The Interaction between the Endothelial Cell Protein C Receptor and Protein C Is Dictated by the \( \gamma \)-Carboxyglutamic Acid Domain of Protein C*

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The endothelial cell protein C receptor (EPCR) binds to both protein C and activated protein C (APC) with similar affinity. Removal of the Gla domain of protein C results in the loss of most of the binding affinity. This observation is compatible with at least two models: 1) the Gla domain of protein C interacts with phospholipid on cell surfaces to stabilize interaction with EPCR or 2) the Gla domain of protein C makes specific protein-protein interactions with EPCR. The latter model predicts that chimeric proteins containing the protein C Gla domain should interact with EPCR. To test this, we constructed a prothrombin chimera in which the Gla domain and the aromatic stack of prothrombin were replaced with the corresponding region of protein C. The \( ^{125} \text{I} \)-labeled chimera (\( K_D = 176 \text{ nm} \)) and \( ^{125} \text{I} \)-APC (\( K_D = 65 \text{ nm} \)) both bound specifically to 293 cells stably transfected with EPCR, but both bound poorly to sham-transfected cells. The chimera also blocked APC binding to EPCR-transfected cells in a dose-dependent fashion (\( K_D \approx 139 \text{ nm} \)) similarly to protein C (\( K_D \approx 75 \text{ nm} \)). No inhibition was observed with the isolated prothrombin Gla domain and aromatic stack. A protein C chimera with the Gla domain was blocked with soluble EPCR, demonstrating direct protein-protein interaction between the chimera and EPCR. Inconsistent with this conclusion, the isolated Gla domain of protein C blocked APC binding to EPCR-transfected cells (\( IC_{50} = 2 \mu \text{m} \)). No inhibition was observed with the isolated prothrombin Gla domain. A protein C chimera with the prothrombin Gla domain and aromatic stack failed to bind to EPCR detectably. These data suggest that the Gla domain of protein C is responsible for much of the binding energy and specificity of the protein C-EPCR interaction.

Protein C plays a major role in the regulation of blood coagulation. The protein C anticoagulant pathway functions in large part on the endothelial cell surface (reviewed in Ref. 1). The pathway is initiated when thrombin binds to thrombomodulin on the endothelium, and this complex catalyzes protein C activation. Activated protein C (APC)\(^1\) is the terminal enzyme of the pathway and functions as an anticoagulant by proteolytic inactivation of the coagulation cofactors, factors Va and VIIIa (2–4). Defects in this pathway are frequently associated with thrombophilia (5, 6). The pathway has also been implicated in regulating the inflammatory response, including providing protection against septic shock (7–9) and modulating tumor necrosis factor production (10, 11).

Recently, the endothelial cell surface was shown to bind protein C and APC with high affinity (12, 13), and the responsible cell-surface glycoprotein, EPCR, was identified by expression cloning (13). EPCR binds directly to protein C and APC with high affinity even when expressed in a soluble form (14). Blocking protein C binding to EPCR with a monoclonal antibody results in an \( \sim 4–5 \)-fold decrease in the rate of protein C activation over the surface of the endothelium, with no change in the rate of activation when substrates lacking the vitamin K-dependent Gla domain are employed (Gla domainless protein C) (15). The interaction of soluble EPCR with APC blocks plasma anticoagulant activity and the ability of APC to inactivate factor Va without altering reactivity with protein C inhibitor or \( \alpha_1 \)-antitrypsin (16).

