IDENTIFICATION OF THE PROTEIN C/ACTIVATED PROTEIN C BINDING SITES ON
THE ENDOTHELIAL CELL PROTEIN C RECEPTOR: IMPLICATIONS FOR A NOVEL
MODE OF LIGAND RECOGNITION BY AN MHC CLASS 1-TYPE RECEPTOR*

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**Running Title: Protein C and Activated Protein C Binding Site on EPCR**
SUMMARY

The endothelial cell protein C receptor (EPCR) is an endothelial cell-specific transmembrane protein that binds both protein C and activated protein C (APC). EPCR regulates the protein C anticoagulant pathway by binding protein C and augmenting protein C activation by the thrombin-thrombomodulin complex. EPCR is homologous to the MHC class 1/CD1 family, members of which contain two $\alpha$-helices that sit upon an eight stranded $\beta$-sheet platform. In this study, we identified ten residues that, when mutated to alanine, result in the loss of protein C/APC binding (R81, L82, V83, E86, R87, F146, Y154, T157, R158, and E160). Glutamine substitutions at the four N-linked carbohydrate attachment sites of EPCR have little affect on APC binding, suggesting that the carbohydrate moieties of EPCR are not critical for ligand recognition. We then mapped the epitopes for four anti-human EPCR monoclonal antibodies (mAbs), two of which block EPCR/Fl-APC interactions whereas two do not. These epitopes were localized by generating human-mouse EPCR chimeric proteins, since the mAbs under investigation do not recognize mouse EPCR. We found that five of the ten candidate residues for protein C/APC binding (R81, L82, V83, E86, R87) co-localize with the epitope for one of the blocking mAbs. 3-D molecular modeling of EPCR indicate that the ten protein C/APC binding candidate residues are clustered at the distal end of the two $\alpha$-helical segments. Protein C activation studies on 293 cells that co-express EPCR variants and thrombomodulin demonstrate that protein C binding to EPCR is necessary for the EPCR-dependent enhancement in protein activation by the thrombin-thrombomodulin complex. These studies indicate that EPCR has exploited the MHC class 1 fold for an alternative and possibly novel mode of ligand recognition. These studies are also the first to identify the protein C/APC binding region of EPCR and may provide useful information about molecular defects in EPCR that could contribute to cardiovascular disease susceptibility.
INTRODUCTION

The protein C anticoagulant pathway serves as the major physiologic control of clot formation (reviewed in (1-3)). The pathway is initiated upon binding of thrombin to the endothelial cell surface protein thrombomodulin (TM) (4). The thrombin-TM complex activates protein C to activated protein C (APC). APC, in conjunction with its cofactor protein S, degrades factors Va and VIIIa on the phospholipid surface, thereby attenuating the coagulation cascade. Defects in the protein C anticoagulant pathway have been implicated as the underlying risk factors for the development of venous and arterial thrombosis (5-11).

Recent studies have demonstrated that human endothelial cells express a transmembrane protein that binds both protein C and APC with high affinity (Kd=30 nM) (12). This molecule, named endothelial cell protein C receptor (EPCR), is an endothelial cell-specific, type 1 transmembrane protein that binds to the Gla-domain of protein C and APC (13). EPCR enhances the rate of protein C activation by the thrombin-thrombomodulin complex on the endothelial cell surface (14, 15) and when reconstituted into phosphatidylcholine liposomes (16), primarily by decreasing the $K_m$ for protein C. A soluble form of EPCR is found in normal human plasma (17) and has been shown to bind to protein C and APC with an affinity similar to that of intact membrane-bound EPCR (15, 17). In contrast to the membrane-bound form, soluble EPCR blocks APC anticoagulant activity (18, 19) by blocking phospholipid interactions (19). Interestingly, sEPCR alters the active site of APC, suggesting that the macromolecular specificity of APC may be altered by complex formation with soluble EPCR (19). EPCR also appears to aid in the host response to sepsis since blocking EPCR-protein C interactions in baboons exacerbates the coagulation and inflammatory responses to Escherichia coli (20). Preliminary clinical studies suggest that protein
C and APC supplementation is beneficial in sepsis or septic shock (21-23).

EPCR has sequence homology to members of the MHC class I/CD1 family of molecules. MHC class I/CD1 molecules are organized into the α1, α2, and α3 domains, followed by a transmembrane region and a short cytoplasmic tail. The α3 domain associates non-covalently with β-2 microglobulin, although in EPCR this domain is absent. The α1 and α2 domains form a ligand binding groove composed of two antiparallel α-helices that sit upon an 8-stranded β-sheet platform. Although most members of the MHC/CD1 family utilize this groove to bind short peptides, it should be noted that there are exceptions. For example, the neonatal Fc receptor, which shares the MHC fold, has a closed groove that is incapable of binding peptides (24). Instead, the ligand binding interface is on the side of the neonatal Fc receptor.

