Expression of the endothelial cell protein C receptor (EPCR) gene in mammalian cells imparts the capacity to bind activated protein C (APC) or protein C. Immunological analysis of CCD41, apparently the murine homologue of EPCR, suggested centrosomal localization, raising questions about the location of the EPCR gene product and its role in protein C binding. In this study, we express a soluble form of EPCR, demonstrate EPCR expression on the cell surface, and direct binding between soluble EPCR and protein C/APC. Affinity purified polyclonal and a monoclonal antibody against EPCR bound to the cell surface of EPCR-transfected cells but not to control cells. A 49-kDa protein, a mass similar to soluble EPCR, was immunoprecipitated from the cell surface of endothelium and cells transfected with human EPCR but not from control cells. The FLAG epitope and APC bound to cells expressing an EPCR construct containing the FLAG epitope located in a putative extracellular domain, whereas an EPCR construct truncated just before the putative transmembrane domain produced only soluble EPCR antigen. Soluble EPCR inhibited APC binding to EPCR expressing cells in a concentration-dependent fashion, KD(app) = 29 nM and bound to immobilized protein C in a Ca2+-dependent fashion. Thus, EPCR is a type 1 transmembrane protein that binds directly to APC.

The protein C anticoagulant pathway is an indispensable, on demand, regulatory mechanism of blood coagulation (1). Thrombin is generated by the coagulation cascade and catalyzes the formation of fibrin and also promotes the activation of many cell types, including platelets (2). Thrombin also binds to the endothelial cell surface receptor, thrombomodulin. After binding to thrombomodulin, thrombin changes enzyme specificity and catalyzes the conversion of the plasma vitamin K-dependent zymogen, protein C, to the anticoagulant serine protease, activated protein C (APC) (3). APC is the terminal enzyme of the protein C pathway and catalyzes the proteolytic inactivation of the coagulation cofactors, factors Va and VIIIa (4). Defects in this pathway are associated with thrombophilia (4, 5).

In addition to modulating the coagulant response, this pathway appears to modulate the inflammatory response. In vivo, APC administration prevents death and organ dysfunction in baboons challenged with lethal numbers of Escherichia coli (6). Humans suffering from severe meningococcemia and an associated acquired protein C deficiency have been reported to exhibit rapid systemic improvement following protein C administration (7, 8). In vitro, APC has been shown to inhibit tumor necrosis factor elaboration by monocytes (9), and protein C has been reported to inhibit leukocyte adhesion to selectins (10). The exact mechanisms by which the protein C pathway modulates inflammatory responses remains obscure.

As one approach, we initiated studies to identify new members of the pathway. Recently, we (11) and others (12) observed specific protein C and APC binding to cultured human umbilical vein endothelium (HUVEC). Binding was Ca2+-dependent and required the presence of the Gla domain of protein C (11, 12).

To gain initial information on the nature of the protein C binding site, we used an expression cloning strategy to isolate a cDNA, which, when expressed in mammalian cells, elicits the formation of specific protein C binding sites with properties similar to those of endothelium (11). A survey of cultured cells indicated that message and binding function was endothelial cell-specific, and therefore the protein was designated endothelial cell protein C/APC receptor (EPCR). Cloning of murine and bovine EPCR demonstrated conserved cellular specificity, structure, and regulation, including down-regulation by tumor necrosis factor (13). The sequence of EPCR predicts it to be a type 1 transmembrane glycoprotein (11), and the functional data would be consistent with EPCR binding directly to protein C.

The unexpected observation that EPCR is homologous, and probably identical, to a previously described intracellular murine protein, CCD41 (14), raised questions about this model of
EPCR function. The CCD41, or centrocyclin, cDNA clone was originally isolated from Ehrlich ascites tumor cells (14). Immunocytochemical analysis using antibodies against a bacterially expressed CCD41 fusion protein revealed nuclear localization and centrosome association of the CCD41 gene product (14). The nucleotide sequence of murine EPCR contains only five nucleotide differences from that of CCD41, and these differences occur in regions of the cDNA that are technically difficult to sequence, suggesting that the differences are due to cloning or sequencing errors (13). If murine EPCR and CCD41 are identical molecules, then the cellular localization studies described above suggest that the EPCR gene product is not a cell surface molecule and would suggest that EPCR elicits protein C binding sites indirectly by induction of another protein that serves as the cell surface receptor for protein C.

