Transfer of Specific Endothelial Cell-Binding Properties from the Procoagulant Protein Human Factor IX into the Anticoagulant Protein Human Protein C†

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ABSTRACT: A series of recombinant (r) chimeric mutants of human coagulation protein C (PC) and activated protein C (APC) containing replacements of homologous PC domains by those of human coagulation factor IX (fIX) were generated, with the intention of determining whether the specific bovine aortic endothelial cell (BAEC) receptor-binding characteristics of fIX could be incorporated into the chimeric r-PC while maintaining the essential properties of PC and APC. Using a competitive BAEC displacement assay with [^{125}I]fIX, we found that a chimeric r-PC (r-[ΔPC$^{1-46}$/∇fIX$^{1-47}$]PC), consisting of the entire γ-carboxyglutamic domain ([GD$_{IX}$], residues 1−38) and helical stack ([HS$_{IX}$], residues 38−47) of fIX as replacements for these same domains of PC, provided an IC$_{50}$ for fIX-related BAEC binding of 13 nM, as compared to 10 nM for that of unlabeled fIX. This showed that all of the BAEC tight binding determinants for fIX existed within the [GD$_{IX}$/HS$_{IX}$]. Additionally, this chimera reacted to the same extent as fIX with the Ca^{2+}-dependent, [GD$_{IX}$]-specific monoclonal antibody H5B7 and lost its reactivity to a similar antibody specific for the [GD$_{PC}$], JTC1. A synthetic peptide containing residues 1−47 of fIX also competed effectively (IC$_{50}$ = 16 nM) with intact fIX for BAEC binding. Displacement of [^{125}I]fIX from BAEC did not occur with a chimera containing the [HS$_{IX}$] alone or with another mutant protein possessing a replacement of the two epidermal growth factor (EGF) homology regions of r-PC (residues 47−137) with those same domains of fIX. Further mutants within [GD$_{PC}$] revealed that replacement of amino acid residues 1−13 of r-PC with the homologous residues 1−14 of fIX (r-[ΔPC$^{1-13}$/∇fIX$^{1-14}$]PC) was sufficient to allow full incorporation of the fIX-related BAEC-binding characteristics into r-PC. Both r-[ΔPC$^{1-46}$/∇fIX$^{1-47}$]APC and r-[ΔPC$^{1-13}$/∇fIX$^{1-14}$]APC, when bound to the fIX receptor on BAEC, possessed activity for the natural APC substrate fVIII. The data obtained in this study confirm that the major binding site for fIX on its BAEC receptor is located within the amino-terminal 14 amino acids of its light chain and show that this property can be fully transferred to PC. The results also further substantiate the approach of using strategically-generated chimeric recombinant molecules to delineate the regions of these types of proteins that are responsible for their functional properties and allow new coagulation proteins to be constructed that contain properties of others. In this case, an endothelial cell population could be made antithrombotic by utilizing specific fIX binding properties of these cells to replace this procoagulant protein with other proteins possessing different functions and also rendered anticoagulant by concomitant incorporation of APC activity at this same site.

The PC[1]-related anticoagulant system is composed of the zymogen (PC), its activated protein (APC), the PC activators (fIIa and the fIIa/Tm complex), and the positive and negative effectors of PC activation and/or APC activity, viz., Ca^{2+}, PS, and PCI. This highly regulated system serves to maintain the fluid state of blood by provision of the serine protease APC, an enzyme that inactivates through limited proteolysis the coagulation cofactors fV/fVa (Kisiel et al., 1977) and fVIII/fVIIIa (Vehar & Davie, 1980). Activation of PC by fIIa is inhibited by Ca^{2+} (Amphlett et al., 1981), whereas this same activation by the fIIa/Tm complex is activated by Ca^{2+} at low cation concentrations and subsequently inhibited at higher levels of Ca^{2+} (Clarke et al., 1993).

PC consists of a series of domain regions (Foster et al., 1985) that are very similar to those present in fVII (O'Hara et al., 1987), fIX (Yoshitake et al., 1985), and fX (Leytus et al., 1986). In each of these proteins, an amino-terminal [GD], consisting of residues 1−37, containing 9−11 Gla residues, exists immediately downstream of a [HS], comprising amino acid residues 38−46, which possesses 0−1 Gla residues. These domains are followed by two [EGF]-related modules, an activation cleavage site(s), and the carboxyl-terminal serine protease chain. Substantial efforts by several laboratories have been centered on discovery of the roles of the domain regions in these proteins. In general, it is believed that the [GD] contains the Ca^{2+} binding sites needed for the conformational change in these proteins that is requisite to PL and cell binding (Nelsestuen et al., 1978; Liebman et al., 1987; Toomey et al., 1992; Liebman, 1993). Additional studies have shown that the [HS] is required for the complete expression of the Ca^{2+}-binding sites located in the [GD] (Colpitts & Castellino, 1994) and the first [EGF]

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domain (Valcarce et al., 1993), as well as for interactions with activators and cofactors for activation (Petersen et al., 1994). In addition, the [EGF] domains have been demonstrated to interact with Ca^{2+} and also with proteins important to the different functions of the molecules in which these modules exist (Ohlin et al., 1988; Astermark & Stenflo, 1991; Astermark et al., 1992, 1994). Lastly, the serine protease region contains the catalytic machinery of the enzymes.

It is of considerable basic and clinical importance to determine whether a strategy involving exchange of the above domains, or regions within these domains, between proteins with different functions could be employed to incorporate desired properties of one protein into another. Some successes have been realized in this regard with chimeric proteins. In one case, this type of research has allowed the transfer of BAEC- (Cheung et al., 1992; Toomey et al., 1992) and platelet- (Ahmad et al., 1994) binding properties of fIX into fVII. In another example, substitution of the [GD/HS] regions from fIX into PC conferred reactivity on the chimeric r-PC for a Ca^{2+}-dependent epitope of a [GD_{IX}]-specific MAb while still allowing the chimera to possess essential functional properties of PC and APC (Christiansen & Castellino, 1994). We thus believed that it would be possible to obtain a chimeric r-PC that, while maintaining its essential anticoagulant properties, would also bind to BAEC via the fIX receptor. If successful, this strategy could then be used to impart antithrombotic and anticoagulant properties in BAEC and attenuate their thrombogenic character. The current manuscript provides a summary of the results of these investigations.

MATERIALS AND METHODS

Proteins and Peptides. wtr-PC (Zhang & Castellino, 1990), r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]PC (Christiansen & Castellino, 1994), r-[$\Delta PC^{38-46}/\nabla fIX^{39-47}$]PC,[2] and r-[EGF1,$2_{IX}$]PC (Yu et al., 1994) were expressed in human kidney 293 cells and isolated as described in the indicated publications. The purifications of r-[∇K^5]PC and r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC were accomplished in the same manner as for the above r-variants of r-PC. Each of the corresponding r-APCs were prepared from the relevant zymogens by activation with the venom protease Protac C (American Diagnostica, New York, NY), as described (Zhang & Castellino, 1990).

Human plasma fIX was obtained from Enzyme Research Laboratories (South Bend, IN). Restriction endonucleases were purchased from Promega (Madison, WI).

The MAb H5B7, which recognizes a Ca^{2+}-dependent epitope in [GD_{IX}] (Velander et al., 1989), was provided by Dr. D. Strickland (Rockville, MD). The MAb JTC1, which recognizes a similar epitope in PC, was a gift of Dr. K. Wakabayashi (Tokyo, Japan). The MAb (C3) to human plasma PC, which possesses a Ca^{2+}-independent epitope within its [EGF] regions (Heeb et al., 1988; Yu et al., 1994), was obtained from Dr. J. Griffin (La Jolla, CA). The MAbs were radiolabeled using the Iodobead (Pierce Chemical Company, Rockford, IL) procedure as published earlier (Zhang & Castellino, 1992).