In this study, we set out to identify the residues within EPCR that are involved in protein C/APC binding. Our approach was to combine data from loss-of-function alanine substitution studies with that obtained from gain-of-function epitope mapping studies. Given that EPCR differs from other members of the MHC class I/CD1 family in that (a) it lacks the α-3 domain and (b) its ligands are 62 kDa proteins rather than short peptides, these studies will also provide insight into how the MHC structural motif may have evolved to serve different modes of ligand recognition.
EXPERIMENTAL PROCEDURES

Materials- Human thrombin (25), human protein C and APC (26), and recombinant Gla-domainless protein C (GDPC) (27) were prepared as described previously. GDPC is a truncated form of protein C lacking residues 1 to 46 of the N-terminus. Oligonucleotides were synthesized by Operon Technologies Inc. Dubecco’s modified Eagle’s medium, fetal bovine serum, and G418 were from Gibco BRL Life Technologies, Inc. (Gaithersburg, MA). Sulfo-NHS-LC-biotin UltraLink and Immobilized NeutrAvidin Plus resin were from Pierce (Rockford, IL). Effectene transfection reagent was from Qiagen Inc. (Valencia, CA). H-D-(γ-carbobenzoxy)-L-prolyl-L-arginine-p-nitroanilide-diacetate (Spectrozyme PCa) was from American Diagnostica (Greenwich, CT). All other chemicals were of the highest grade commercially available.

DNA construction and mutagenesis- Human (12) and murine EPCR (28) cDNAs were cloned as XhoI/NotI fragments into the multiple cloning site of the eukaryotic expression vector pcDNA3.1 (-) (Invitrogen, San Diego, CA). In vitro mutagenesis to generate and select point mutations was performed using the QuickChange site-directed mutagenesis system as described by the supplier (Stratagene, La Jolla, CA). DNA manipulations to generate deletion mutants or human-murine chimeric cDNA molecules were carried out using standard DNA cloning techniques (29). Double-stranded DNA sequencing was used to verify the authenticity of the mutations.

Transient expression of wild-type and variant forms of EPCR in 293T cells- In the pcDNA3.1(-) vector, expression of EPCR cDNA is under the control of the human cytomegalovirus immediate-early promoter. Transient transfection of 293T cells was performed in 6-well dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum using Qiagen-purified pcDNA3.1(-) constructs employing the Effectene transfection reagent as described by the supplier (Qiagen).
Stable expression of wild-type and variants forms of EPCR in 293 cells- 293 cells were transfected as described above. 48 hours post-transfection, the medium was changed to Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 400µg/ml G418 (Gibco BRL). After 2 weeks of drug selection, in which the medium was changed every 3 days, drug-resistant colonies were isolated and the levels of cell-surface EPCR expression were determined by flow cytometric analysis as described below.

Fluorescent labelling of APC- APC labelled at the active site with fluorescein (Fl-APC) was prepared as described by Bock (30, 30). Briefly, 6.5 ml of 0.2 mg/ml APC was incubated with 6-fold molar excess of N^α-[acetlythio)acetyl]-FPR-chloromethylketone (ATA-PPACK) (Molecular Innovations Inc., Royal Oak MI) in 100 mM Hepes pH 7.5, 100 mM NaCl, 1mM EDTA. The labelling reaction was allowed to proceed until the APC was 99% inactive as monitored by the loss of enzymatic activity using Spectrozyme PCa (typically 1 h at room temperature). Excess ATA-PPACK was removed by centrifuging the sample in a molecular weight 10 kDa-cut off Centricon 10 filter (Amicon Inc.) for 15 min at 6,000 x g. All subsequent steps were performed in the dark. 10-fold molar excess of 5-iodoacetamidofluorescein and 1/10 volume of 1 M hydroxylamine ( in 1M Hepes, pH 7.4) was added to the ATA-PPACK-APC and incubated at room temperature for 2 h. Free fluorescein was removed by gel filtration on a PD-10 column (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and the labeled APC was stored at -70°C.

Fluorescent labelling of protein C, JRK1494, JRK 1535, JRK 1500, and JRK1513 monoclonal antibodies- Human protein C, and four monoclonal antibodies against human EPCR (JRK1535, JRK1494, JRK1500, and JRK 1513) were labelled with fluorescein 5-isothiocyanate as described by Goding (31).
Flow cytometric analysis of fluorescein-labelled APC, protein C, JRK 1535, JRK 1494, JRK 1500, and JRK 1513 binding to transfected cells - Adherent transfected cells were harvested by incubation at room temperature for 5 min in phosphate-buffered saline (PBS) (137 mM NaCl, 8 mM Na₂HPO₄•7H₂O, 2.7 mM KCl, 1.5 mM KH₂PO₄) containing 0.02% EDTA. Cells were resuspended in Hank’s balanced salt solution (HBSS) containing 1% bovine serum albumin, 25 mM Hepes pH 7.5, 3 mM CaCl₂, 0.6 mM MgCl₂, and 0.02% sodium azide (binding buffer). Cells (1x10⁵) were incubated at room temperature with 30 nM of Fl-APC or Fl-protein C, or 5µg/ml fluorescein-labelled monoclonal anti-EPCR antibodies for 15 min in the dark. After washing, the cells were resuspended in binding buffer. Bound Fl-APC, Fl-protein C, or fluorescein-labelled was detected on the fluorescence-1 channel on a FACsCalibur (Becton Dickinson). The fluorescence intensity of each sample was analyzed twice.