In this study, we demonstrate that human EPCR is expressed on the surface of endothelium and cells transfected with the EPCR cDNA and that a soluble form of EPCR can bind APC directly.

**EXPERIMENTAL PROCEDURES**

Cell Culture—All human cell lines were maintained as described previously (11). HUVEC were kindly provided by Dr. Craig Carson. Stable transformants expressing EPCR were established as follows. The human EPCR cDNA construct in the mammalian expression vector pEF-BOS (15), was co-transfected into human kidney 293 cells (ATCC CRL 1573) with another plasmid, pBK-CMV (STRATAGENE), carrying the neomycin-resistance gene. After G418 selection, EPCR-negative and -positive clones were isolated from cells transfected with the same expression vector but without the insert. EPCR-negative and -positive clones of murine NIH3T3 were also generated and selected as described above.

Expression and Purification of the Soluble EPCR Fusion Protein—A cDNA fragment coding for a soluble EPCR protein, starting from residue 16, corresponding to a potential signal peptidase cleavage site (11), and truncated immediately above the putative transmembrane spanning domain at residue 210, was amplified from a human EPCR cDNA clone by the polymerase chain reaction (PCR) and ligated into the mammalian expression vector pCDM8. This construct codes for an EPCR fusion protein containing the transferrin signal sequence followed by the HPC4 epitope at the amino terminus (16, 18). Transfection, selection, and affinity purification with HPC4 antibody were as described (16). In some experiments, the s-HPC4-EPFR was further purified by Mono-Q FPLC column chromatography. The accuracy of all EPCR constructs generated in this study were confirmed by sequencing (17).

An alternative soluble fusion protein with the HPC4 epitope site on the carboxyl-terminal of the protein was prepared by PCR methods essentially as described above. The resultant construct consisted of the native signal peptide, the EPCR coding region truncated to delete the transmembrane and cytosolic tail, a factor Xa cleavage site, and the HPC4 epitope. This construct was ligated to the pGt-H expression vector (a kind gift from Drs. Brian Grinnell and David Berg) to allow hybridoma selection in human 293 cells (19) and the resultant fusion protein isolated as described above, except that 0.6 mM MgCl2 was included in wash buffers.

A soluble form of EPCR was also expressed in E. coli using the ThioFusion™ Expression system (Invitrogen). The cloning strategy was essentially as described above, except that 0.6 mM MgCl2 was included in wash buffers.

**Fig. 1. SDS-PAGE analysis of soluble EPCR.** s-HPC4-EPCR (2.5 μg) before (A) or after (B) mononQ chromatography and s-EPCR-HPC4 (2.5 μg) (C) were analyzed by SDS-PAGE on 10% polyacrylamide gels without reduction. Samples of these preparations in the order above were run after reduction (D-F). Chromatography was performed on a 0.5 x 5.2-cm MonoQ column in TBS with a 20-ml linear gradient from 0.1 to 1.0 mM NaCl. The s-HPC4-EPCR monomer was found in the breakthrough.

**Fig. 2. Effects of magnesium ions on Fl-APC binding to EPCR-positive cells.** A, E-7 cells (upper panel) and N-1 cells (lower panel) were incubated with 160 nM of Fl-APC in the presence of 1.3 mM calcium alone (solid lines) or with 1.3 mM calcium and 0.6 mM magnesium in 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.5 (bold solid lines). Dotted lines show Fl-APC binding in the presence of EDTA instead of divalent cations. B, E-7 cells were incubated with the indicated concentrations of Fl-APC in the binding buffer containing 1.3 mM calcium without (open circles) or with 0.6 mM magnesium (closed circles). As negative controls, the same experiments were carried out in the same buffer containing EDTA (closed squares), or a single point at 160 nM Fl-APC was not above the EDTA control at Mg2+ concentrations ranging from 0.1 to 5 mM Mg2+.
to insert the coding sequence of the His tag at the 3'-end of the molecule before the stop codon and a factor Xa cleavage site (IEGR) immediately before the stop codon and a factor Xa cleavage site (IEGR) immediately after PCR amplification of EPCR cDNA with appropriate primers, the PCR product was digested by EcoRV and Sall (these sites were included at the 5'- and 3'-ends, respectively) and was subcloned into the Smal and Sall sites of the pTrxFus expression vector. The insert codes for a fusion protein starting with thioredoxin followed by soluble EPCR, a factor Xa cleavage site, and the Histag (TFT201His). The fusion protein was expressed in GI724 cells and purified from the cell lysate by column chromatography on a TALON™ metal affinity column (Clontech) by elution with 0.3 M imidazole according to the manufacturer's protocol.

