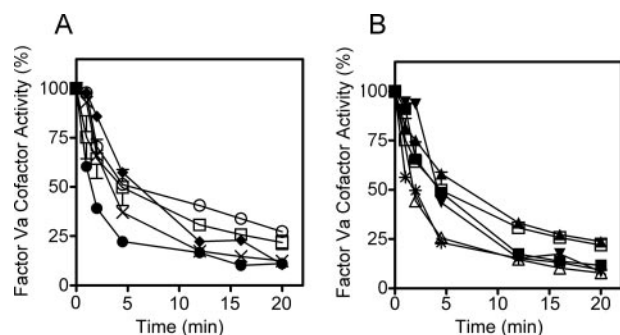


FIGURE 3. **FVa proteolysis by recombinant APC variants.** FVa (1 nM) was incubated with APC (0.08 nM) in the presence of phospholipids (PC/PS, 80%:20%; 19 μM). At specified time points, an aliquot was removed and the FVa cofactor activity determined using a prothrombinase assay (phospholipids (25 μM), FXa (1 nM), and prothrombin (0.5 μM)) as described under "Experimental Procedures." Wild-type APC (\square) in both panels. *A*, APC variants with substitutions in the N-terminal half of the Gla domain: APC(PT3), (\blacklozenge); APC(PT8/10), (\circ); APC(PT11/12), (\bullet); APC(PT18), (\times). *B*, APC variants with substitutions in the C-terminal half of the Gla domain: APC(PT28/30), (\blacktriangle); APC(PT33–39), (\triangle); APC(PT35/36), (*); APC(PT36/38/39), (\blacktriangledown); APC(PT42/44/45), (\blacksquare). Values correspond to the mean of at least two determinations.

Protein S-independent FVa Proteolysis by APC Variants—Each protein C variant was activated by Protac, and the amidolytic activity of each APC variant against a short chromogenic substrate (S-2366) was tested and found to be identical to that of wild-type APC (data not shown). To determine whether the introduced prothrombin residue

substitutions influenced FVa proteolysis in the absence of protein S, each APC variant was investigated for its ability to proteolyze FVa in a phospholipid-dependent time course reaction (Fig. 3). Under these conditions, wild-type APC reduced FVa cofactor activity to $21 \pm 5\%$ after 20 min. APC variants were found to inactivate FVa at effectively the same rate as wild-type APC. Some variants exhibited mildly increased FVa proteolysis, including APC(PT11/12) (S11G/S12N), APC(PT33–39) (N33S/V34S/D35T/D36A/L38D/A39V), and APC(PT35/36) (D35T/D36A) (Fig. 3, *A* and *B*), possibly as a result of the slightly increased affinity for phospholipids of these variants (Fig. 2).

Protein S Does Not Enhance FVa Proteolysis by APC Variants APC(PT33–39), APC(PT35/36), and APC(PT36/38/39)—To examine the influence of substituted protein C Gla domain residues in mediating protein S cofactor activity, the ability of each APC variant to proteolyze FVa in the presence of varying protein S concentrations was determined. Wild-type APC reduced FVa cofactor activity to $27 \pm 8\%$ of its original activity in the presence of 25 nM protein S (Fig. 4). All N-terminal Gla domain APC variants tested were stimulated by protein S normally (Fig. 4A), as were half of the C-terminal variants (Fig. 4B). However, FVa proteolysis by APC variants APC(PT33–39) and APC(PT36/38/39) (D36A/L38D/A39V) was not enhanced by protein S. The remaining FVa activity in the presence of 25 nM protein S with variants APC(PT33–39) and APC(PT36/38/39) was 92 ± 7 and $91 \pm 2\%$, respectively (Fig. 4B). The APC(PT35/36) variant also exhibited a significantly impaired response to protein S compared with wild-type APC, only reducing FVa cofactor activity to $73 \pm 9\%$ of its original value in the presence of 25 nM protein S (Fig. 4B).

APC Variants Containing Residue Substitutions between Positions 33–39 Are Not Enhanced by Protein S in Plasma—To assess whether the observed reduced sensitivity to protein S exhibited by APC variants APC(PT33–39), APC(PT36/38/39), and APC(PT35/36) using purified plasma proteins was replicated in the plasma milieu, an assay measuring tissue factor-induced thrombin generation in protein C-deficient plasma was used. Thrombin generation in protein C-deficient plasma was initiated by the presence of tissue factor and anionic phospholipid vesicles, generating an estimated thrombin potential (ETP) of 1720 ± 82 nM. When wild-type APC was incubated in the protein C-deficient plasma, the ETP diminished in a concentration-dependent manner (Fig.