Triton X-100 lysis of transfected 293T cells - Confluent transfected 293T cells in a 6-well dish were harvested as described above. The cells were lysed in 50 µl of PBS containing 1% Triton X-100 at room temperature for 10 min. The levels of EPCR in the cell lysates were analyzed by electrophoresis and immunoblotting as described below.

Biotinylation of cell surface proteins and precipitation of biotinylated EPCR - Confluent transfected 293T cells in a 6-well dish were harvested as described above and resuspended in 90µl of HBSS. The cells were surface biotinylated with 10µl of 5 mg/ml sulfo-NHS-LC-biotin (Pierce) at room temperature for 10 min. After pelleting, the cells were lysed in 200 µl of PBS containing 1% triton X-100 for 10 min at room temperature. To precipitate the biotinylated EPCR, 20µl of UltraLink Immobilized NeutrAvidin Plus resin (Pierce) was added to the lysate and mixed for 1 h at room temperature. The resin was washed 3 times with 0.5M NaCl, 20 mM Tris-HCl, pH 7.5, 0.5% Triton X-100.
x-100, then washed once with TBS. Cell surface levels of EPCR were analyzed by electrophoresis of the resin and immunoblotting as described below.

Electrophoresis and Immunoblotting- Electrophoresis was performed according to the method of Laemmli (32) using 4-20% SDS polyacrylamide gels. Immunoblotting was performed using JRK 1513 anti-EPCR monoclonal antibody.

Flow Cytometric Analysis of Fl-APC Binding to EPCR Mutants- The affinities of Fl-APC for EPCR mutants expressed on the surface of 293 cells were determined as follows. Briefly, cells were grown to confluency in T-75 flasks, detached with 0.53 mM EDTA, and suspended in 3 ml of complete HBSS buffer (HBSS containing 3 mM CaCl₂, 0.6 mM MgCl₂, 1% bovine serum albumin, and 0.02% NaN₃). The cells were diluted 1:20 in complete HBSS buffer and incubated with increasing concentrations of Fl-APC at 4°C for 15 min in the dark. Binding was analyzed on a FACS Calibur flow cytometer (Becton Dickinson). Values of Kₐ were determined by fitting binding isotherms with a hyperbolic equation using the TableCurve™ program (Jandel Scientific, San Rafael, CA). Previous studies demonstrate that this approach yielded values similar to those obtained with direct radioligand measurement (12).

Molecular Modelling of hEPCR- The hEPCR model was constructed by homology modeling following the method of Greer (33) using the graphics program MAIN (34). The template structure was murine MHC class I H-2Kb (35) (PDB accession 1VAB), with which there was 51 percent amino acid similarity and 22 percent identity. The insertion loops present in EPCR between residues A31-E42, C101-E106, and R156-L161 in EPCR were not modeled and a turn was constructed between R10 and Q15 where MHC-I has a 10 residue insert.

Protein C Activation on Transfected 293 Cells- Stably transfected 293 cells in 24-well plates were
washed 3 times with PBS. The cells were preincubated for 5 min at room temperature with 0.5 ml of HBSS containing 25 mM Hepes pH 7.5, 0.1% BSA, 3 mM CaCl$_2$, and 0.6 mM MgCl$_2$ before the addition of 0.2 µM human protein C or 0.2 µM GDPC. Protein C or GDPC activation was initiated by addition of 10 nM thrombin. In some cases, 0.5 µM of anti-EPCR mAb JRK 1494 was added before protein C and preincubated with the cells for 10 min at room temperature. After 30 min at 37°C, 100µl of the reactions were stopped by the addition of 20µl of 1.66 mg/ml antithrombin containing 20 mM EDTA. 50 µl of the supernatant was transferred into a 96 well microplate and amidolytic activities of APC were determined toward 0.2 mM Spectrozyme PCa substrate in 20 mm Tris-HCl, pH 7.5, 150 mM NaCl. The rates of substrate cleavage were measured in a Vmax microplate reader (Molecular Devices). All determinations were performed in duplicate. Under the conditions employed in this assay, less than 10% of the protein C was activated during the assay as determined by reference to a standard curve of fresh fully activated protein C versus mOD/min.
RESULTS

Expression of EPCR variants in 293 Cells- Human, bovine, and murine EPCR (hEPCR, mEPCR, and bEPCR, respectively) are single-chain transmembrane glycoproteins containing 221, 222, and 225 amino acids, respectively. The amino acid comparisons of human, bovine, and murine EPCR are shown in Figure 1. cDNAs encoding human and mouse EPCR were cloned into the eukaryotic expression vector pcDNA3.1(-) and expressed in 293 cells. The apparent molecular mass of wild-type hEPCR, as determined by SDS-PAGE and immunoblot analysis (~46 kDa), is approximately twice that of its predicted molecular mass (~24 kDa), consistent with the presence of carbohydrate moieties on EPCR (Figure 2). Glutamine substitutions at the four N-glycosylation consensus sites (N30, N47, N119, N155), result in EPCR variants with decreased molecular masses (Figure 2). The electrophoretic mobility of the mutant proteins increases proportionally with the number of N-glycosylation sites ablated. In contrast, hEPCR variant L1, which contains alanine substitutions at five residues (S71, Q75, T145, R156, E163), exhibits the same electrophoretic mobility as wild-type hEPCR (Figure 2).