Previous studies have shown that recombinant Gla domainless protein C cannot inhibit the binding of APC to human umbilical vein endothelial cells (13). This suggests that the Gla domain is required either for binding to the membrane phospholipid, thereby promoting binding to EPCR, or for binding to EPCR itself. The observation that the affinity of soluble recombinant EPCR (sEPCR) for protein C/APC is comparable to the affinity for cell-surface EPCR suggested that phospholipid-protein C interaction contributes little to the affinity of protein C for the endothelial cell surface (14). The inability of Gla domainless protein C to inhibit APC binding could reflect either a difference in global folding, particularly \( \text{Ca}^{2+} \)-induced conformational changes (17, 18), or the loss of direct protein C Gla domain interaction with EPCR. Either function differs from the membrane phospholipid binding activity normally ascribed to the Gla domain of vitamin K-dependent proteins (19, 20). The major exception to this function of Gla domains is the binding of factor IXa to the factor Xa receptor on endothelial cells. This interaction was shown to be mediated by the Gla domain (21, 22).

In this study, we investigated the role of the Gla domain of protein C in binding to EPCR using a prothrombin chimera in which the Gla domain and aromatic stack of prothrombin were replaced with the corresponding region of protein C (PT-PC replaced with the corresponding region of protein C; PT-PC Gla, prothrombin Gla, sEPCR, soluble recombinant EPCR; PT-PC Gla, prothrombin with the Gla domain and hydrophobic stack replaced with the corresponding region of prothrombin; Fl-APC, APC modified in the active site by fluorescein; PCR, polymerase chain reaction; EGF, epidermal growth factor.)

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§ The abbreviations used are: APC, activated protein C; EPCR, endothelial cell protein C/APC receptor; sEPCR, soluble recombinant EPCR; PT-PC Gla, prothrombin with the Gla domain and hydrophobic stack.
Gla). We find that this domain mediates direct protein-protein interactions with the receptor.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human protein C (23), human prothrombin (24), and soluble recombinant EPCR (14) were prepared as described previously. The Gla domains from bovine protein C and prothrombin were prepared by limited proteolysis of the plasma zymogens with chymotrypsin as described previously for bovine protein C (25).

The extinction coefficients (mg/ml) -1 cm -1 and molecular weights used in this study were 1.45 and 62,000 for APC and protein C, 1.55 and 72,000 for prothrombin, 1.55 and 72,000 for PT-PC Gla, 1.45 and 62,000 for the protein C-prothrombin Gla domain chimera (PC-PT Gla), 1.45 and 55,000 for Gla domainless protein C, 1.45 and 4,000 for the protein C Gla domain peptide, 1.55 and 4,000 for the prothrombin Gla domain peptide, and 1.0 and 42,000 for sEPCR.

**Modification of APC—Iodo-GEN (Pierce) was used to label APC and PT-PC Gla with NaCl and then reacting the modified enzyme with 5-(iodoacetamido)fluorescein (Molecular Innovations) by a modification (13) of the method of Bock et al. (26).**

**Construction of the Prothrombin-Protein C Gla Domain Chimera—**Prothrombin cDNA was ligated into the Smal and EcoRI restriction sites of pUC19 (pUC-II). Using the unique Xhol restriction enzyme site in exon IV of the prothrombin cDNA (base 287), a double digestion of pUC-II with XhoI and XbaI (a site on the polylinker cloning site on pUC) released the first three exons of the prothrombin cDNA as well as 21 base pairs of the beginning of exon IV. Two PCR primers were synthesized. The sense primer (5'-GTCGTCTAGACCCGGGATGTG-GTAGTGG-3') starts at the initiation codon of the protein C cDNA and contains Xhol and Smal restriction sites at the 5'-end (underlined). The antisense primer (5'-GCGTCTCGAGGCGTCCTCGGACAGGTCAAGCAT CCT-3') starts at the last nucleotide of exon III in the protein C cDNA and contains an XhoI restriction site along with 21 base pairs of exon IV of the prothrombin cDNA (underlined). After PCR amplification of the protein C cDNA with these primers and a double digestion with XhoI and XbaI, the DNA fragment was ligated into the identical sites of pUC-II. The modified prothrombin cDNA was cleaved by Smal and EcoRI from the pUC-II plasmid and ligated into the identical sites on the pSC101 vector. After sequencing, the DNA was transfected into baby hamster kidney cells as described previously for the wild-type counterpart (27).