The synthetic peptide containing the amino acid sequence of the [GD/HS] region of fIX (residues 1−47 of the light chain), [GD_{IX}/HS_{IX}]$^{1-47}$, was a gift from Dr. B. C. Furie (Boston, MA). Its properties have been described (Jacobs et al., 1994).

cDNAs. Descriptions of the cDNAs that encode human fIX (p119-[fIX]) and a restriction site-modified human PC in pUC119 (p119-[PC]) (Zhang & Castellino, 1990) have been provided in earlier publications (Yu et al., 1994). The mutant cDNAs encoding r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]PC (Christiansen & Castellino, 1994), r-[$\Delta PC^{38-46}/\nabla fIX^{39-47}$]PC[2], and r-[$\Delta PC^{47-137}/\nabla fIX^{48-128}$]PC (Yu et al., 1994) have been generated as described.

The mutagenesis strategies used for construction of the cDNAs encoding r-[∇K^5]PC and r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC were PCR-based, involving gene splicing by overlap extension. In the case of r-[∇K^5]PC, the following two PCR primers (the fIX nucleotides and amino acids are italicized), viz., (1) 5′-forward primer 5′-ACT TGC AGT ATC TCC ACG and (2) 3′-reverse primer 5′-ACG GAG CTC-CTC CAG *CTT* GAA GGA GTT GGC ACG were used with p119-[PC] as a template to generate fragment 1. This latter segment is a portion of chimeric cDNA beginning at nucleotide −171 of PC, which is 45 bases upstream of the codon for the signal Met residue (this begins at nucleotide −126), and terminating at base 30, which is within the codon for Arg^9 of PC (Arg^{10} of this new chimera). This construct also includes the codon for the newly inserted Lys^5 residue.

Primers 3 and 4, viz., (3) 5′-forward primer 5′-CGT GCC AAC TCC TTC *AAG* CTG GAG GAG CTC CGT and (4) 3′-reverse primer 5′-CCT GGT CAT CTT CCC ATC were used with p119-[PC] as a template to generate fragment 2. This region of cDNA begins at nucleotide −3 of PC and terminates at nucleotide 531. Fragment 2 includes the necessary bases to encode the Lys^5 insertion. Fragments 1 and 2 were annealed through their overlapping sequences and amplified by PCR using primers 1 and 4. This provided

[1] Abbreviations: PC/APC, human protein C/activated human protein C; fIIa, thrombin; fV/fVa, human coagulation factor V/activated factor V; fVII, human coagulation factor VII; fVIII/fVIIIa, human coagulation factor VIII/activated factor VIII; fIX/fIXaβ; human coagulation factor IX/activated factor IX; fX/fXa, human coagulation factor X/activated factor X; PS, protein S; PCI, protein C inhibitor; Tm, thrombomodulin; [GD_{PC}], the γ-carboxyglutamic rich domain, residues 1−37, of PC; [GD_{IX}], the γ-carboxyglutamic rich domain, residues 1−38, of fIX; [HS_{PC}], the helical stack region, residues 38−46, of PC; [HS_{IX}], the helical stack region, residues 39−47, of fIX; [EGF1/2_{PC}], the epidermal growth factor homology regions, residues 47−137, of PC; [EGF1/2_{IX}], the epidermal growth factor homology regions, residues 48−128, of fIX; [GD_{IX}/HS_{IX}]$^{1-47}$, a synthetic peptide containing residues 1−47 of fIX; r-[∇K^5]PC/APC, a recombinant mutant PC/APC in which a lysine residue has been inserted at amino acid sequence position 5 of the [GD_{PC}]; r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC/APC, a recombinant chimeric PC/APC wherein the amino-terminal 13 residues in the [GD_{PC}] have been replaced by the amino-terminal 14 residues of the [GD_{IX}]; r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]PC/APC, a recombinant chimeric PC/APC in which the [GD_{PC}/HS_{PC}] (residues 1−46) has been replaced by the [GD_{IX}/HS_{IX}] (residues 1−47); r-[$\Delta PC^{38-46}/\nabla fIX^{39-47}$]PC/APC, a recombinant chimeric PC/APC in which the helical stack domain of protein C (residues 38−46) has been replaced by that of factor IX (residues 39−47); r-[$\Delta PC^{47-137}/\nabla fIX^{48-128}$]PC/APC, a recombinant chimeric PC/APC in which the two EGF-like domains of PC (residues 47−137) have been replaced by those same regions of fIX (residues 48−128); BAEC, bovine aortic endothelial cells; BSA, bovine serum albumin; FBS, fetal bovine serum; Gla, γ-carboxyglutamic acid; Hya, β-hydroxyaspartic acid; PL, phospholipid vesicles containing a relative ratio of 60% chicken egg phosphatidylcholine/40% bovine brain phosphatidylserine; wt, wild-type; r, recombinant; FFQ, fast flow Q resin; IC_{50}, the total concentration of competitor required to diminish the measured ligand-induced property by 50%; C_{50}, the total concentration of ligand required to alter the property by 50%.

[2] Manuscript submitted for publication.

a chimeric cDNA fragment (fragment 3) spanning nucleotides −171−531 of modified p119-[PC] (base 1 in this plasmid is defined as the first nucleotide encoding the amino-terminal Ala[1] residue of mature PC), with nucleotides 13−15 containing the codon for *Lys*[5]. Fragment 3 contains a unique *Nhe*I-sensitive cleavage site in its 5′-region (between bases −153 and −152) and a unique *Sac*II restriction site in its 3′-region (between nucleotides 508 and 509). Digestion of fragment 3 with *Nhe*I/*Sac*II liberated a cDNA fragment (fragment 4) containing bases −152−508. Fragment 4 was then inserted into the same restriction fragment of the modified p119-[PC], generating plasmid p119-[∇K[5]]PC.

A similar strategy was used to construct the cDNA that codes for r-[ΔPC[1−13]/∇fIX[1−14]]PC. The template used was pUC119-[ΔPC[1−46]/∇fIX[1−47]]PC. This construct contained the 5′-noncoding sequences of the signal sequence, the propeptide sequence of PC, and the [GD_{IX}] and [HS_{IX}] modules (amino acid residues 1−47, nucleotides 1−141), and the remainder of the cDNA possessed the PC sequence. Primers 1 (above) and 5, viz., (1) 5′-forward primer 5′-ACT TGC AGT ATC TCC ACG and (5) 3′-reverse primer 5′-CCG CTC *CAG GTT CCC TTG AAC AAA CTC* were employed in the first PCR reaction, generating fragment 5. This cDNA segment spans the sequence from base −171, as above, to nucleotide 48, with bases 1−42 (amino acids 1−14) containing sequences from fIX (nucleotides 43−48 encode identical amino acids at positions 15 and 16 in PC and fIX).

The second PCR reaction, used to generate fragment 6, employed pUC119-[PC] as the template with primers 4 (above) and 6, viz., (6) 5′-forward primer 5′-*CAA GGG AAC CTG* GAG CGG GAG TGC ATA and (4) 3′-reverse primer 5′-CCT GGT CAT CTT CCC ATC. Fragment 6 is a cDNA segment spanning nucleotides 31−534, with nucleotides 31−42 (encoding amino acids 11−14) containing sequences found in fIX. Fragments 5 and 6 were annealed though their overlapping sequences and amplified by PCR using primers 1 and 4, as above. This resulted in fragment 7, which is a segment of chimeric cDNA spanning bases −171−534, with nucleotides 1−42 identical with those in fIX. Upon digestion of fragment 7 with *Nhe*I/*Sac*II, fragment 8 was generated. This oligonucleotide contained bases −152−508. Upon insertion into p119-[PC] via these same restriction sites, the plasmid p119-[ΔPC[1−13]/∇fIX[1−14]]-PC resulted. DNA sequences were obtained through the regions that were amplified by PCR.

The cDNAs that contained the complete coding sequences for r-[∇K[5]]PC and r-[ΔPC[1−13]/∇fIX[1−14]]PC were excised from p119-[∇K[5]]PC and p119-[ΔPC[1−13]/∇fIX[1−14]]PC by use of a *Nhe*I/*Xho*I restriction digestion (the cDNA for PC is excised between the 5′-nontranslated and 3′-nontranslated regions) and inserted into these same restriction sites in the multiple cloning region of the mammalian cell expression vector pCIS2M (Zhang & Castellino, 1990). This provided pCIS2M[∇K[5]]PC and pCIS2M[ΔPC[1−13]/∇fIX[1−14]]PC, respectively. These latter plasmids were then transfected into human kidney 293 cells (ATCC CRL 1573). Positive clones were selected by assay with the MAb C3.