Selection of amino acid residues on EPCR for mutagenesis- Human, bovine, and murine EPCR all bind saturably and in a Ca^{2+}-dependent manner to Fl-APC (28). Figure 1 shows the locations of the residues in the extracellular domain of EPCR that we selected for individual or multiple mutations to alanine. Most of the alanine substitutions were directed at conserved residues, but some were also directed at non-conserved regions of EPCR.

Interaction of EPCR alanine mutants with Fl-APC and fluorescein-labelled anti-hEPCR monoclonal antibodies- The affinities of EPCR variants for Fl-APC were first qualitatively assessed by flow cytometric analysis. The cDNAs of the variants were transiently transfected into 293 cells,
and Fl-APC binding was monitored on a FACs Calibur flow cytometer. The analyses were performed in the presence of 30 nM Fl-APC, which is the dissociation constant for the interaction of hEPCR with APC (36). The ligand binding properties of the cell surface EPCR variants were compared to 293 cells transfected with hEPCR (positive control) and pcDNA3.1(-) vector (negative control). Western blotting analysis of whole cell lysates and cell surface proteins was performed in parallel to qualitatively monitor the EPCR antigen levels of the variants.

The transiently transfected cells were also screened for the ability to bind FITC-labelled anti-human EPCR monoclonal antibodies. Our laboratory has raised a panel of anti-human EPCR monoclonal antibodies, four of which are used in this study. These antibodies recognize human EPCR but not its mouse counterpart. As shown in Figure 3, JRK 1494 and JRK 1535 mAbs block hEPCR/Fl-APC interactions whereas JRK 1500 and JRK 1535 mAbs do not. As expected, protein C also blocks hEPCR/Fl-APC interactions, consistent with previous studies demonstrating that EPCR binds to the Gla domain of both protein C and APC (13). The effect of these four mAbs on the interaction between hEPCR and Fl-protein C is identical to that observed on the interaction between hEPCR and Fl-APC (data not shown).

The results of the flow cytometric analyses and Western blotting analyses are summarized in Figure 1. Residues enclosed in green circles are those that, when mutated to alanine, do not affect the binding of Fl-APC to the transfected cells. These residues include the four N-linked carbohydrate attachment sites at N30, N47, N119, and N115, suggesting that the carbohydrate moieties of EPCR are not critical for protein C/APC interactions. The ten residues marked with red asterisks (R81, L82, V83, E86, R87, F146, Y154, T157, R158, and E160) are those that when mutated to alanine, result in cell surface expression but no detectable Fl-APC binding, suggesting
that these residues are involved in EPCR-APC interactions. As expected, these ten mutants do not bind to Fl-protein C. Residues enclosed in blue circles are those that, when mutated to alanine, result in the loss of detectable intracellular and cell surface expression, suggesting that these residues are critical for secondary structure integrity of EPCR. Figure 1 also shows residues that, when mutated to alanine, result in the loss of binding to FITC-labelled anti-EPCR monoclonal antibodies (denoted by arrows).

_Determination of the affinities of EPCR variants for Fl-APC_- As mentioned above, the initial flow cytometric characterization of the variants was performed in the presence of 30 nM Fl-APC. Thus, to determine qualitatively the affinities of EPCR variants for Fl-APC, stably transfected 293 cells were generated. The affinities of eleven EPCR variants for Fl-APC were determined by monitoring the changes in cell fluorescence during Fl-APC titration. These variants were chosen to represent a wide range of ligand binding properties. Binding was analyzed by flow cytometry and $K_d$ values were determined by fitting binding isotherms to a hyperbolic equation. To correct for non-specific binding, 293 cells transfected with pcDNA3.1(-) vector alone were utilized.

As shown in Table 1, hEPCR, mEPCR, and clone 64-3 (a hEPCR-mEPCR chimera) all have similar binding affinities to Fl-APC ($K_d=31 \pm 28$, 46 $\pm$ 15, and 51 $\pm$ 22 nM, respectively). Alanine substitutions that result in the removal of the epitope for JRK 1500 do not influence the binding of hEPCR to Fl-APC ($K_d=55 \pm 11$ nM). Alanine substitution of 5 residues in the groove of hEPCR (clone L1) or removal of two N-linked carbohydrate attachment sites (clone A/D sugar) result in a modest decrease in binding affinity to Fl-APC ($K_d=161 \pm 52$ and 87 $\pm$ 4 nM, respectively). In contrast, the mutations E86A, R87A, F146A, Y154A, and R158A, all of which result in variants that do not bind Fl-APC as demonstrated in the initial qualitative screens (Figure 1), decreased the
affinity of hEPCR for Fl-APC greater than 30-fold ($K_d$ values of $\geq 1000$ nM).