**Construction of the Protein C-Prothrombin Gla Domain Chimera—**A protein C chimera was constructed in which the first three exons of prothrombin (i.e. coding for the signal peptide, the Gla domain, and the aromatic stack regions) replaced the corresponding regions of protein C. Mutagenesis was performed by PCR methodology. The wild-type protein C cDNA was ligated into the HindIII and XhoI sites of pBS/RSV (Invitrogen, San Diego, CA) to form RSV-PC as described previously (28). There is a unique BstEII restriction site in the protein C cDNA in the beginning of exon IV, which encodes the N-terminal EGF domain. Double digestion of RSV-PC with HindIII and BstEII removed the DNA sequences of the first three exons of protein C as well as the first exon of exon IV, which is an Asp. To exchange exons I-III of protein C with those of prothrombin, two PCR primers were synthesized. The prothrombin sense primer (5'-CGTCAAGCTTCTAGTCGCCCTAGTCGCAGGTCT-3') starts at the initiation codon of the prothrombin cDNA and contains a HindIII restriction enzyme site at the 5'-end of the primer (underlined). The prothrombin antisense primer (5'-GAGGTGTCTAGGGTCCAGGCGGAGGGTCT-3') starts at the 72th base of exon II in the prothrombin cDNA and also contains the native BstEII restriction enzyme site containing the missing Asp codon in the beginning exon IV of protein C cDNA. Following PCR amplification of prothrombin cDNA with these two primers and double digestion with HindIII and BstEII, the DNA fragment was ligated into the identical sites of the wild-type protein C expression vector described above.

After sequencing, the mutant construct was transfected into human 293 cells, and chimeric protein C was purified from the cell culture supernatants by immunoaffinity chromatography using the calcium-dependent monoclonal antibody HPC4 as described previously (28, 29). PC-PT Gla was further purified by Mono Q chromatography essentially as described (23).

**Gla Determination—**Carboxyglutamic acid content was determined by the base hydrolysis method described by Smalley and Preusch (30) with modifications. Briefly, proteins (0.5 nmol each) were hydrolyzed with 2 N NaOH, in the absence of oxygen, for 16 h at 110 °C. Samples were desalted over Dowex resin (Sigma) and dried. Samples were then derivatized with phenyl isothiocyanate (Pierce) and dried. Samples were then rehydrated with 50 μl of 0.1% xylene (pH 7.5), 0.05% triethylamine, and 1% aceticacetic acid, and glutamate were resolved in this buffer on an Alltech C18 high pressure liquid chromatography column.

**Radioligand Binding Assays—**Cells were incubated in suspension as indicated in the figure legends. Samples were then layered over 100 μl of spigean oil (31, 32) and centrifuged for 2 min at 13,000 rpm. Tubes were cut, and the cell pellet was counted (32). For direct binding experiments, increasing concentrations of 125I-APC or 125I-PT-PC Gla were incubated with the cells in Hanks' balanced salt solution containing 3 mM CaCl2, 0.6 mM MgCl2, 1% bovine serum albumun, and 0.02% sodium azide (binding buffer) for 30 min at room temperature. Samples were treated as described above. Ki values were determined by Scatchard analysis. The regression analyses were those of Marquardt (33) as those provided by Bevington (34) using data analysis software kindly provided by J. Jesty (State University of New York, Stony Brook, NY). Some experiments were performed using 125I-1-APC modified in the active site with d-Phe-Pro-Arg-chloromethyl ketone (Calbiochem), which binds identically as the unmodified form to EPCR-transfected cells. For these experiments, increasing concentrations of sEPCR were incubated with cells in the presence of 60 μM 125I-PT-PC-Gla or 125I-APC in binding buffer for 30 min at room temperature. Samples were treated as described above.