Construction and Expression of Recombinant EPCR Molecules Containing the FLAG Epitope—Several EPCR constructs were generated by the PCR mutagenesis method in which the FLAG epitope was fused at different sites of the EPCR cDNA sequence. The DNA sequence coding for the FLAG epitope (DYKDDDK) was included at the 3'-end of the PCR primers. The first construct, mFL1, contains the FLAG epitope that replaces eight amino acid residues of EPCR between residue 198 and 206 (see Fig. 6 below for a schematic of the FLAG mutants described in this section). In the second full-length mutant (mFL2), the sequence between 146 and 153 was replaced by the FLAG epitope by the same methods. In the third construct (smFL3), the sequence between 211 and 218 corresponding to the amino-terminal sequence of the putative transmembrane domain was replaced with the FLAG epitope sequence followed by a stop codon. The PCR products were digested with XhoI and NotI (included at the 5'- and 3'-ends of primers, respectively) and were subcloned into a mammalian expression pEF-BOS vector (15). The constructs were transfected into 293T cells by the calcium phosphate method. After 48 h, the cells were analyzed by flow cytometry using M2 (an anti-FLAG monoclonal antibody, Eastman Kodak Co.) staining. Fl-APC binding to the transfectants was also detected using fluorescein isothiocyanate-labeled goat anti-mouse Ig (Becton Dickinson) as the second antibody at a 1:100 dilution. Antibodies were purified from ascites by ammonium sulfate precipitation, QAE-Sephadex chromatography, and gel filtration as described (6). Antibodies were biotinylated with NHS-LC-Biotin (Pierce) according to the manufacturer's protocol.

EPCR Expression and Function

EPCR, the molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the extinction coefficient was calculated based on the predicted amino acid composition.

Preparation of Antibodies—The preimmune serum was collected from a goat. The goat was immunized subsequently with 0.5 mg of the purified s-HPC4-EPCR in Freund's complete adjuvant followed by 0.25 mg of s-HPC4-EPCR in Freund's incomplete adjuvant. The serum was collected weekly after the second immunization. Polyclonal immunoglobulin was prepared by standard methods including 50% ammonium sulfate precipitation, dialysis, and ion-exchange chromatography on DE52 (Whatman). For some experiments, the ammonium sulfate precipitated EPCR was resuspended in TBS (20 mM Tris, 150 mM NaCl, 0.02% sodium azide, pH 7.5), gel-filtered and affinity-purified on bacterially expressed EPCR (TFT210H15, 5 mg), adsorbed to a 1.5-×-3-cm TALON™ column equilibrated in 6 M guanidine HCl, 20 mM Tris-HCl, pH 8.0. The column was washed extensively with the same buffer, and bound immunoglobulin (11 mg) was eluted with 6 M guanidine HCl, 20 mM Tris-HCl, pH 8.0, and subsequently dialyzed against TBS.

Monoclonal antibodies against s-HPC4-EPCR were obtained as described previously for other proteins (20). The hybridoma supernatants were screened for the ability to bind to cell surface EPCR by fluorescence-activated cell sorter (FACS) analysis of cell surface staining using the EPCR-negative and -positive stably transfected cell lines. Monoclonal antibody binding was detected using fluorescein isothiocyanate-labeled goat anti-mouse Ig (Becton Dickinson) as the second antibody at a 1:100 dilution. Antibodies were purified from ascites by ammonium sulfate precipitation, QAE-Sephadex chromatography, and gel filtration as described (6). Antibodies were biotinylated with NHS-LC-Biotin (Pierce) according to the manufacturer's protocol.

Flow Cytometric Analysis of Cell Surface Antibody Binding—EPCR-negative (N-1) and -positive (E-7) stably transfected 293T cells were incubated at 4 °C for 30 min with various concentrations of the affinity purified goat anti-s-HPC4-EPCR antibody in 138 mM NaCl, 2.7 mM KCl,
8.1 mM Na2HPO4, pH 7.1 (PBS) containing 5% fetal bovine serum and 10 mM EDTA). The bound antibody was stained at 4 °C for 30 min with fluorescein-labeled affinity purified rabbit anti-goat IgG (Kirkegaard & Perry Laboratories, Inc.). FACS analysis was carried out as described previously (11) using a FACSCalibur (Becton Dickinson).