*Mutagenesis to map monoclonal antibody epitopes on hEPCR*- A potential weakness of alanine substitution mutagenesis is that lack of ligand recognition may be a consequence of destabilizations in tertiary structure, or loop conformations of the native molecule rather than in the removal of ligand-binding residues. We thus aimed to merge the above loss-of-function data with data obtained from gain-of-function studies. Since our anti-hEPCR mAbs do not recognize mEPCR, we generated human/mouse chimeric proteins to delineate the mAb epitopes. The overall goal is to see if the epitopes for the blocking mAbs co-localize with the 10 residues that, when mutated to alanine, lose Fl-APC binding ability. If so, this would increase our confidence in the definitive assignment of a role for R81, L82, V83, E86, R87, F146, Y154, T157, R158, and E160 in protein C/APC binding.

The cDNAs of five human-mouse chimeras were transfected into 293 cells and ligand binding was monitored by flow cytometry. Figure 4A shows the results of this epitope-mapping strategy. The epitope for JRK 1494, a blocking mAb, is localized to hEPCR (W26-V116). Consistent with this map, mutation of R81 to alanine abolishes Fl-JRK 1494 and Fl-APC binding, but does not affect the binding of the other three mAbs (Figure 1). Residues V25 to L52 contains the epitope for JRK 1513, a non-blocking mAb. Mutations of L37, T38, and H39 to alanine abolishes Fl-JRK 1513 binding but does not affect the binding of Fl-APC nor the other three mAbs (Figure 1). The C-terminal half of hEPCR (F113 to C222) contains the epitopes for both JRK 1500 (non-blocking) and JRK 1535 (blocking). The epitope for JRK 1500 likely included residues R127, E129, and R130 since clone 64-3 (R127A, E129A, R130A) does not bind Fl-JRK 1500 but does bind to Fl-APC as well as to the other three mAbs (Figure 1).

Figure 4B summarizes the results of the loss-of-function and gain-of-function studies. Five
of the ten candidate residues for protein C/APC binding (R81, L82, V83, E86, R87) co-localize with
the epitope for the blocking mAb JRK 1494. In contrast, the epitopes for JRK 1513 and JRK 1500,
both of which are non-blocking mAbs, do not co-localize with any of the 10 candidate residues for
APC/protein C binding. Western blot analysis revealed that hEPCR is immunoreactive only with
JRK 1513 mAb, suggesting that JRK 1494, JRK 1500, and JRK 1535 mAbs recognize
conformation-dependent epitopes (data not shown).

Figure 5 shows a 3-dimensional ribbon model of the extracellular domain of human EPCR
built using mouse CD1 as a structural template. The domain consists of an eight-stranded
antiparallel ß-pleated sheet with two antiparallel α-helices (helix 1= residues 58 to 83, helix 2=
residues137 to 179). In the left panel, areas shown in green are residues that can be mutated to
alanine without loss of Fl-APC binding. Regions in blue, when mutated, result in loss of cell surface
expression and hence are likely mutations that influence protein folding. Regions in red are the
amino acid side chains of seven of the ten protein C-APC binding candidate residues. T157, R158,
and E159 are not shown since they reside in a region of EPCR that was not modeled due to lack of
homology to mouse CD1. The right panel shows the epitopes for JRK 1494 (red), JRK 1535 (red),
JRK 1513 (white), and JRK 1500 (pink). Based on our 3-dimensional molecular model, the
candidate residues for protein C/APC binding are located at the distal end of the two α-helical
segments that form the putative ligand binding groove.

Influence of EPCR variants on protein C activation rates- Previous studies demonstrated that
binding of protein C to EPCR enhances the rate of protein C activation by the thrombin-TM complex
(14-16). In this study, we co-expressed EPCR variants and TM in 293 cells to confirm the
requirement for protein C binding in the EPCR-dependent enhancement in protein C activation. The
following cell lines were used in these studies: (a) 293 cells, (b) 293 cells stably transfected with TM cDNA, (c) 293 cells stably transfected with both TM and hEPCR, (d) 293 cells stably transfected with both TM and E86A hEPCR, and (e) 293 cells transfected with both TM and A/D sugar hEPCR. Protein C activation was performed on the cell surface in the absence or presence of JRK 1494, a mAb that blocks protein C binding to EPCR. We also determined the activation rates of gla-domainless protein C, which is a derivative of protein C that cannot bind to EPCR, but in solution is activated by the thrombin-TM complex at the same rate as full-length protein C (37). As shown in Figure 6, the levels of protein C and GDPC activation on 293 cells is low, and protein C activation is not influenced by pre-incubation with JRK 1494. In 293 cells expressing either TM alone or TM and EPCR variants, the activation rate of GDPC is approximately 7-fold higher than that observed in 293 cells. This rate is relatively constant between the four TM-expressing cell lines as is to be expected since the cell lines express similar levels of TM as measured by $^{125}$I-radiolabelled CTM 1009 anti-human TM Fab fragments (Figure 5, bottom of graph). Interestingly, addition of JRK 1494 to 293 cells expressing TM alone inhibited protein C activation rates approximately two-fold. A likely explanation for this observation is that transfection of 293 cells with TM cDNA also increases the levels of cell surface EPCR. Indeed, cell surface EPCR antigen is higher in TM-expressing cells compared with 293 cells as measured by $^{125}$I-radiolabelled JRK 1535 anti-human EPCR Fab fragments (Figure 6, bottom of graph), and by flow cytometry using fluorescently-labelled JRK 1500 and JRK 1535 mAbs (data not shown).