For competition experiments using sEPCR, Ki determinations were performed by fitting data points using the equation $Y = AB/(B + X)$ for simple competitive inhibition, where $A$ is % APC bound in the absence of inhibitor (100%), $B = K_i$, and $X$ is concentration of inhibitor in the reactions. Curves were then fitted with $100%$ at zero inhibitor. Regression procedures were performed as described above.

For competition experiments using unlabeled PT-PC Gla and protein C, Ki values were determined with the same equation, but since the concentration of labeled protein was at or above $K_i$, $B = K_i + 1/S_{max}$, where $S_{max}$ is concentration of APC added to the reaction (60 nm) and $K_{i} = 65$ nm for 125I-APC experiments.

**Flow Cytometric Analysis—**Fluorescence-activated cell sorter (Becton Dickinson). The amount of F1-APC that bound to N1 cells was taken as nonspecific binding and subtracted from all data points. Ki values were determined as described above using $K_{i} = 30$ μM (13) and confirmed by direct binding studies with the lot of F1-APC used in the present analysis.

**RESULTS**

The PT-PC Gla chimera was purified as described under "Experimental Procedures" and was homogenous on SDS gel electrophoresis (data not shown). When the chimera was chromatographed on Mono Q, two peaks were partially resolved. They correspond to the trailing fractions of the second peak bound most tightly to EPCR-expressing cells and contained 9 mol of Gla residues/mol of protein. The chimera in the first peak bound less tightly (see below) and contained 8 mol of Gla residues/mol of protein.

**Direct Binding of PT-PC Gla to E7 Cells—**To determine whether the protein C Gla domain conferred specific binding of the chimera to E7 cells, the chimera was iodinated and incubated at increasing concentrations with E7 cells (Fig. 1A).

The small amount of binding of the chimera to N1 cells was sub-
Scatchard analysis of the data resulted in $K_d = 176 \pm 26$ nM and $(13.7 \pm 3.9) \times 10^6$ sites/cell. A similar experiment using $^{125}$I-APC resulted in $K_d = 65 \pm 6$ nM and $(8.0 \pm 2.7) \times 10^6$ sites/cell.

Inhibition of PT-PC Gla Binding to E7 Cells—To determine if the Gla domain was involved in phospholipid-protein interactions or protein-protein interactions, we investigated the effect of sEPCR, which does not bind to phospholipid, on the binding of $^{125}$I-APC and $^{125}$I-PT-PC Gla to E7 cells. sEPCR inhibited chimera binding in a concentration-dependent fashion similar to that observed with APC. We assumed that this inhibition was due to a decrease in free ligand caused by complex formation with sEPCR. Using this assumption and the concentration dependence of binding seen in Fig. 1A, we calculated $K_i$ values of sEPCR of 109 ± 20 nM for the chimera and 141 ± 39 nM for APC (Table I). This suggests that EPCR can bind the Gla domain of both APC and PT-PC Gla in solution, and therefore, the Gla-dependent binding is mainly through protein-protein interactions.

Inhibition of APC Binding to E7 Cells—Prothrombin, Gla domainless protein C, protein C, and the chimera were used to compete with the binding of either Fl-APC (Fig. 2A) or $^{125}$I-APC (Fig. 2B) to E7 cells. As shown in Fig. 2A, prothrombin and Gla domainless protein C failed to compete effectively with Fl-APC for binding to EPCR expressed on the cell surface. In contrast, protein C and PT-PC Gla inhibited Fl-APC binding to these cells effectively and with similar dose-response curves. Analysis of the competition indicated that the $K_i$ for protein C was 75 ± 16 nM and for the chimera was 139 ± 43 nM (Table I). PT-PC Gla with an incomplete complement of Gla residues (8) was not nearly as effective as fully carboxylated PC-PT Gla in blocking Fl-APC binding to the cells. The ability of the Gla domain of protein C to impart to prothrombin the capacity to compete effectively for binding to EPCR-expressing cells suggests that the Gla domain of protein C provides the majority of the binding energy of the protein C/APC-EPCR interaction.
The observation that the chimera could compete effectively with APC for binding to the cells eliminates the possibility that chimera binding to EPCR-transfected cells was due to the presence of more prothrombin-specific binding sites on these cells than on the sham-transfected cells.