Molecular Recognition of Protein S and EPCR by Protein C/APC

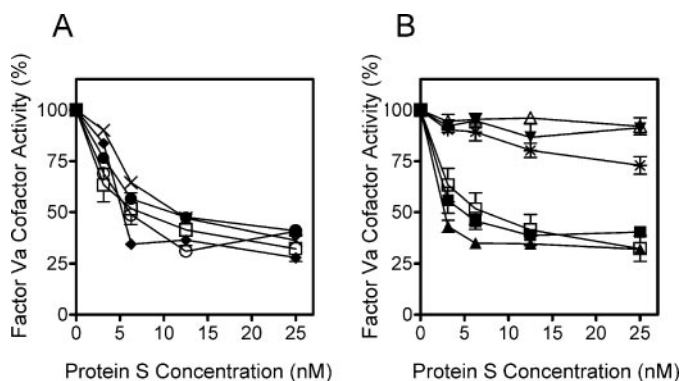


FIGURE 4. Protein C Gla domain residues 33–39 are important for protein S cofactor activity. Human protein S (3.25–25 nM) was incubated with APC (0.2 nM), 2 nM FVa, and phospholipids (PC/PE/PS, 60%:20%:20%; 19 μ M) for 2 min and then added to a prothrombinase mixture (FXa, 0.1 nM; prothrombin, 0.5 μ M; phospholipids (as above, 25 μ M)) to assess FVa cofactor activity as described under “Experimental Procedures.” Wild-type APC (\square) in both panels. **A**, APC variants with substitutions in the N-terminal half of the Gla domain: APC(PT3), (\blacklozenge); APC(PT8/10), (\circ); APC(PT11/12), (\bullet); APC(PT18), (\times). **B**, APC variants with substitutions in the C-terminal half of the Gla domain: APC(PT28/30), (\blacktriangle); APC(PT33–39), (\triangle); APC(PT35/36), ($*$); APC(PT36/38/39), (\blacktriangledown); APC(PT42/44/45), (\blacksquare). Values correspond to the mean and S.E. of at least three determinations.

5A, top panel). At the highest APC concentration tested (7 nM), the ETP was reduced to $15 \pm 1\%$ of the ETP determined with no APC present (Fig. 5, A and C). However, when a polyclonal anti-protein S antibody known to inhibit APC-dependent protein S cofactor function (36) was incubated with 7 nM wild-type APC, the ETP was not diminished and the anticoagulant effect of wild-type APC was effectively lost (Fig. 5B). In this way, the assay was shown to be completely dependent upon protein S to facilitate APC down-regulation of thrombin generation. Variants APC(PT33–39), APC(PT35/36), and APC(PT36/38/39) were tested in both the presence and absence of the inhibitory anti-protein S antibody. In the presence of the anti-protein S antibody, minimal ETP reduction (<10%) was observed for each of the APC variants tested (data not shown). In the absence of the anti-protein S antibody, APC(PT35/36) reduced thrombin generation ~ 2 -fold less effectively than wild-type APC (Fig. 5, A and C). Variants APC(PT33–39) and APC(PT36/38/39) failed to inhibit thrombin generation at any of the APC concentrations tested (Fig. 5, A and C), highlighting their inability to interact with protein S to reduce thrombin generation. The phospholipid PE has been previously shown to significantly enhance APC anticoagulant activity (18). To confirm that the results observed for variants APC(PT33–39), APC(PT35/36), and APC(PT36/38/39) were not a consequence of defective interaction with PE, the same experiments were performed in the presence of phospholipid vesicles composed of PC/PS only. The results of these experiments were found to replicate those performed in the presence of PC/PS/PE (data not shown). These data confirm that APC Gla domain residues between 33–39 (and 36–39 in particular) are critical in mediating protein S cofactor activity.

EPCR Recognition by Protein C Is Mediated by Leu-8—Using surface plasmon resonance, human prothrombin was tested for sEPCR binding and was found to have essentially no sEPCR affinity (Fig. 6A). Wild-type protein C bound sEPCR with high affinity ($K_d = 117 \pm 36$ nM) as reported previously (32) (Fig.