In the presence hEPCR and A/D sugar hEPCR, the protein C activation rate by the thrombin-TM complex is increased by 12.1-fold and 10.5-fold, respectively (Figure 6). Preincubation with JRK 1494 blocked protein C activation rates to near that observed with 293 cells transfected with
TM cDNA alone. In contrast, even at an EPCR:TM ratio of 8:1, E86A hEPCR does not augment protein C activation by the thrombin-TM complex on the cell surface. Taken together, these studies confirm that binding of protein C to EPCR is necessary for the EPCR-dependent enhancement in protein C activation by the thrombin-TM complex.

**DISCUSSION**

Although EPCR has been implicated as an important regulatory protein of coagulation and inflammation, until now no information was available related to the critical contact sites between EPCR and protein C/APC. In this study, we have identified 10 amino acids (R81, L82, V83, E86, R87, F146, Y154, T157, R158, and E160) that, when individually mutated to alanine residues, result in EPCR variants that lose protein C/APC binding ability. We believe that these alanine substitutions reflect the removal of ligand-binding residues, rather than the disruption of structural integrity of EPCR for the following reasons. First, using human-mouse chimeric EPCR constructs, half of the point mutations responsible for loss of protein C/APC binding were mapped to the epitope responsible for binding one of the inhibitory antibodies (Figure 4). Second, each of the ten EPCR variants was screened for the ability to bind four anti-EPCR mAbs, three of which recognize conformation-dependent epitopes (Figure 1). All of these EPCR variants retain the ability to bind the anti-EPCR mAbs, suggesting that the mutations have not perturbed the 3-dimensional conformation of EPCR. The only exception is hEPCR R81A, which does not bind to JRK 1494, suggesting that alanine substitution of R81 removes both protein C/APC and JRK 1494 binding. Third, molecular modelling of EPCR indicates that the 10 candidate residues are clustered in the distal end of the two α-helical segments of EPCR.

This study has also identified several residues in EPCR that, when mutated, result in the loss
of intracellular and cell surface expression (Figure 1). Given the importance of the protein C pathway and the roles that EPCR plays in this pathway, it follows that mutations in EPCR that impair protein C/APC binding or impair EPCR expression would likely increase thrombotic risk. This assumption appears to be supported by preliminary clinical studies by Merati G. et al.(38), in which an EPCR loss-of-function mutation has been identified that is more prevalent in patients with deep vein thrombosis compared to controls.

We have also provided evidence of glycosylation at all four N-glycosylation consensus sites of hEPCR (N30, N47, N119, and N155). The glycosylation contributes to nearly half of the apparent molecular mass of the molecule. Human EPCR variants containing mutations in the carbohydrate attachment sites exhibited decreased molecular masses compared with wild-type EPCR (Figure 2). These findings are consistent with previous studies in our laboratory showing that treatment of hEPCR with endoglycosidase F/Peptide-N-glycosidase reduces the apparent molecular mass from 46 kDa to 28.5 kDa on SDS-PAGE (36). Since glutamine substitutions at the glycosylation sites do not affect Fl-APC nor Fl-protein C binding significantly (Figure 1), it suggests that the carbohydrate moieties of EPCR are not critical for APC/protein C recognition. Instead, the N-glycosylation of hEPCR may contribute to the protection of EPCR from proteolytic degradation or may serve as specific recognition domains for other, as yet unidentified, ligands.

Our 3-dimensional molecular model of hEPCR was constructed by homology modeling using the murine MHC class 1 H-2Kb as the structural template (35). The model suggests that hEPCR is folded into a ligand binding groove composed of two anti-parallel α-helices that sit upon an eight-stranded β-sheet platform (Figure 5), a fold that is characteristic of members of the MHC class1/CD1 family of receptors. The overall structure of our hEPCR model is similar to that developed by
Villouteix et al. (39) using the X-ray structure of mouse CD1 as template.