Inhibition of Fl-APC Binding with s-EPCR-HPC4—The binding of Fl-APC to E-7 cells was performed essentially as described (11). Fl-APC (80 nM) was incubated for 30 min at room temperature with s-EPCR-HPC4 (25–1000 nM) in Hank’s balanced salt solution (HBSS) that was supplemented with 3 mM CaCl2, 0.6 mM MgCl2, 1 mg/ml bovine serum albumin, and 0.02% NaN3. The mixtures were added to E-7 cells and incubated for 30 min on ice. The samples were washed with the same buffer, centrifuged, and cell-bound fluorescence was determined on a FACSCalibur. Nonspecific binding of Fl-APC (0–80 nM) was determined in the presence of 3.44 mM protein C and was subtracted from the total bound fluorescence. Assuming the decrease in mean channel fluorescence was due to s-EPCR-HPC4 binding to Fl-APC and a resultant decrease in free Fl-APC concentration, the free Fl-APC concentration was calculated by reference to the standard curve of Fl-APC concentration versus mean channel fluorescence. The concentrations of the s-EPCR-HPC4-Fl-APC complex were obtained by subtracting the free Fl-APC concentrations from total Fl-APC (80 nM). Free s-EPCR-HPC4 concentrations were calculated by subtracting the complex concentrations from added s-EPCR-HPC4 concentrations. These values were used to calculate Kd (app) at each concentration of s-EPCR-HPC4 added.

Western Blotting—Samples were electrophoresed in Laemmli buffers (23), and the gels were transferred to Immobilon-P membranes (Millipore) in a semi-dry apparatus (Bio-Rad). Membranes were blocked with non-fat milk incubated with HPC4 (0.5 μg/ml) in buffer containing 5 mM Ca2+, washed, and incubated with goat-anti-mouse IgG conjugated with horseradish peroxidase (Pierce). The membranes were washed and developed with an enhanced chemiluminescence substrate (Pierce).

Immunoprecipitation—Cell surface labeling with biotin and immunoprecipitation was performed as described (24). Harvested cells (1 × 10⁶) were washed with PBS and resuspended in 1 ml of 100 mM HEPES, 150 mM NaCl, pH 8.0, and incubated at room temperature for 30 min with 0.2 mg/ml sulfo-NHS-biotin (Pierce). After washing once in serum-free medium and twice in medium containing 10% fetal bovine serum, cells were lysed in 1 ml of 20 mM Tris-HCl, 150 mM NaCl, 3 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 0.5% Nonidet P-40, pH 7.5. Samples (100 μl) were pretreated for 3 h with 100 μl of a 50% slurry of Affi-Gel Protein A followed by a 16-h incubation of the supernatants (50 μl) with 50 μl of Affi-Gel Protein A containing nonimmune IgG (100 μg/50 μl packed gel). These supernatants were incubated with 50 μl of Affi-Gel Protein A containing either goat anti-EPCR or goat anti-mouse IgG as control for 3 or 8 h in two separate experiments. Gels were washed five times with 1 ml of 10 mM Tris-HCl, 140 mM NaCl, 3 mM MgCl2, 2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, pH 8.0, and once with 1 ml of HBSS. Adsorbed protein was eluted by boiling in 50 μl of electrophoresis sample buffer and analyzed by Western blotting using streptavidin-horseradish peroxidase conjugate (Amersham Corp.) and the enhanced chemiluminescence system.