These results were confirmed with a similar assay using 125I-APC binding to E7 cells (Fig. 2B). The $K_i$ for protein C was found to be $100 \pm 7 \text{nM}$, whereas the $K_i$ for PT-PC Gla was $139 \pm 10$. Again, prothrombin and Gla domainless protein C did not inhibit APC binding effectively. Under-carboxylated PT-PC Gla again failed to compete as effectively as the fully carboxylated chimera (data not shown).

Inhibition of 125I-APC Binding to E7 Cells by Gla Domain Peptides—Since the Gla domain of protein C appears to be required for binding to EPCR, we examined the possibility that the isolated Gla domain (residues 1–41) might be able to compete with APC for binding to EPCR-transfected cells. As a control for specificity, we employed the isolated Gla domain of prothrombin (Fig. 3). Consistent with the predictions based on the chimera, the isolated Gla domain of protein C exhibited concentration-dependent inhibition of APC binding ($IC_{50} = 2 \text{ } \mu\text{M}$). In contrast, the Gla domain of prothrombin was not an effective competitor. Since these two Gla domains are structurally similar, we conclude that the isolated Gla domain of protein C is capable of specific interaction with EPCR on cell surfaces.

Binding Interactions between EPCR and a Protein C-Prothrombin Gla Domain Chimera—To assess the possibility that EPCR-binding sites on protein C outside of the Gla domain and hydrophobic stack might contribute significantly to the binding interaction, we examined the ability of the PC-PT Gla chimera to compete with Fl-APC for binding to E7 cells (Fig. 4). Unlike the PT-PC Gla chimera, the PC-PT Gla chimera failed to compete for binding to these cells. The competition studies were chosen for this analysis since it is easier to detect low affinity binding by competition than by direct binding analysis. Failure to bind the E7 cells was not due to improper folding of the chimera since it inactivates factor Va on phospholipid vesicles as does wild-type APC.

**DISCUSSION**

Vitamin K-dependent coagulation proteins possess a Gla domain that binds Ca\(^{2+}\) and imparts to these proteins the ability to bind to negatively charged phospholipid surfaces. These binding interactions are critical to the assembly of physiologically relevant coagulation complexes. One of the major regulatory events that controls the coagulation cascade appears to be the expression of negatively charged phospholipids on the surface of activated cells, particularly platelets. Explo-
The protein C Gla domain peptide (●) requires negatively charged phospholipid surfaces. Proteins proceed in a Gla domain-dependent fashion, but without a requirement for exogenous phospholipids (38, 39). Recent studies have implicated a second vitamin K-dependent proteins (36). Studies have shown that thrombin infusion can rapidly activate protein C without a requirement for exogenous phospholipids. In vivo potentiating factors such as factor Xa has little coagulant activity unless negatively charged phospholipids are infused simultaneously (37). This implies that the normal vasculature has very little negatively charged phospholipid exposed under physiological circumstances. The observation reported here suggest that activation of the vitamin K-dependent anticoagulant protein C can proceed in a Gla domain-dependent fashion, but without a requirement for negatively charged phospholipid surfaces. In vivo thrombin infusion can rapidly activate protein C without a requirement for exogenous phospholipids (38, 39). Recent studies have implicated a second endothelial cell receptor, EPCR, in protein C activation (15), and the present study suggests that the binding interaction involved in the activation involves primarily the vitamin K-dependent Gla domain of protein C. The Gla domain involvement in protein-protein interactions between protein C and EPCR is consistent with the previous observation that binding to EPCR is Ca\(^{2+}\)-dependent and abolished by proteolytic removal of the Gla domain (12, 13). The observation that the under-carboxylated forms of the chimera interact weakly with EPCR-expressing cells and compete poorly with Fl-APC for binding to the receptor suggest that the Ca\(^{2+}\)-mediated conformational changes in the Gla domain of protein C are important for optimal binding interactions with EPCR. These properties are similar to the requirements of the factor IX receptor, the other receptor constitutively expressed on the endothelium (21, 40).