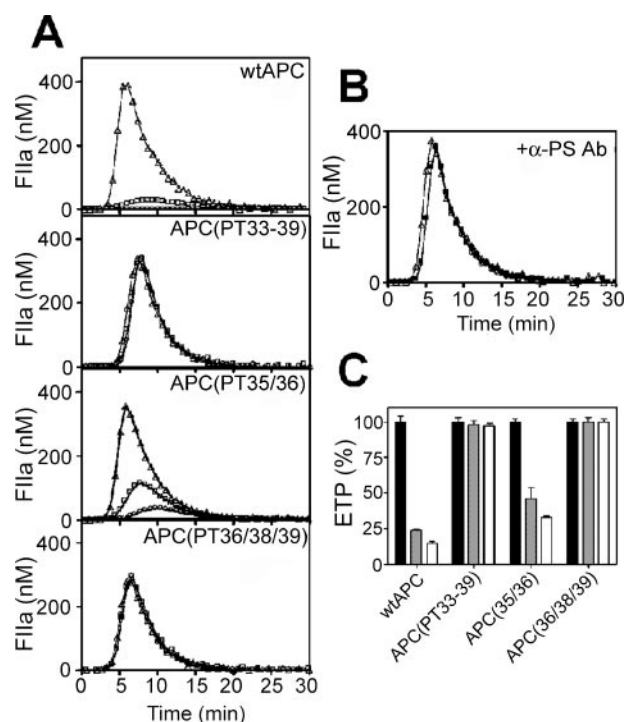


FIGURE 5. APC variants APC(PT33–39) and APC(PT36/38/39) have no protein S-dependent anticoagulant activity in protein C-deficient plasma. **A**, 7 nM APC, (\circ); 3.5 nM APC (\square), or HEPES buffer (no APC), (\triangle) was incubated with the protein C-deficient plasma. Thrombin generation in protein C-deficient plasma was initiated by 16 pM tissue factor, 17.5 μ M phospholipid vesicles (PC/PS/PE, 60%:20%:20%), 16.7 mM CaCl_2 , and CTI (42 μ g/ml). **B**, thrombin generation in protein C-deficient plasma in the presence of an inhibitory anti-protein S polyclonal antibody (240 μ g/ml) with 7 nM APC (\blacksquare) and in the absence of APC (\triangle). **C**, the ETP for each variant in the presence of 7 nM APC (white), 3.5 nM APC (gray), and with APC absent (black) was determined using Thrombinoscope software. For each sample, the anticoagulant effect in the presence of the inhibitory anti-protein S antibody (<10%) has been subtracted. Values correspond to the mean and S.E. of at least three determinations.

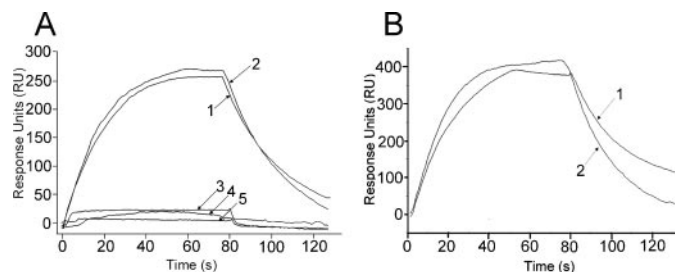


FIGURE 6. Leu-8 is required for sEPCR binding. Recombinant protein C affinity for sEPCR was assessed by surface plasmon resonance (see “Experimental Procedures”). **A**, an anti-EPCR monoclonal antibody was immobilized onto both flow cells of a CM5 sensor chip. sEPCR (800–900 RU) was then bound to the second flow cell, the other flow cell acting as a “no sEPCR” control for nonspecific binding. sEPCR binding of wild-type recombinant protein C (1), PC(PT10) (2), PC(PT8) (3), PC(PT8/10) (4), and human plasma-purified prothrombin (5) (all 80 nM) was assessed. **B**, 100 nM human protein C (1) and human FVIIa (2) were assessed for sEPCR binding as above.