RESULTS

Soluble, recombinant s-HPC4-EPCR was produced from a construct in which the putative transmembrane region of EPCR was deleted using the protocols described under “Experimental Procedures.” Addition of the HPC4 epitope tag facilitates detection, purification, and analysis of recombinant proteins since the HPC4 antibody binds to this P7-P5 region in protein C in a calcium-dependent fashion (18). Although the peptide binds to HPC4 in the presence of calcium, the peptide itself does not bind calcium (18), and the recombinant proteins can be easily removed from an HPC4 affinity column with buffer containing EDTA. The affinity purified fractions were analyzed by SDS-PAGE and Western blotting. The mass of the
major protein was 46 kDa under both nonreducing and reducing conditions (Fig. 1). Mono Q chromatography separated most of the high molecular weight material from the s-HPC4-EPCR. The major protein was also blotted by HPC4 antibody (data not shown). The amount of aggregate varied between preparations, but some aggregated, very high molecular weight protein was always detected, and this did not usually react with HPC4. This contaminating protein may be due to either the presence of free sulfhydryl(s) in EPCR (25) or the low expression level (20 mg/liter). The amino-terminal sequence of the purified protein was determined as XXQVDPRLIDGKIEG which was identical to the sequence of the HPC4 epitope. The 46-kDa molecular mass is considerably higher than predicted based on protein sequence (24 kDa), possibly due to the presence of N-linked sugars at the four potential N-glycosylation sites (11). This possibility was supported by the observation that endoglycosidase F/Peptide-N-glycosidase (Oxford GlycoSystems, Inc.) reduced the apparent molecular mass on SDS-PAGE from 46 kDa to a major band at 28.5 kDa (data not shown). In addition, s-HPC4-EPCR was transferred to a polyvinylidene difluoride membrane and treated with reagents to generate aldehydes by cleaving carbohydrate vicinal alcohol groups. The modified carbohydrate was biotinylated and the product detected with a streptavidin detection system and enhanced chemiluminescence substrate (Glycoprotein Detection System, Amersham Corp.). s-HPC4-EPCR, but not bacterially expressed TFT210HIS EPCR, was detected by this method (data not shown). The s-EPCR-HPC4 fusion protein with the HPC4 epitope on the carboxyl terminus had a similar SDS-PAGE pattern. The amino-terminal sequence of the s-EPCR-HPC4 was QXQASDGQR which indicates a preference of cleaving the signal peptide at residue 18 rather than residue 16 as predicted (11). From this sequence, we infer that EPCR has 3 Cys residues in the putative extracellular domain and hence at least one must be unpaired.

To analyze the function and expression of EPCR, stably transfected human 293 and murine NIH3T3 cells expressing EPCR were selected. E-7 and N-1 cells were EPCR-positive and -negative clones of human 293 cells, respectively. HEN-1 (EPCR-positive) and NN-1 (negative control) were derived from murine NIH3T3 cells. Analysis by flow cytometric methods indicated that Fl-APC binding to E-7 and HEN-1 cells was saturable and calcium-dependent. Based on the recent observation that Mg²⁺ enhances factor IX activity (26), we explored the possibility that Mg²⁺ would influence APC binding to EPCR. We observed that in the presence of optimal Ca²⁺, low levels of Mg²⁺ enhanced binding (Fig. 2A) about 2-fold and decreased the Kd(app) from 266 to 74 nM (Fig. 2B). Mg²⁺ (2.6 mM) did not support binding in the absence of Ca²⁺ (Fig. 2B). In the experiment shown, in the presence of both metal ions the Kd(app) was slightly higher than that observed previously with HUVEC (30–50 nM). However, other experiments with HEN-1 and E-7 cells gave Kd(app) values of 21.0 ± 2.9 and 25.8 ± 0.48 nM, respectively. Hence, we conclude that the APC affinity for

![Fig. 6. Properties of FLAG™-tagged EPCR mutants.](image-url)
EPCR-transfected cells and endothelium are similar. The increased cell-associated fluorescence was not due to Mg$^{2+}$-induced changes in quantum yield since the fluorescence emission of Fl-APC, measured as described previously (27), was insensitive to the addition of Mg$^{2+}$ at these concentrations (data not shown).

To determine whether EPCR antigen was expressed on the cell surface, we analyzed antibody binding to EPCR-transfected cells. To ensure specificity of the polyclonal anti-s-HPC4-EPCR antibody, the antibody was affinity-purified on the bacterially expressed form of EPCR. The cell surface of the E-7 cells stained brightly with this affinity-purified polyclonal antibody, whereas little staining of the control N-1 cells was detected (Fig. 3).