MHC class 1 molecules bind to octamer and nonamer peptides (35, 40), whereas CD1 molecules recognize lipids and glycolipids (reviewed in (41)). In the case of MHC class 1 receptors, peptide ligands are tethered by hydrogen bonds between backbone atoms of the peptide and side chain residues in the α-helices that line the groove (42). These interactions are further supplemented by contacts of polymorphic MHC groove side chains with a few “anchor” side chains in the peptide (40). The anchor residues of the peptide occupy depressions within the MHC groove. In contrast, the interaction between CD1 molecules and lipids involves extensive hydrophobic interactions in deeply buried depressions within the CD1 groove (43).

Unlike conventional MHC class 1/CD1 proteins, EPCR lacks the α-3 domain and its ligands are large proteins rather than short peptides or lipids. The results of this study indicate that the APC/protein C binding region of EPCR is located in the distal end of the two α-helical segments that form the putative binding groove. To our knowledge, this is the first report of such a binding motif for a member of the MHC class 1/CD1 family of molecules. There are other examples of ligand binding versatility of the MHC class 1/CD1 fold. For example, the neonatal Fc receptor has a proline residue in the α2 helix which produces a kink in the α2 helix, resulting in a closed groove (24). Although the peptide-binding groove is lost, the neonatal Fc receptor has evolved to recognize the Fc portion of immunoglobulins on the side of the Fc receptor. In the case of MIC-A, a stress-inducible antigen restricted to gut epithelium, the peptide-binding groove is absent due to disordering in one of the groove-defining helices (44). Potential receptor interaction surfaces are on the “underside” of the β-sheet platform.

The present study is the first to identify the protein C/APC binding region of EPCR. This
information may provide a framework to help guide interpretation of future genetic screening studies. This work also suggests that EPCR has exploited the MHC class 1 fold for an alternative and possibly novel mode of ligand recognition.
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**FOOTNOTES**

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1The abbreviations used are: TM, thrombomodulin; activated protein C, APC; endothelial cell protein C receptor, EPCR; gla-domainless protein C, GDPC; Fl-APC, APC labelled at the active site with fluorescein; ATA-PPACK, Nα-[(acetylthio)acetyl]-FPR-chloromethyl ketone.

**ACKNOWLEDGEMENTS**

We thank Dr. Naomi L. Esmon for critical reading of the manuscript and for many helpful discussions. We are grateful to Nici Barnard in the preparation of this manuscript.
FIGURE LEGENDS

FIG. 1. Amino acid sequence comparisons of human, bovine, and murine EPCR. Residues enclosed in green circles are those that, when mutated to alanine, retain APC binding activity. Residues enclosed in blue circles are those that, when mutated to alanine, result in the loss of all mAb epitopes as well as APC binding, and hence, appear to have global effects on EPCR conformation. The ten residues marked with red asterisks are those that when mutated to alanine, resulted in loss of APC binding but retain mAb epitopes. The only exception is R81 which resulted in the loss of APC as well as JRK 1494 binding. The seven residues marked with arrows are alanine substitutions that result in the loss of either the JRK 1513 epitope (L37, T38, H39), or the JRK 1494 epitope (R81), or the JRK 1500 epitope (R129, E130, R131).

FIG. 2. Immunoblot analysis of cell surface proteins of 293 cells transfected with hEPCR variants. 293 cells were transiently transfected with cDNAs encoding wildtype hEPCR (WT), hEPCR mutant L1, hEPCR mutant N47Q, hEPCR mutant N119Q, and hEPCR mutant N30Q/N155Q. 48 hrs post transfection, the cells were harvested and cell surface proteins were isolated as described under “Experimental Procedures”. The proteins were subjected to electrophoresis in a 4-20% SDS-polyacrylamide gel under non-reducing conditions and transferred to nitrocellulose. The immunoblot was probed with JRK 1513, a monoclonal antibody against hEPCR. Molecular weight standards are on the left as indicated.

FIG. 3. Flow cytometric analysis of Fl-APC binding to hEPCR in the presence of protein C and anti-hEPCR mAbs. 293 cells stably expressing hEPCR were pre-incubated at room
temperature with 500 nM of protein C, JRK 1494, JRK 1535, JRK 1500, or JRK 1513 in the presence of 3 mM CaCl₂ and 0.6 mM MgCl₂. After 15 min, 30 nM of Fl-APC was added to the cells and incubated at room temperature for an additional 15 min. Binding of Fl-APC to the cells was analyzed by flow cytometry.

FIG.4. **Gain-of-function mutagenesis to map mAb epitopes on hEPCR.** *A*, Schematic representation of human-mouse EPCR chimeras. The amino acid residues at the junction between the human and mouse cDNAs are shown. The cDNAs of hEPCR, mEPCR, and the six human-mouse EPCR chimeras were transfected into 293 cells and binding to Fl-APC and FITC-labelled mAbs was monitored by flow cytometry as described under “Experimental Procedures”. The symbols “+” and “−” designates binding and lack of binding to the ligands, respectively. *B*, Summary of the results of the loss-of-function alanine mutagenesis studies and the gain-of-function epitope mapping studies. The 10 candidate residues for protein C/APC binding are shown in the top schematic diagram. The epitopes for the four mAbs are shown below.