BAEC Manipulations. A previous published method for BAEC handling (Cheung et al., 1991) was used with minor operational modifications. BAECs (third passage) were thawed rapidly in warm water and added to 10 mL of Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO), supplemented with 10% (v:v) FBS, 4 mM L-glutamine, 200 units/mL penicillin, 200 μg of streptomycin, 2 mM sodium pyruvate, and MEM amino acids (Hyclone Labs, Logan, UT). This mixture was centrifuged at 1400 rpm for 10 min in an IEC Centra-7 centrifuge (IEC, Needham Heights, MA). The resulting pellet was resuspended in this medium and grown in a 75 cm^2 flask at 37 °C, under an atmosphere of 5% CO_2. After confluence was reached, the medium was removed and the flask was washed with 10 mL of a buffer containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM NaH_2PO_4, pH 7.3. The cells were then detached as a result of a 5 min incubation with 5 mL of a solution composed of 0.25% (w:v) trypsin in Hank's balanced salt solution containing 0.2 g/L EDTA. The cells were suspended in an equal volume of growth medium and centrifuged (1400 rpm) for 15 min at room temperature. The resulting cell pellet was resuspended in 1 mL of growth medium, diluted 1:100 (v:v), and the cells were counted. This typically provided 4 × 10^6 cells/mL. This mixture was diluted to 3 × 10^4 cells/mL after which 0.4 mL was added to each well of two 48-well microtiter plates. These were then grown to confluence (ca., 4 days), after which time binding assays were conducted.

Competitive Binding to BAEC. For binding analyses, the culture medium was removed from the wells and each was washed four times with 0.5 mL of a solution of 0.01 M Na-Hepes, 0.137 M NaCl, 0.004 M KCl, and 0.011 M glucose, pH 7.5 (buffer A). The cells were then incubated for 15 min in 0.5 mL/well buffer A, supplemented with 2 mg/mL BSA and 2 mM $CaCl_2$ (buffer B). This solution was then replaced with 0.2 mL of this same buffer containing 2 nM [^{125}I]fIX and various concentrations of competitive proteins or peptides. After incubation for 2.5 h at 4 °C, the cells were washed five times with 0.5 mL of a solution containing chilled 5 mM Tris-HCl, 0.14 M NaCl, 2 mM $CaCl_2$, and 2 mg/mL BSA. After each treatment, the cells remained in a confluent monolayer as assessed microscopically and as determined by trypan blue exclusion. Control studies demonstrated that release of all [^{125}I]fIX occurred when this same incubation was carried out in the presence of 5 mM EDTA. After this washing process, a volume of 0.5 mL of a solution consisting of 0.2 M NaOH, 0.01 M EDTA, and 1% (w:v) $NaDodSO_4$ was added to the adherent cells. The extracted cell solution was then subjected to γ-counting.

The inhibitor dissociation constants (IC_{50}) were calculated by plotting the relative percent of [^{125}I]fIX bound to the cells (cpm of [^{125}I]fIX in the absence of competitor = 100% bound) against the log of the concentration of the total added competitive protein. The IC_{50} values for the competition were calculated from plots of this type through regression analyses of nonlinear least-squares fit of the data.

Interaction of Ca^{2+} with the [GD]s of r-[∇K[5]]PC and r-[ΔPC[1−13]/∇fIX[1−14]]PC. Intrinsic fluorescence (quenching) titrations accompanying the binding of Ca^{2+} to the [GD] of the r-PC mutants were conducted as described earlier (Zhang & Castellino, 1992). Fluorescence measurements were carried out at 20 °C with use of a SLM-Aminco 8100 (SLM-Aminco Instruments, Urbana, IL) recording spectrofluorometer. The excitation and emission wavelengths used were 283 and 340 nm, respectively.

MAb Bind to r-[∇K[5]]PC and r-[ΔPC[1−13]/∇fIX[1−14]]PC. The dependencies on the Ca^{2+} concentration of the binding to the r-PC mutants of the Ca^{2+}-dependent, [GD_{IX}]-directed MAb [^{125}I]H5B7 and of the corresponding Ca^{2+}-dependent,

[GD_{PC}]-directed MAb [^{125}I]JTC1 were determined as detailed earlier (Zhang & Castellino, 1992).

Plasma-Based Anticoagulant Assays. Plasma APTT anticoagulant assays at 37 °C were employed to determine the functional activities of the r-APC mutants. Our methodologies have been published (Zhang et al., 1992; Jhingan et al., 1994).

In Vitro fVIII Inactivation Assays. A two-stage assay was employed for these measurements (Jhingan et al., 1994), the details of which differed when PL vesicles or BAECs were used a the source of membrane PL.

In the case of PL vesicles, the assay was conducted as follows. In stage 1 of the assay, a solution of fVIII (6 nM final concentration) was incubated with the desired r-APC (0.5 nM final concentration), in the presence of (final concentrations) PL (40 μM) and Ca^{2+} (2.5 mM). Next, fIXaβ (6.7 nM) and fIIa (0.12 unit/mL) were added in order to activate the remaining fVIII. Following this, in stage 2 of the assay, the amount of fVIIIa present was determined in the tenase system. For this, the fVIIIa mixture was added to a solution containing final concentrations of 40 μM (in phosphate) PL, 2.5 mM $CaCl_2$, and 180 μM of the fXa chromogenic substrate S2222. After recording the base line for 1 min, substrate hydrolysis was accelerated by addition of fX (32 μM final concentration). The rate of amidolysis of S2222 by the generated fXa was determined spectrophotometrically at 405 nm. The relative amount of fVIII (%) remaining was taken as the absorbance at 405 nm/min in the final stage of the assay relative to that same measurement for the control experiment with wtr-APC present as the inactivating enzyme in stage 1 of the assay (100% activity). An incubation of fVIII with r-PC demonstrated that the loss of activity of fVIII was <10% over the time period examined. The buffer used contained 25 mM Hepes-NaOH/150 mM NaCl, pH 7.4, at 37 °C.

The strategy of the assay was modified with use of BAEC as the membrane PL source and performed in a two-stage competitive mode. In the first stage, the ability of BAEC-bound chimeric proteins to inactivate r-fVIII was examined. Here, wtr-APC (control), r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]APC, or r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]APC (ca. 2.0 nM final concentration), in the presence of various concentrations of $[GD_{IX}/HS_{IX}]^{1-47}$, each dissolved in buffer B, was added to prewashed (as in the BAEC binding section) adherent cells in separate wells of a 48-well microtiter plate and allowed to incubate for 2.5 h at 4 °C. The cells were then washed five times with a solution of 0.5 mL of chilled 5 mM Tris-HCl, 0.14 M NaCl, 2 mM $CaCl_2$, and 2 mg/mL BSA. Next, r-fVIII (2.5 nM final concentration) dissolved in 200 μL of this same buffer was added and allowed to incubate for an additional 6 min at 37 °C. To activate the remaining r-fVIII prior to assay in the fX system, a total of 150 μL of this mixture was then removed and transferred to an Eppendorf tube containing 20 μL of a solution of fIXaβ (6.3 nM final concentration—used for protection of fVIIIa activity) and thrombin (0.12 unit/mL final concentration). Incubation was allowed to proceed at 37 °C for 1.5 min. The amount of r-fVIIIa present was then determined by its ability to stimulate the activation rate of fX (stage 2). This latter part of the assay was conducted as above. The data obtained in this competitive assay were plotted as the amount of r-fVIII remaining in the first stage of the assay as a function of the competing peptide concentration. The IC_{50} value for the peptide was determined from this plot against each of the mutants tested, as well as against wtr-APC.