To further characterize the cell surface expression of EPCR, we utilized a monoclonal antibody, JRK-1, prepared against s-HPC4-EPCR. JRK-1 bound to the cell surface of EPCR-positive E-7 and HEN-1 cells (Fig. 4, A and C). No binding was detected to the control, EPCR-negative N-1 cells or NN-1 cells (Fig. 4, B and D). Fl-APC and JRK-1 binding to cells transiently transfected with either human, bovine, or murine EPCR were also compared (Fig. 4, E–G). Fl-APC bound almost equally well to 293T cells transfected with human, bovine, and murine EPCR. In contrast, JRK-1 bound only to cells transfected with human EPCR (Fig. 4E) and not to those transfected with bovine or murine EPCR (Fig. 4, F and G). The Fl-APC and JRK-1 pattern was complex in these experiments, probably because the transient expression system resulted in variable EPCR expression levels among the cells. It is of note that the distribution of cells with high and low levels of Fl-APC and JRK-1 binding sites are similar. Biotinylated JRK-1 bound to s-HPC4-EPCR in a saturable and specific manner in an enzyme-linked immunosorbent assay format, but JRK-1 did not bind to control recombinant proteins containing the HPC4 epitope (28) (data not shown).

The antigen distribution was examined using JRK-1 and confocal microscopy. Immunofluorescence was detected on the cell surface of HEN-1 cells and not on NN-1 cells (Fig. 5).

To analyze the requirements for cell surface expression and Fl-APC binding, we prepared the three FLAG® epitope-tagged forms of EPCR illustrated in Fig. 6. The FLAG® epitope was detected on the surface of cells transiently transfected with mFL-1 or mFL-2, but not with sFL-3 (Fig. 6). Fl-APC bound to mFL-1, but not to mFL-2 or sFL-3-transfected cells. mFL-1 and wild type EPCR-transfected cells bound Fl-APC similarly. Transfection with sFL-3 resulted in the appearance of immunoreactive forms of the antigen in the cell supernatant (Fig. 6B), a result consistent with deletion of the transmembrane region of EPCR. Under nonreducing conditions 42-, 84-, 130-, 170-kDa forms were detected (Fig. 6B). These aggregates were probably due to the presence of free sulfhydryl group(s) in EPCR (25). Under reducing conditions, a 45-kDa protein was detected (Fig. 6B). No antigen was detected in the cell supernatants from cells transfected with either mFL-1 or mFL-2.

As another approach to determine the cell surface expression and properties of EPCR, the cell surfaces of HUVEC, E-7, and N-1 cells were biotinylated using a membrane-impermeable reagent as described under "Experimental Procedures." The
polyclonal anti-EPCR antibody precipitated a 49-kDa protein from HUVEC and EPCR-positive E-7 cells and not from control N-1 cells (Fig. 7). The mass of the protein was similar to the 46-kDa observed with s-HPC4-EPCR and consistent with a glycosylated form of EPCR. Like s-HPC4-EPCR, the apparent mass of the immunoprecipitated protein was not influenced by reduction.

The above experiments indicate that EPCR is a cell surface protein, but they do not demonstrate that EPCR binds directly to APC. To test this possibility, we examined the ability of soluble EPCR to inhibit Fl-APC binding to E-7 cells by flow cytometry. We employed both s-HPC4-EPCR and s-EPCR-HPC4 in these experiments. Initially, cells were incubated with various concentrations of Fl-APC to establish a standard curve relating mean channel fluorescence intensity to free Fl-APC concentration (Fig. 8A). Next, a single concentration of Fl-APC (80 nM) was mixed with different concentrations of s-EPCR-HPC4, and these mixtures were allowed to bind to the cell surface. s-EPCR-HPC4 blocked Fl-APC binding in a concentration-dependent fashion (Fig. 8B). Assuming that inhibition is caused by complex formation between EPCR and Fl-APC in solution and the decrease in Fl-APC binding is due to the reduction in the free Fl-APC concentration, the free Fl-APC concentration can be estimated based on the standard curve of mean channel fluorescence intensity versus Fl-APC concentration, which can be used to calculate the $K_{d(app)}$. Using this approach, a $K_{d(app)} = 29.2 \pm 5.7$ nM using s-EPCR-HPC4 concentrations ranging from 100 to 1000 nM with the range from 16 to 38 nM was determined. s-HPC4-EPCR inhibited Fl-APC somewhat more weakly ($K_{d(app)} \approx 170$ nM) (data not shown), and inhibition was independent of whether or not the preparation was further purified on a Mono Q column. A recombinant prethrombin-1 fusion protein which also contains the HPC4 epitope at the amino-terminal had little effect on Fl-APC binding at concentrations as high as 5 μM (data not shown).