6A). Each protein C variant was tested for sEPCR binding using a range of protein C concentrations (12.5–200 nM). The vast majority of protein C variants exhibited sEPCR binding affinity similar to that of wild-type protein C (data not shown). However, the protein C variant PC(PT8/10) (L8V/H10K) showed no EPCR affinity, eliciting minimal response upon passing the sEPCR surface (Fig. 6A). No specific binding response was

observed even at high concentrations (up to 1.5 μM) (data not shown). To determine the relative contributions of residues Leu-8 and His-10 in mediating ligand recognition, individual variants containing either L8V or H10K substitutions were generated and tested in the same manner. Although variant PC(PT10) (H10K) bound sEPCR with similar affinity (85 nM) to wild-type protein C, no sEPCR binding was observed with PC(PT8) (L8V), highlighting the critical nature of this residue in EPCR recognition by protein C (Fig. 6A).

Sequence alignment of human vitamin K-dependent coagulation protein Gla domains indicated that the protein C/APC Gla domain ω -loop residues required for EPCR binding are entirely conserved in FVII(a). FVIIa binding to sEPCR was examined by surface plasmon resonance. FVIIa concentrations between 12.5–200 nM were passed over an sEPCR surface, and binding was assessed. FVIIa was found to bind sEPCR with comparable affinity to protein C (~ 150 nM) (Fig. 6B), confirming the importance of Leu-8 for EPCR recognition.

DISCUSSION

Using recombinant protein C/APC Gla domain variants, we have elucidated the role of protein C/APC Gla domain residues in mediating protein S cofactor function and EPCR recognition. 13 recombinant protein C/APC variants were generated in which the 22-protein C Gla domain residues not shared with prothrombin were substituted with those of prothrombin (Fig. 1). This approach was used to enable Gla domain structure to be maintained while probing protein C-specific interactions. The majority of variants were expressed normally and exhibited normal phospholipid binding (Fig. 2) and amidolytic activity once activated.

Despite numerous studies describing the important protein S domains/residues for protein S cofactor function with APC (22, 26, 28, 37), the mapping of APC residues involved in mediating the interaction with protein S has been limited. One study, however, broadly implicated residues 22–45 of the APC Gla domain in enabling protein S to act as an APC cofactor (18). Using variants with substitutions within this region, we have mapped the residues responsible for mediating protein S cofactor function upon APC. APC variants containing prothrombin substitutions at positions 33, 34, 35, 36, 38, and 39 were not effectively stimulated by protein S in anticoagulant assays, highlighting the critical importance of this small cluster of APC residues in defining protein S cofactor activity (Figs. 4 and 5). APC variants containing substitutions in this region exhibited normal FVa proteolysis in the absence of protein S (Fig. 3), demonstrating that the loss of cofactor activity exhibited in the presence of protein S was not caused by disruption of any site of FVa interaction within the APC Gla domain. Most of the residues in this region are either hydrophobic or acidic in nature and are surface exposed on a model of the complete protein C Gla domain with Ca^{2+} bound (Fig. 7A) (38, 39). Consequently, several of these residues form part of a putative protein-protein binding site predicted using molecular modeling based on surface hydrophobicity (40).

APC variants APC(PT35/36) and APC(PT36/38/39) provided a useful insight into the relative importance of some of these residues in mediating protein S stimulation of APC anti-