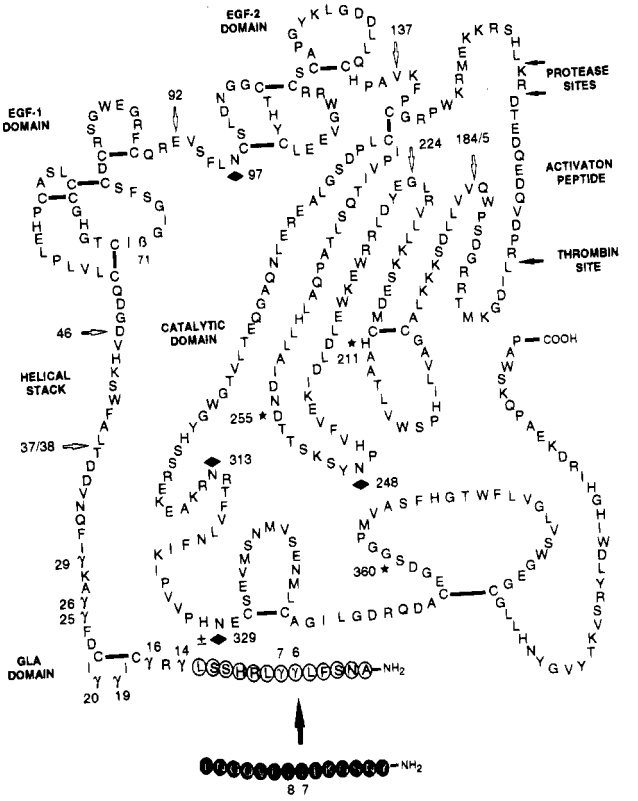

FIGURE 1: Amino acid sequence of r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC. The amino acid sequence regions of the various domains in PC are indicated, along with corresponding positions of introns in the gene (open arrows). The filled stars are locators of the catalytic triad of amino acids in the serine protease domain of APC, and the filled diamonds indicate the sites of consensus sequences for N-linked glycosylation. The first 13 amino acids of the light chain of PC (in open circles) have been replaced with the corresponding 14 amino acids of fIX (in filled circles) to generate the chimera r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC.

Analytical Methods. The methods that were employed for DNA manipulations, which included oligonucleotide synthesis, cDNA sequencing, bacterial cell transformations, mammalian cell transfections, plasmid minipreparations, large-scale plasmid preparations, generation of single-stranded DNA, purification of DNA fragments, and PCR amplifications, were accomplished as described in previous publications (Menhart et al., 1991; De Serrano & Castellino, 1992; De Serrano et al., 1992). Gla (Zhang & Castellino, 1990) and Hya (Yu et al., 1994) determinations, as well as amino acid compositional and sequence analyses (Chibber et al., 1990), were conducted as described. Western immunoblotting with MAb C3 was accomplished as previously published (Zhang & Castellino, 1990).

RESULTS

Two new r-PC mutants were generated in this study, one in which a Lys at amino acid sequence position 5 was inserted into r-PC (r-[∇K^5]PC) and another in which the first 13 amino acids of the light (non-protease) chain of PC were replaced with the first 14 amino acids of that same region of fIX (r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC). These changes in the PC amino acid sequence are illustrated in Figure 1. The rationale for selection of these mutations was to attempt to incorporate

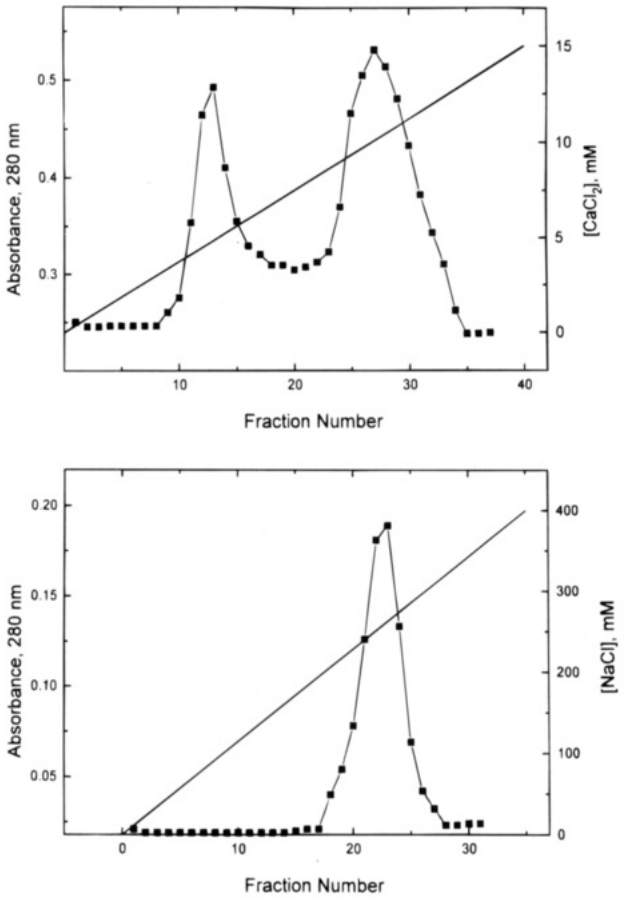

FIGURE 2: Purification of r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC. (Top) A volume of 800 mL of culture medium was percolated over a 5 mL column of FFQ that had been pre-equilibrated with 20 mM Tris-HCl/150 mM NaCl/4 mM EDTA, pH 7.4, at 4 °C (TBS/EDTA). After washing the column with this same buffer, followed by 20 mM Tris-HCl/150 mM NaCl, pH 7.4 (TBS), a $CaCl_2$ gradient was applied (120 mL total volume). Fractions (1.6 mL) were collected at a flow rate of 0.4 mL/min. The major peaks were collected and pooled. (Bottom) Peak 1 of the top panel (subsequently found to contain the desired material) was dialyzed against TBS and reapplied to a 3 mL column of FFQ equilibrated in this same buffer. The indicated gradient of NaCl was applied (50 mL total volume), and 1 mL fractions were collected. The flow rate was 0.25 mL/min. The major fraction was pooled and employed for further studies.

functional [GD_{IX}]-associated properties into r-PC while making minimal alterations in the [GD_{PC}]. Since it is believed that the specific binding site of fIX to BAEC is resident in amino acids 3−11 (Cheung et al., 1992), we replaced this region of PC by that of fIX, and since Lys^5 is the extra amino acid in fIX in this region of the [GD_{IX}], we constructed another mutant wherein this single amino acid was inserted into r-PC.

Purification of these new mutants proteins was accomplished using two FFQ chromatography steps, one involving Ca^{2+} elution and the other based on elution with NaCl (Figure 2). As was the case with several other r-PC mutants and chimeras previously reported (Zhang & Castellino, 1990, 1991; Zhang et al., 1992; Christiansen & Castellino, 1994; Yu et al., 1994), these two steps were sufficient to yield highly purified preparations of the two mutants. In each case, the usual doublet band pattern for the purified proteins was observed on $DodSO_4$/PAGE (Figure 3), which had been determined previously to be due to the presence of two populations of r-PC glycoforms (Grinnell et al., 1991). The mutants were present mainly in their two-chain forms, showing that the Lys^{155}-Arg^{156} (Lys^{156}-Arg^{157} in the two mutants) dipeptide had been effectively processed out of the proteins. The purified proteins contained 8.7 (r-[∇K^5]PC) and 9.1 (r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC) mol of Gla/mol of protein and 1.01 (r-[∇K^5]PC) and 0.98 (r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC) mol of Hya/mol of protein, thus demonstrating that these posttranslational processing events were not affected by the mutations incorporated into r-PC. The only unusual aspect of the purification was the fact that two peaks of r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC were obtained on elution from the first FFQ column (Figure 2). Similar to the findings for purification of r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]PC (Christiansen & Castellino, 1994), the second fraction contained protein material that had not been effectively cleaved at the junction of the propeptide and amino-terminus of the mature protein, since amino acid sequence analysis showed amino-terminal sequences that exist within the propeptide. It thus appears as though the Tyr^1 residue in fIX that replaces the Ala^1 residue in r-PC does affect processing at this location when the propeptide of PC is present as the cleavage partner for a fIX-based amino-terminus. However, the first fraction from this column did contain properly excised signal/propeptide, as determined by automated amino-terminal sequencing of the mature proteins through 30 amino acid residues.