FIG. 5. **Molecular model of hEPCR based on the murine MHC class 1 H-2Kb structure.**

*Left panel*, Regions in red are the amino acid side chains of seven of the ten protein C/APC binding candidate residues. T157, R158, and E159 are not shown since they reside in a region of EPCR that was not modeled due to lack of homology to mouse MHC class 1 H-2Kb. Areas shown in green, many of which are in the groove region, could be mutated to alanine without loss of APC binding. Regions in blue, when mutated, result in loss of cell surface expression and hence are likely mutations that influence protein folding. *Right panel*, the epitopes for the non-
blocking antibodies are shown in white and pink (JRK 1513 and JRK 1500, respectively) and the epitopes for the blocking antibodies are shown in red (JRK 1494 and JRK 1535). Black line, with competitor; grey area, without competitor.

FIG. 6. **Protein C activation of 293 cells expressing EPCR variants and TM.** Protein C activation was performed on confluent 293 cells and on confluent 293 cells expressing (a) TM and (b) TM and EPCR variants as described under “Experimental Procedures”. Grey bars, activation rate of protein C; black bars, activation rate of protein C in the presence of 500 nM inhibitory anti-hEPCR mAb JRK 1494; white bars, activation rate of GDPC. The bars represent the mean, while the lines above the bars reflect the standard error of the mean of at least two determinations. The number of EPCR and TM molecules expressed per cell surface are indicated on the figure, as are the EPCR: TM ratio of each cell line.
TABLE 1
Dissociation Constants for the Interaction of Fl-APC with EPCR variants expressed in 293 cells

The affinities of Fl-APC for EPCR variants were measured by flow cytometry as described under “Experimental Procedures”. The experiments were performed in HBS containing 3 mM CaCl₂ and 0.6 mM MgCl₂. The values correspond to the mean and the standard error of the mean of the mean of at least two determinations.

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<thead>
<tr>
<th>Name of Clone</th>
<th>Mutation</th>
<th>Properties</th>
<th>Kd (nM)</th>
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<td>hEPCR</td>
<td>None</td>
<td>Human EPCR</td>
<td>31 ± 28</td>
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<tr>
<td>mEPCR</td>
<td>None</td>
<td>Mouse EPCR</td>
<td>46 ± 15</td>
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<tr>
<td>64-3</td>
<td>Human (1-116), mouse (113-225)</td>
<td>Human-mouse chimera</td>
<td>51 ± 22</td>
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<tr>
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<td>R127A, E129A, R130A in hEPCR</td>
<td>Does not bind JRK 1500</td>
<td>55 ± 11</td>
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<td>A/D sugar</td>
<td>N30Q, N155Q in hEPCR</td>
<td>Lacks two N-linked carbohydrate attachment sites</td>
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<tr>
<td>L-1</td>
<td>T145A, R156A, E163A, S71A, Q75A in hEPCR</td>
<td>Contains 5 point mutations in the groove regions</td>
<td>161 ± 52</td>
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<td>E86A</td>
<td>E86A in hEPCR</td>
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<td>R87A</td>
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Liaw et al., Figure 2
Liaw Figure 3
A.

Liaw et al., Figure 4A

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**Figure 4A**

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**Annotations**

- **FL-APC**: 1494 1535 1500 1513
- **V116/F113**: (mutations)
- **V80/K80**: (mutations)
- **V25/W26**: (mutations)
- **V116/F113**: (mutations)
- **V144/N149**: (mutations)
- **R127A, E129A, R130A**: (mutations)
B.

Liaw et al., Figure 4B

```
N
R  L  V  E  R
81  82  83  86  87

C
F  Y  T  R  E
146 154 157 158 159

hEPCR

JRK 1513 (non-blocking)

V25  L52

JRK 1500 (non-blocking)

R127  R130

JRK 1494 (blocking)

W26  V116

JRK 1535 (blocking)

V116  V144
```
Liaw et al., Figure 5
Liaw et al. Figure 6

![Graph showing APC activity with different conditions.

<table>
<thead>
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<th># of TM/Cell</th>
<th>EPCR:TM Ratio</th>
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<td>293</td>
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<td>7.8 x10^4</td>
<td>0.4 : 1</td>
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<tr>
<td>TM only</td>
<td>6.4 x10^5</td>
<td>7.6 x10^4</td>
<td>8.4 : 1</td>
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<tr>
<td>91E/TM</td>
<td>1.0 x10^6</td>
<td>1.2 x10^5</td>
<td>8.3 : 1</td>
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<td>WT hEPCR/TM</td>
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<tr>
<td>AD sugar/TM</td>
<td>3.81 x10^5</td>
<td>9.2 x10^4</td>
<td>4.1 : 1</td>
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Identification of the protein C/activated protein C binding sites on the endothelial cell protein C receptor: Implications for a novel mode of ligand recognition by an Mhc class I-type receptor
Patricia C.Y. Liaw, Timothy Mather, Natalia Oganesyan, Gary L. Ferrell and Charles T. Esmon

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