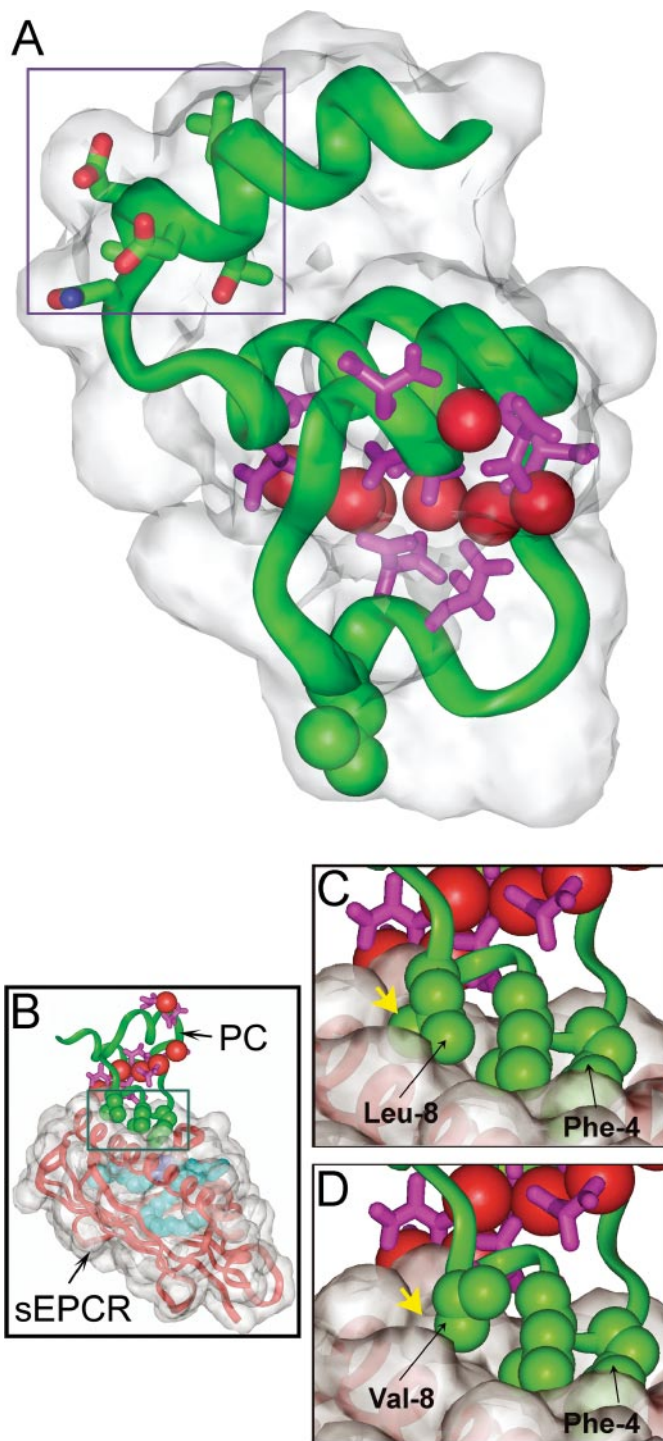


FIGURE 7. Position of APC Gla domain residues critical for protein S enhancement of APC activity and EPCR recognition. A, residues 33–39 (green, in stick representation within blue box) appear largely surface exposed on a model of the full protein C Gla domain with Ca^{2+} ions (red spheres) coordinated by Gla residues (purple, stick representation) (38). Side chains of residues 33–39 are colored by atom: carbon (green), oxygen (red), and nitrogen (blue). Leu-8, important for EPCR recognition (see “Results”), is highlighted on the ω -loop (green, Corey-Pauling-Koltun representation). B, the protein C Gla domain (green) binds to sEPCR (gray) primarily via hydrophobic residues (green) in the ω -loop when Ca^{2+} (red spheres) is bound by Gla residues (purple) (30). C, Phe-4 and Leu-8 (yellow arrow) of protein C are primarily responsible for binding and become buried in the sEPCR surface. D, PC(PT8) binding to sEPCR is impaired by the inability of Val-8 (yellow arrow) to make full hydrophobic contacts with Leu-82 of sEPCR.

Molecular Recognition of Protein S and EPCR by Protein C/APC

coagulant activity. Residues Asp-36, Leu-38, and Ala-39 appear critical in mediating protein S cofactor function, given the inability of protein S to enhance APC(PT36/38/39) activity (Figs. 4 and 5), despite normal phospholipid binding (Fig. 2) and FVa proteolytic activity in the absence of protein S (Fig. 3). Furthermore, the reduced sensitivity to protein S stimulation exhibited by APC(PT35/36) (Figs. 4 and 5) highlights the importance of one or both of acidic residues Asp-35 and Asp-36 in enabling protein S enhancement of APC activity.

Our findings suggest surface residues in positions 33–39 of APC determine protein S cofactor activity, most likely via a direct binding interaction through these residues. Interestingly, a cluster of 9 protein S Gla domain residues has recently been identified that are crucial in mediating protein S cofactor activity, despite having no influence upon normal protein S phospholipid binding (28). These residues (at positions 21, 23, 24, 28, 33, 34, 35, 41, and 45) are situated toward the C-terminal end of the protein S Gla domain and are predicted to be surface exposed. Furthermore, the majority of these residues possess either hydrophobic or basic properties. Such protein S Gla domain residues may offer a complementary binding site for APC residues 33–39. However, direct binding between APC and protein S has been difficult to demonstrate. A single report using fluorescence-activated cell sorter analysis determined a K_d of 75 nM between APC and protein S in the presence of phospholipids (18). Further studies are now required to establish the precise role of individual APC residues in binding to protein S.