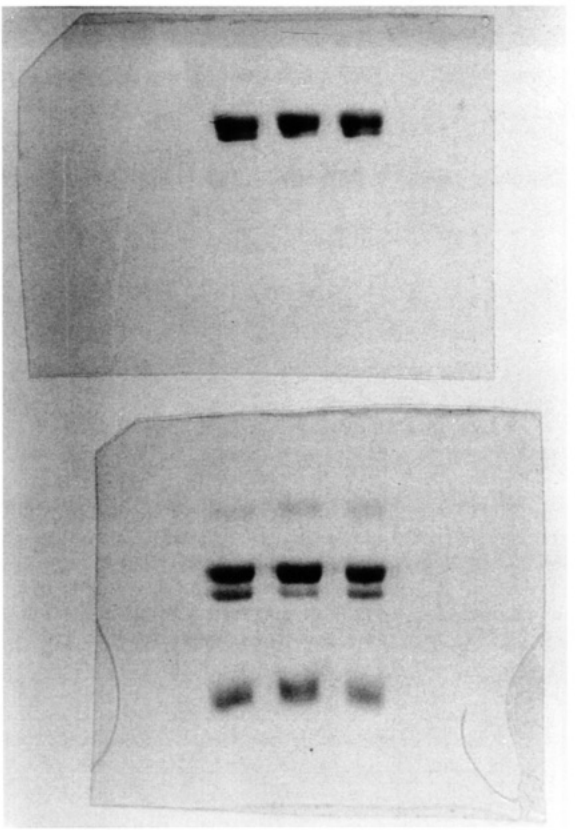

FIGURE 3: $DodSO_4$/PAGE analysis of the newly constructed r-PC mutants. (Top) Left-to-right: nonreduced wtr-PC, nonreduced r-[∇K^5]PC, and nonreduced r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC. (Bottom) Left-to-right: reduced wtr-PC, reduced r-[∇K^5]PC, and reduced r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC.

The [GD]-associated quenching of fluorescence induced by Ca^{2+} has been employed to assess the integrity of Ca^{2+} binding in r-[∇K^5]PC and r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC. Titrations with Ca^{2+} of the change in fluorescence of these two mutants are illustrated in Figure 4. The C_{50} values (and the maximal fluorescence change) for Ca^{2+} have been deter-

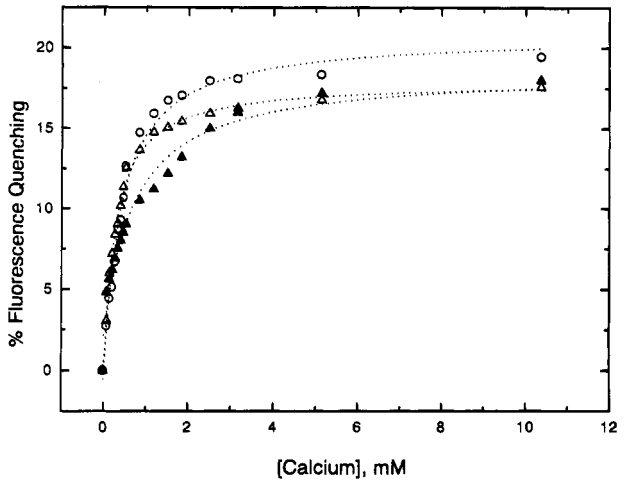

FIGURE 4: Titration of the effects of Ca^{2+} on the intrinsic fluorescence of r-PC mutants. Incremental additions of Ca^{2+} were made to the proteins, and the intrinsic fluorescence intensities were measured at 20 °C. The fluorescence (F_o) of the proteins obtained in the absence of Ca^{2+} was arbitrarily adjusted to 1.0, and fluorescence values (F) were obtained after addition of Ca^{2+}. The fluorescence quenching (%) was calculated as $(F_o - F)/F_o \times 100$ and plotted as a function of the total Ca^{2+} concentration. The C_{50} for Ca^{2+} and the ΔF_{max} (the total fluorescence change at saturation with Ca^{2+}) were determined by nonlinear least-squares iterative fitting of the data. The excitation and emission wavelengths were 283 and 340 nm. Slit widths of 1 and 16 nm were used for excitation and emission, respectively. The buffer was 20 mM Tris-HCl/100 mM NaCl, pH 7.4, at 20 °C. The protein concentrations were ca. 1 μM: (○) wtr-PC, (△) r-[∇K^5]PC, and (▲) r-[ΔPC$^{1-13}$/∇fIX$^{1-14}$]PC.

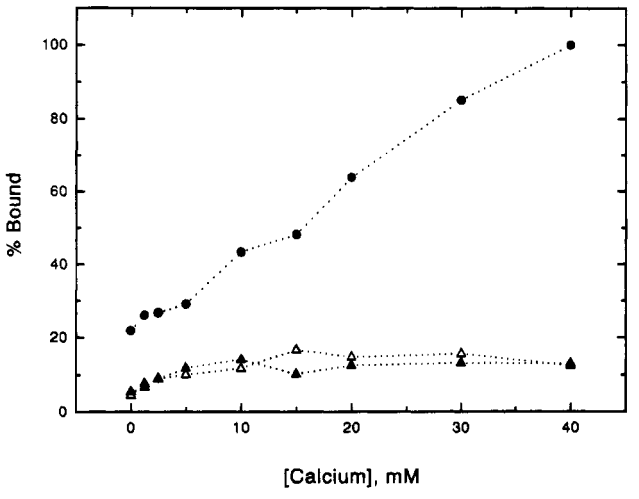

FIGURE 5: Binding of r-PC mutants to the [GD_{IX}]-directed, Ca^{2+}-dependent monoclonal antibody H5B7. The proteins in the presence of the MAb [^{125}I]H5B7 were titrated in microtiter plates with sequential additions of Ca^{2+}, and the amount of MAb bound was measured (in cpm) relative to the binding of the MAb bound to fIX in the presence of 40 mM Ca^{2+} (taken as 100%): (●) human plasma fIX, (△) r-[∇K^5]PC, and (▲) r-[ΔPC$^{1-13}$/∇fIX$^{1-14}$]PC.

mined from these plots to be 0.28 mM (18.0%) and 0.79 mM (16.6%) for r-[∇K^5]PC and r-[ΔPC$^{1-13}$/∇fIX$^{1-14}$]PC, respectively. These values are similar to those for wtr-PC, 0.40 mM (17.4%).

To determine whether the Ca^{2+}-dependent epitope for MAb H5B7, which has been shown to reside in the [GD_{IX}] (Velander et al., 1989), had been incorporated into r-[∇K^5]-PC and r-[ΔPC$^{1-13}$/∇fIX$^{1-14}$]PC, we titrated with Ca^{2+} the binding of this MAb to these mutant proteins. The results shown in Figure 5 demonstrate that the Ca^{2+}/protein com-

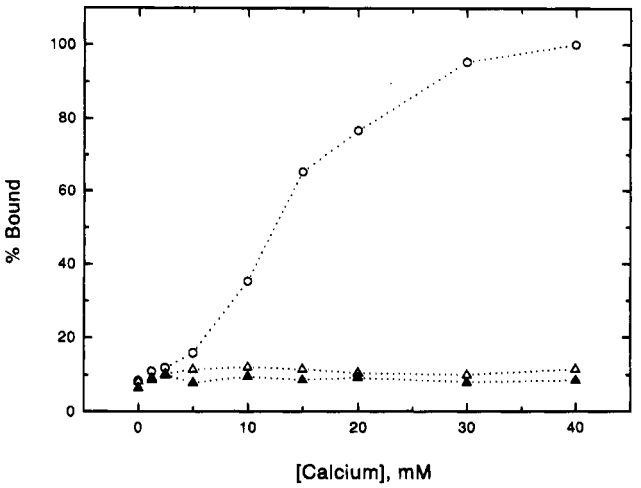

FIGURE 6: As in Figure 5, except that the [GD_{PC}]-directed, Ca^{2+}-dependent monoclonal antibody JTC1 was employed, and binding was relative to that of PC at 40 mM Ca^{2+}: (○) wtr-PC, (△) r-[∇K^5]-PC, and (▲) r-[ΔPC$^{1-13}$/∇fIX$^{1-14}$]PC.