Protein C is most homologous to factors X, IX, and VII since all contain a Gla domain, a hydrophobic stack, two EGF domains, and a protease domain. EGF domains have been implicated in many protein-protein interactions involved in blood coagulation (41–47). Prothrombin is structurally distinct from these coagulation factors in that the EGF domains are replaced by kringle domains. Therefore, to eliminate the possibility of EGF domain contribution to EPCR binding and to test the importance of the protein C Gla domain, we prepared the PT-PC Gla chimera described herein. The observation that this chimera binds to EPCR with affinities very similar to those of protein C strongly supports the conclusion that the binding is mediated primarily by the Gla domain. The kinetic parameters for protein C were 2–3-fold higher than the K\(_{d}\) observed in earlier binding studies (13, 14) and by competition in solution. The basis for this discrepancy is unclear, but may be due to some complex binding processes on the cell surface, differences in protein preparations, or simple error in the measurements. It is clear, however, that protein C and the PT-PC Gla chimera compete effectively with Fl-APC or Fl-APC for binding to the EPCR-transfected cells and that sEPCR can block binding of both molecules with similar dose-response relationships. These data unambiguously demonstrate that the major binding energy for the protein C-EPCR interaction is mediated by the protein C Gla domain. This conclusion is bolstered by the observation that the isolated Gla domain can block the reaction, albeit with lower efficiency than the intact chimera. The decreased affinity for EPCR is probably due to the fact that the isolated Gla domain prepared by chymotryptic digestion is not in an optimal conformation (48, 49). For instance, Colpitts and Castellino (48) have shown that the aromatic stack, in addition to the Gla domain of protein C, is essential for phospholipid binding. It would appear based on this study and the results reported herein that the aromatic stack plays a more important role in phospholipid binding than in binding to EPCR.

While the Gla domain appears to be the major determinant of binding to EPCR, this does not rule out a contribution of other parts of protein C to the binding. The thrombin-thrombomodulin interaction is largely determined by EGF domains 5 and 6, but requires EGF-4 to effect the substrate specificity switch of thrombin from a procoagulant to an activator of protein C anticoagulant activity (Refs. 50 and 51; reviewed in Ref. 1). Therefore, it could be expected that another part of the protein C molecule can bind to EPCR and in that way affect the ability of APC to cleave its substrates and/or allow APC to cleave new substrates.

Requirement for γ-carboxylation in the Gla domain also has clinical implications. Coumadin inhibits the vitamin K-dependent γ-carboxylation of coagulation proteins. This will inhibit APC binding not only to negatively charged surfaces, but also to endothelial cells through interaction with EPCR. The physiological significance of this effect is not known at this time. However, it should be clarified once we have a better under-

![Figure 3](image3.png)

**Fig. 3. Inhibition of 125I-APC binding to E7 cells by Gla domain peptides.** E7 cells were incubated in binding buffer for 30 min at room temperature with 125I-APC (60 nM) and the indicated concentrations of the protein C Gla domain peptide (●) or the prothrombin Gla domain peptide (■). Samples were then layered over oil, centrifuged, and counted. Each point is the average of five sets of duplicate samples.

![Figure 4](image4.png)

**Fig. 4. Inhibition of Fl-APC binding to E7 cells.** The indicated concentrations of protein C (●), prothrombin (■), and PC-PT Gla (□) were added to E7 cells in the presence of Fl-APC (60 nM) in binding buffer. Samples were treated as described for Fig. 2A. Each point is the average of three sets of duplicate samples. MCF, mean fluorescence.
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standing of the in vivo function of the protein C/APC-EPCR complex.

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