EPCR binding by protein C/APC has been reported to be specific to the protein C Gla domain (29). The majority of vitamin K-dependent proteins are unable to bind to EPCR (31).³ From the crystal structure, it appears that only 5 protein C Gla domain residues mediate EPCR binding. Of these, 3 are Gla residues that are entirely conserved across all other vitamin K-dependent coagulation proteins (Gla-7, Gla-25, and Gla-29). The remaining residues (Phe-4 and Leu-8) are hydrophobic and are predicted to contribute the majority of the binding energy required for the protein C/APC-EPCR interaction (30). These residues form part of a hydrophobic ω -loop structure present in all vitamin K-dependent coagulation proteins and important in mediating phospholipid binding (41, 42). Prothrombin, like protein C/APC, also possesses 4/5 EPCR binding residues, including Phe-4 in its ω -loop, yet still cannot bind EPCR (29). Consequently, we investigated whether specific structural motifs within the protein C Gla domain were responsible for defining protein C/APC specificity for EPCR or whether the subtle residue differences between the protein C/APC ω -loop and that of other vitamin K-dependent coagulation proteins were sufficient to prevent EPCR binding.

The majority of the protein C variants tested bound to sEPCR with similar affinity as wild-type protein C, excluding the possibility of protein C Gla domain-specific structural motifs distal from the EPCR binding residues influencing EPCR binding specificity. However, protein C variants containing the L8V substitution were completely unable to bind to sEPCR (Fig. 6),

highlighting the importance of Leu-8 in enabling EPCR recognition and subsequent high affinity binding. Molecular modeling of PC(PT8) binding to sEPCR using the protein C/sEPCR crystal structure provides a potential explanation for the loss of EPCR binding in this variant (Fig. 7, B–D). When wild-type protein C binds EPCR, Leu-8 of the protein C/APC ω -loop makes close hydrophobic contacts with Leu-82 of sEPCR and becomes buried in the EPCR surface (Fig. 7C). Modeling of the PC(PT8) variant suggests that the shorter valine R group is unable to contact Leu-82 of EPCR (Fig. 7D). Consequently, despite retaining a similarly hydrophobic residue at position 8, PC(PT8) and prothrombin are unable to bind EPCR.

Sequence alignment of the human protein C Gla domain with that of other species indicates that Leu-8 is conserved in the bovine sequence but not in rat and mouse, which have a valine and methionine, respectively, at this position. EPCR residues that directly contribute to protein C binding are completely conserved across human, bovine, rat, and mouse (43). Human and bovine protein C bind to human sEPCR with equal affinity.³ Our study has shown that Val-8 in the ω -loop cannot sustain human sEPCR binding (Fig. 6) and Met-8 in murine protein C possesses an identical ω -loop to that of human FX, which has been shown in binding competition experiments (31) to exhibit minimal affinity for human EPCR. It seems likely that small EPCR sequence variations close to the protein C/APC binding residues on EPCR must alter the binding surface to accommodate protein C/APC binding in these species.

The identical FVII(a) ω -loop sequence to protein C/APC has been shown to contribute to the low phospholipid affinity of both proteins in comparison to other vitamin K-dependent proteins (17). Our data show that the functional similarity of the Gla domains of protein C/APC and FVII(a) extends further, as FVII(a) binds sEPCR with comparable affinity to protein C/APC (117 and 150 nM, respectively; Fig. 6B). The physiological consequences of FVII(a) ability to bind EPCR are uncertain. FVII circulates in plasma at a 6-fold lower concentration than protein C, making it unlikely that FVII(a) would be able to effectively compete with protein C/APC for EPCR binding under normal physiological conditions. However, under conditions where FVII(a) is elevated or administered therapeutically, FVIIa antigen levels may more closely approach those of protein C, making competition for EPCR binding a possibility.

This report further demonstrates that the function of the Gla domain is not restricted to phospholipid binding. Selective mutation has enabled the development of a compact multifunctional domain that interacts directly with a receptor (EPCR) and a cofactor (protein S). The protein C Gla domain therefore represents a paradigm for adaptive interactions of other Gla domains.

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