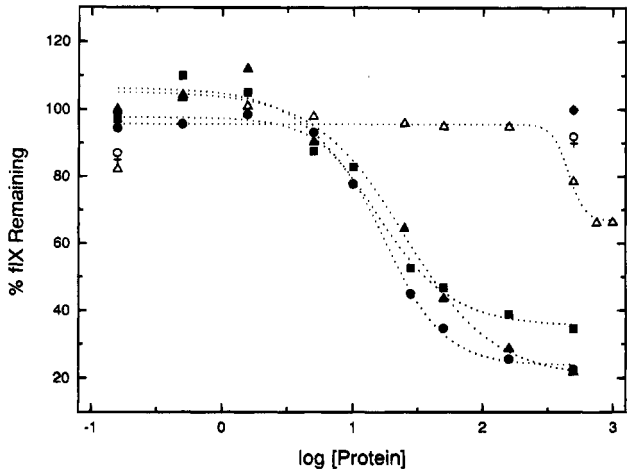

FIGURE 7: Competition with [^{125}I]fIX of r-PC mutants for binding to BAEC. The percent of fIX bound to the cells was taken as the radioactivity at any concentration of competitor relative to the radioactivity present in the absence of the competitor (taken as 100%). The temperature of the cell-binding phase of the experiment was 4 °C. The proteins used as competitors for [^{125}I]fIX binding were (○) wtr-PC, (●) human plasma fIX, (△) r-[∇K^5]PC, (■) r-[ΔPC$^{1-46}$/∇fIX$^{1-47}$]PC, (+) r-[ΔPC$^{47-137}$/∇fIX$^{48-128}$]PC, (◆) r-[ΔPC$^{38-46}$/∇fIX$^{39-47}$]PC, and (▲) r-[ΔPC$^{1-13}$/∇fIX$^{1-14}$]PC.

plexes do not interact with this MAb, at least with an affinity within 10−100-fold of the strength of that of fIX. A similar result has been obtained with MAb JTC1 (Figure 6), which possesses a corresponding Ca^{2+}-dependent epitope within the [GD_{PC}] (Wakabayashi et al., 1986). In this latter case, even increasing the JTC1 concentrations 3-fold yielded protein titration results similar to those in Figure 6, suggesting that the Ca^{2+}/mutant protein complexes do not significantly interact with MAb JTC1.

The ability of these and other previously described chimeras of r-fIX/PC to displace [^{125}I]fIX from its receptor on BAECs has been evaluated. The results are illustrated in Figure 7. Neither r-PC, r-[ΔPC$^{38-46}$/∇fIX$^{39-47}$]PC, nor r-[ΔPC$^{47-137}$/∇fIX$^{48-128}$]PC were capable of significant displacement of [^{125}I]fIX at the low concentrations of unlabeled fIX (IC_{50} = 9 nM), r-[ΔPC$^{1-46}$/∇fIX$^{1-47}$]PC (IC_{50} = 12 nM), or r-[ΔPC$^{1-13}$/∇fIX$^{1-14}$]PC (IC_{50} = 13 nM) that were very effective in this regard. The mutant r-[∇K^5]PC displaced [^{125}I]fIX only at very high concentrations of the competitor

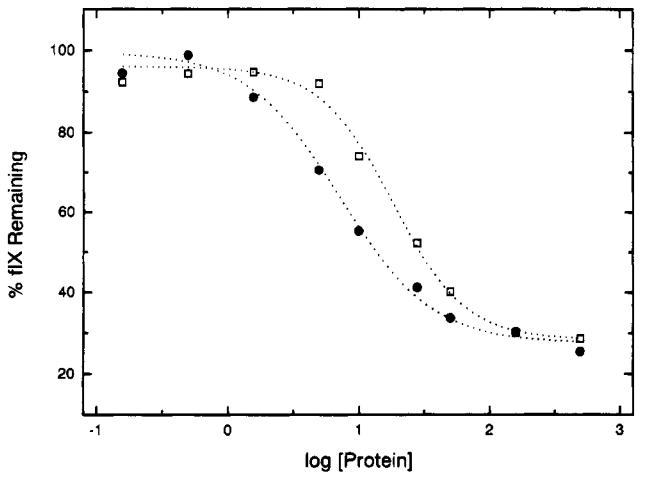

FIGURE 8: Competition with [^{125}I]fIX for binding to BAEC at 4 °C, as in Figure 7: (●) human plasma fIX and (□) $[GD_{IX}/HS_{IX}]^{1-47}$.

(IC_{50} > 500 nM), while, as seen in Figure 8, the synthetic peptide $[GD_{IX}/HS_{IX}]^{1-47}$ also was nearly as effective (IC_{50} of 16 nM) as fIX in competitive BAEC binding.

Both mutants were converted to their corresponding r-APCs by Protac C and assayed for their enzymatic activities toward r-APC substrates. The plasma-based APTT activity of r-[∇K^5]APC was 70% of that of wtr-APC when present at the same amidolytic activity of the wt-enzyme. Similarly, for r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]APC, the APTT activity was 83%of that of wtr-APC. *In vitro* assays of the abilities of these two mutants to inactivate fVIII using synthetic acidic PL as substitutes for cell membranes demonstrated that this activity of r-[∇K^5]APC was 76% of that of wtr-APC, again at the same amidolytic activities of the two enzymes, while that for r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]APC was 90% of that of wtr-APC under these conditions. These latter results are illustrated in Figure 9.

Finally, it was of considerable interest to determine whether chimeric r-APCs, when bound to BAEC via the fIX receptor, would provide anticoagulant activity to these cells. To address this question, r-fVIII inactivation assays were employed, with the strong binding r-PC chimeras r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]APC and r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]APC. While r-APC did not yield significant (<5%) activity under the conditions of this assay (because it did not interact with BAEC it was washed out of the assay system prior to addition of fVIII), both mutants that bound to BAEC did display r-fVIII inactivation activity, nearly to the same degree as when bound to acidic PL (the variance in this assay is approximately 10%). This activity was progressively eliminated as the synthetic cell-binding peptide $[GD_{IX}/HS_{IX}]^{1-47}$ was titrated into adherent BAEC containing r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]APC or r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]APC prior to addition of r-fVIII. The IC_{50} of this peptide for displacement of either of the chimeras was approximately 19 nM, a value similar to that found in the competitive BAEC-binding experiments. The titrations are illustrated in Figure 10.

DISCUSSION

We have found previously that substitution of the entire $[GD_{IX}]$ and $[HS_{IX}]$ modules for those same domains of PC led to a chimeric r-PC, r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]PC, that was functional in terms of its ability to be converted to APC by fIIa and fIIa/Tm. In addition, the resultant chimeric r-APC

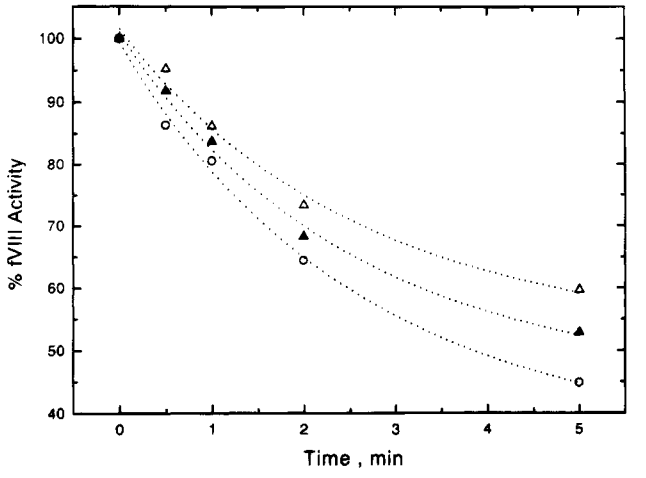

FIGURE 9: Kinetics of inactivation of human r-fVIII by r-APC mutants at 37 °C using phospholipid vesicles as the substitute for membrane lipid. All r-APC mutants were present at the same amidolytic activities. A two-stage assay for fVIII inactivation kinetics was employed, the details of which are described in the methods section. In stage 1 of the assay, fVIII was inactivated with r-APC in the presence of Ca^{2+}/PL for various periods of time and the remaining activatable fVIII was converted to fVIIIa with thrombin/Ca^{2+}/PL. Next, in stage 2 of the assay, the amount of fVIIIa present was determined by its ability to stimulate fX activation in the complete tenase system, additionally consisting of fX/fIXaβ/Ca^{2+}/PL. The amount of fXa generated in stage 2 was determined in rate assays using S2222 as the chromogenic substrate. The ln of the amount of fVIII remaining in stage 1 is plotted against the time of incubation of fVIII with the relevant r-APC mutant. The buffer employed contained 20 mM Hepes-NaOH/150 mM NaCl, pH 7.4, at 37 °C: (○) wtr-APC, (△) r-[∇K^5]APC, and (▲) r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]APC.