Since Fl-APC binding to the cell surface is metal-dependent, it was not possible to use this system to examine the metal dependence of the interaction with soluble EPCR. To address this question, we examined s-HPC4-EPCR binding to immobilized protein C. A noninhibitory antibody (HPC2) was used to immobilize protein C. HPC2 is a calcium-independent, anti-human protein C IgG1 monoclonal antibody that also reacts with human APC. HPC2 does not inhibit Fl-APC binding to E-7 cells. s-HPC4-EPCR did not bind to the HPC2 column in the absence of protein C (Fig. 9A, upper panel). When human protein C was adsorbed to the column prior to application of the s-HPC4-EPCR, most of the s-HPC4-EPCR bound in the presence of metal ions and was eluted with EDTA (Fig. 9A, lower panel). The eluted fraction contained the 46-kDa s-HPC4-EPCR protein (Fig. 9B) that bound HPC4 on Western blots (Fig. 9C). Most of the contaminating protein broke through the column (Fig. 9B). When the column was run in buffers with Ca$^{2+}$, but without Mg$^{2+}$, approximately 30–50% of the EPCR eluted before application of the EDTA elution buffer (data not shown).

![Affinity chromatography of s-HPC4-EPCR on protein C.](image)

Fig. 9. Affinity chromatography of s-HPC4-EPCR on protein C. A noninhibitory murine IgG1 monoclonal antibody to human protein C, HPC2 (5 mg), was adsorbed to 2 ml of Affi-Gel Protein A (Bio-Rad) in a 0.6 x 5-cm column. A, s-HPC4-EPCR (250 μg) was chromatographed on this column without protein C adsorbed (control). B, 998 I.U. (≈5 mg) of protein C concentrate (Immuno Ag) was adsorbed to the column, before s-HPC4-EPCR was applied. A and B, the column was equilibrated with HBSS containing 1.3 mM CaCl$_2$ and 0.6 mM MgCl$_2$. After s-HPC4-EPCR application, the column was washed with the same buffer and eluted (arrow) in HBSS in which the Ca$^{2+}$ and Mg$^{2+}$ were replaced with 1 mM EDTA. The column was run at room temperature with a flow rate of 0.5 ml/min, and 0.7-ml fractions were collected. The fractions were analyzed by Colloidal Gold Total Protein Stain (Bio-Rad) (B) and by Western blotting using the HPC4 antibody (C). For B and C, lanes 1 and 4 were the sample applied, lanes 2 and 5, breakthrough, fraction 6 from A bottom, and lanes 3 and 6, the column eluate, fraction 17. The lower lane numbers were unreduced samples and the higher numbers were reduced.
This study provides many lines of evidence that indicate EPCR is expressed as a cell surface protein and that it is directly involved in protein C/APC binding. First, a monoclonal antibody and affinity purified polyclonal antibodies raised against recombinant EPCR bound to HUVEC and to human or murine cell lines stably transfected with human EPCR but not to the same sham-transfected cell lines. Second, APC and the monoclonal antibody bound to cells transiently transfected with human EPCR, but only APC bound to cells transfected with murine or bovine EPCR. Thus, binding of the antibody is species-specific and cell line-independent. Third, when the FLAG epitope was inserted into an EPCR construct and expressed in 293T cells, the cells bound both the FLAG antibody and affinity purified polyclonal antibodies raised against recombinant s-HPC4-EPCR (46 kDa) and considerably larger than predicted based on the amino acid sequence (25 kDa). The increased mass was probably due to the presence of carbohydrate on EPCR, which contains four potential N-glycosylation sites (11). Fifth, truncation immediately above the putative membrane spanning domain leads to the formation of soluble EPCR (s-HPC4-EPCR or the form with the FLAG epitope). Taken together, these studies demonstrate that EPCR is a type 1 transmembrane cell surface glycoprotein.

EPCR is homologous to the CD1/MHC class 1 family of molecules (11). Many members of this family function as heterodimers (29). It was therefore of importance to determine if EPCR was sufficient by itself to allow protein C binding. The observation that s-EPCR-HPC4 inhibits binding of APC to the cell surface in a concentration-dependent fashion supports the concept that EPCR can bind to APC in solution. The development of the expression system and preparation of antibodies provide key reagents for elucidation of EPCR function.

Acknowledgments—We thank Barbara Carpenter, Teresa Burnett, Jeff Malea, Shu Chen, Sondra Coughlin, and Gary Ferrer for their excellent technical assistance; Jeff Box for assistance with the figures; Dr. Naomi Esmon for helpful experimental and editorial suggestions; and Julie Wiseman for the final preparation of the manuscript.