still possessed significant activity toward its natural substrates in the presence of PL (Christiansen & Castellino, 1994). This, coupled with a previous study indicating that the BAEC receptor-binding properties of fIX resided within amino acid residues 3−11 of $[GD_{IX}]$ (Cheung et al., 1992), suggested the possibility that r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]PC would contain these cell-binding properties of fIX. We demonstrate herein that r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]PC was fully competitive with fIX for BAEC binding (Figure 7), a property not found in PC itself or in a chimeric r-PC construct wherein only $[HS_{IX}]$ was exchanged with that same region of PC. An additional chimera in which $[EGF1/2_{IX}]$ was inserted in PC in place of $[EGF1/2_{PC}]$ also did not yield a protein capable of displacement of [^{125}I]fIX from BAEC (Figure 7). These results show that the BAEC-binding properties of fIX can be transferred into PC by insertion of regions within the $[GD_{IX}]$, a conclusion that is even more strongly fortified by the finding that a synthetic peptide containing the $[GD_{IX}/HS_{IX}]$ domains, viz., $[GD_{IX}/HS_{IX}]^{1-47}$, also fully and effectively displaced fIX from BAEC (Figure 8).

In order to determine whether the BAEC-binding properties of fIX could be conveyed into PC with a more minimal change in this latter protein, we generated a chimeric r-PC that contained a replacement of amino acids 1−14 of fIX with the homologous residues 1−13 of PC. This chimera, r-[$\Delta PC^{1-13}/\nabla fIX^{1-14}$]PC, did in fact function nearly as effectively as fIX and r-[$\Delta PC^{1-46}/\nabla fIX^{1-47}$]PC in competition with [^{125}I]fIX for binding to BAEC. This observation supports the finding that a chimeric r-fVII containing replacements of residues 3−11 by those of fIX also competed effectively with fIX for BAEC binding (Cheung et al., 1992).

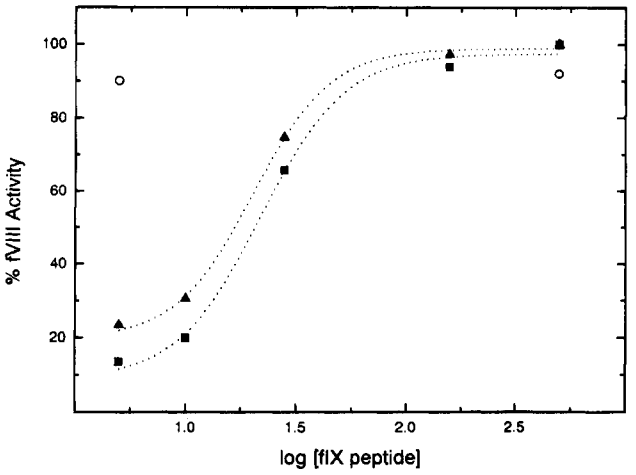

FIGURE 10: Activity of r-PC mutants bound to BAEC toward inactivation of r-fVIII at 37 °C using a competitive two-stage assay. The desired r-APC mutant, in the presence of various concentrations of $[GD_{IX}/HS_{IX}]^{1-47}$, was added to BAEC (stage 1). Next, r-fVIII was incubated with the cells to allow r-fVIII inactivation by the r-APC mutants that remained bound to BAEC. The remaining activatable r-fVIII was converted to fVIIIa using fIIa/Ca^{2+}/PL, and the amounts of r-fVIIIa present were then determined in stage 2 of the assay by its ability to stimulate the activation rate of fX in the complete tenase system, additionally consisting of fX/fIXaβ/Ca^{2+}/PL. The amount of fXa generated in stage 2 was determined in rate assays using S2222 as the chromogenic substrate. The graphs illustrate the velocity of S2222 hydrolysis (in absorbancy at 405 nm/min) versus the logarithm of the concentration of peptide added to stage 1. Since wtr-APC does not interact with BAEC, it is washed from the cells in stage 1, and thus fVIII inactivation was not observed in stage 2: (○) wtr-APC, (■) r-$[\Delta PC^{1-46}/\nabla fIX^{1-47}]$-APC, and (▲) r-$[\Delta PC^{1-13}/\nabla fIX^{1}1-^{14}]$APC.

In order to consider these results more thoroughly, alignment of the amino acid sequences for the amino-terminal 13 residues of the light chain of PC and fVII with the amino-terminal 14 residues of fIX is shown below:

PC:	A	N	S	F	-	L	γ	γ	L	R	H	S	S	L
fVII:	A	N	A	F	-	L	γ	γ	L	R	P	G	S	L
fIX:	Y	N	S	G	K	L	γ	γ	F	V	Q	G	N	L

One major difference between the three proteins is the addition in fIX of a Lys residue at amino acid position 5. In order to determine whether this single amino acid inserted into PC would confer fIX-associated BAEC binding to the resulting mutant, we generated r-$[\nabla K^5$/PC and determined the ability of this mutant to displace [^{125}I]fIX from BAEC. Some displacement was observed at very high concentrations of the mutant, suggesting that Lys^5 is a likely part of the receptor-binding site of fIX. Other amino acids in this region of the $[GD_{IX}]$ that might play a specific role in fIX-related BAEC binding would be those between residues 3 and 11 that are unique to fIX. In addition to Lys^5, these amino acids would be Gly^4, Phe^9, Val^{10}, and Gln^{11} of fIX, and these residues are the focus of our current attention.

Not only are the mutants r-$[\nabla K^5]$PC and r-$[\Delta PC^{1-13}/\nabla fIX^{1-14}]$PC of interest regarding BAEC binding but are also of interest with respect to structure–function relationships of PC. In the case of r-$[\nabla K^5]$PC, the insertion of a hydrophilic side chain in the midst of an important grouping of PL-binding hydrophobic residues, viz., Phe^4, Leu^5, and Leu^8, might result in reduction of PL-dependent anticoagulation activity, as was the situation when the r-$[L^5Q]$PC/APC (Zhang & Castellino, 1994) and r-$[L^8Q]$PC/APC[3] mutants were examined. In these cases, the placement of hydrophilic for hydrophobic head groups at these locations led to a near total loss of PL-dependent APC anticoagulant activity. Regarding the mutant r-$[\Delta PC^{1-13}/\nabla fIX^{1-14}]$APC, it is of considerable importance to determine whether the anticoagulant properties remain intact, since if this was the situation it would be possible to generate an anticoagulant population of endothelial cells. When the entire $[GD_{IX}/HS_{IX}]$ was inserted in a chimeric r-PC, anticoagulant activity was maintained; however, it is possible that this is the case only with the intact $[GD_{IX}]$ substitution and not with a small fragment thereof, which might be disruptive of local $[GD_{PC}]$ conformations.

As seen from the illustrations of Figure 4, both r-$[\nabla K^5]$-PC and r-$[\Delta PC^{1-13}/\nabla fIX^{1-14}]$PC underwent the Ca^{2+}-induced intrinsic fluorescence alterations characteristic of wtr-PC and provided C_{50} values for Ca^{2+} and relative maximal fluorescence changes that were typical for those of wtr-PC. This indicated that both of these mutants adopted the Ca^{2+}-dependent conformations of PC. In order to determine whether a $[GD_{IX}]$-directed, Ca^{2+}-dependent MAb would recognize these Ca^{2+}-induced conformations, we examined the cross-reactivities of these mutants against the MAb H5B7. As seen by the data of Figure 5, such reactions were not observed with these two mutant proteins, suggesting that this epitope was not concomitantly transferred into the chimeric r-PC. Considering that previous studies demonstrated full expression of this epitope in r-$[\Delta PC^{1-46}/\nabla fIX^{1-47}]$PC (Christiansen & Castellino, 1994), the present work indicates that the epitope for this antibody on fIX either is located outside of residues 1–14 of $[GD_{IX}]$ or has major contributions from other amino acids found in $[GD_{IX}]$ that are not present in $[GD_{PC}]$. In addition, the MAb JTC1, specific to a functionally similar Ca^{2+}-dependent epitope on $[GD_{PC}]$, was employed to assess whether the Ca^{2+}-induced conformations of mutants r-$[\nabla K^5]$PC and r-$[\Delta PC^{1-13}/\nabla fIX^{1-14}]$PC still recognized this antibody. The graphs of Figure 6 show that neither the Ca^{2+}/r-$[\nabla K^5]$PC complex nor that of Ca^{2+}/r-$[\Delta PC^{1-13}/\nabla fIX^{1-14}]$PC were reactive with this MAb. These latter data are in agreement with other investigations that address this point and which demonstrate that mutations in the amino-terminal region of r-PC greatly influence its reactivity toward MAb JTC1 (Zhang & Castellino, 1992, 1994; Christiansen & Castellino, 1994), despite adoption of the Ca^{2+}-dependent conformations of the mutant proteins employed. From this and other work, we believe that there are essential amino acids needed for formation of a complete epitope, in addition to the proper Ca^{2+}-induced conformation. In particular, any substitutions between residues 1 and 7 seem particularly detrimental in this regard.

After complete activation of the chimeric PC zymogens, r-$[\nabla K^5]$APC possessed approximately 70% of the *ex vivo* plasma-based anticoagulant activity of wtr-APC and r-$[\Delta PC^{1-13}/\nabla fIX^{1-14}]$APC contained approximately 83% of the relative activity of its wild-type counterpart. Similar activities were found for the mutants in the *in vitro* r-fVIII inactivation assays. These results not only indicated that the mutations likely did not disrupt proper Ca^{2+} and PL binding but also that alignments of the mutant r-APCs on the PL

[3] R. M. Robertson, unpublished experiments.

vesicle with that of its protein substrates, fV and fVIII, were also similar to those of wtr-APC.

The results of this study show that the BAEC-binding region of fIX can be clearly identified and transferred in a very short peptide segment to proteins such as PC and APC. Thus, an endothelial cell population that is nonthrombogenic and anticoagulant can be generated. Using strategies such as these, chimeric coagulation, anticoagulation, and thrombolytic proteins could be constructed that interact with specific surfaces, thereby allowing molecules of choice to be placed on such biological surfaces. This approach could aid design of new agents on cells that alter their hemostatic properties in predesigned manners.

REFERENCES

Ahmad, S. S., Rawala-Sheikh, R., Cheung, W.-F., Jameson, B. A., Stafford, D. W., & Walsh, P. N. (1994) *Biochemistry 33*, 12048−12055.

Amphlett, G. W., Kisiel, W., & Castellino, F. J. (1981) *Biochemistry 20*, 2156−2161.

Astermark, J., & Stenflo, J. (1991) *J. Biol. Chem. 266*, 2438−2443.

Astermark, J., Hogg, P. J., Bjork, I., & Stenflo, J. (1992) *J. Biol. Chem. 267*, 3249−3256.

Astermark, J., Hogg, P. J., & Stenflo, J. (1994) *J. Biol. Chem. 269*, 3682−3689.

Cheung, W. F., Straight, D. L., Smith, K. J., Lin, S. W., Roberts, H. R., & Stafford, D. W. (1991) *J. Biol. Chem. 266*, 8797−8800.

Cheung, W. F., Hamaguchi, N., Smith, K. J., & Stafford, D. W. (1992) *J. Biol. Chem. 267*, 20529−20531.

Chibber, B. A. K., Urano, S., & Castellino, F. J. (1990) *Int. J. Pept. Protein Res. 35*, 73−80.

Christiansen, W. T., & Castellino, F. J. (1994) *Biochemistry 33*, 5901−5911.

Clarke, J. H., Light, D. R., Blasko, E., Parkinson, J. F., Nagashima, M., McLean, K., Vilander, L., Andrews, J. H., Morser, J., & Glaser, C. B. (1993) *J. Biol. Chem. 268*, 6309−6315.

Colpitts, T. L., & Castellino, F. J. (1994) *Biochemistry 33*, 3501−3508.

De Serrano, V. S., & Castellino, F. J. (1992) *Biochemistry 31*, 3326−3335.

De Serrano, V. S., Menhart, N., & Castellino, F. J. (1992) *Arch. Biochem. Biophys. 294*, 282−290.

Foster, D. C., Yoshitake, S., & Davie, E. W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4673−4677.

Grinnell, B. W., Walls, J. D., & Gerlitz, B. (1991) *J. Biol. Chem. 266*, 9778−9785.

Heeb, M. J., Schwartz, P., White, T., Lammle, B., Berrettini, M., & Griffin, J. (1988) *Thromb. Res. 52*, 33−43.

Jacobs, M., Freedman, S. J., Furie, B. C., & Furie, B. (1994) *J. Biol. Chem. 269*, 25494−25501.

Jhingan, A., Zhang, L., Christiansen, W. T., & Castellino, F. J. (1994) *Biochemistry 33*, 1869−1875.

Kisiel, W., Canfield, W. M., Ericsson, L. H., & Davie, E. W. (1977) *Biochemistry 16*, 5824−5831.

Leytus, S. P., Foster, D. C., Kurachi, K., & Davie, E. W. (1986) *Biochemistry 25*, 5098−5102.

Liebman, H. A. (1993) *Eur. J. Biochem. 212*, 339−345.

Liebman, H. A., Furie, B. C., & Furie, B. (1987) *J. Biol. Chem. 262*, 7605−7612.

Menhart, N., Sehl, L. C., Kelley, R. F., & Castellino, F. J. (1991) *Biochemistry 30*, 1948−1957.

Nelsestuen, G. L., Kisiel, W., & DiScipio, R. G. (1978) *Biochemistry 17*, 2134−2138.

O'Hara, P. J., Grant, F. J., Haldeman, B. A., Gray, C. L., Insley, M. Y., Hagen, F. S., & Murray, M. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5158−5162.

Ohlin, A.-K., Linse, S., & Stenflo, J. (1988) *J. Biol. Chem. 263*, 7411−7417.

Petersen, L. C., Schiodt, J., & Christensen, U. (1994) *FEBS Lett. 347*, 73−79.

Toomey, J. R., Smith, K. J., Roberts, H. R., & Stafford, D. W. (1992) *Biochemistry 31*, 1806−1808.

Valcarce, C., Selander-Sunnerhagen, M., Tamlitz, A. M., Drakenberg, T., Bjork, I., & Stenflo, J. (1993) *J. Biol. Chem. 268*, 26673−26678.

Vehar, G. A., & Davie, E. W. (1980) *Biochemistry 19*, 401−410.

Velander, W. H., Orthner, C. L., Tharakan, J. P., Madurawe, R. D., Ralston, A. H., Strickland, D. K., & Drohan, W. N. (1989) *Biotechnol. Prog. 5*, 119−125.

Wakabayashi, K., Sakata, Y., & Aoki, N. (1986) *J. Biol. Chem. 261*, 11097−11105.

Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W., & Kurachi, K. (1985) *Biochemistry 24*, 3736−3750.

Yu, S., Zhang, L., Jhingan, A., Christiansen, W. T., & Castellino, F. J. (1994) *Biochemistry 33*, 823−831.

Zhang, L., & Castellino, F. J. (1990) *Biochemistry 29*, 10828−10834.

Zhang, L., & Castellino, F. J. (1991) *Biochemistry 30*, 6696−6704.

Zhang, L., & Castellino, F. J. (1992) *J. Biol. Chem. 267*, 26078−26084.

Zhang, L., & Castellino, F. J. (1994) *J. Biol. Chem. 269*, 3590−3595.

Zhang, L., Jhingan, A., & Castellino, F. J. (1992) *Blood 80*, 942−952.

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