Construction, Expression, and Properties of a Recombinant Chimeric Human Protein C with Replacement of Its Growth Factor-like Domains by Those of Human Coagulation Factor IX†

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ABSTRACT: The cDNA encoding a chimeric human protein C (PC), in which its epidermal growth factor-(EGF) like regions have been replaced with equivalent structures from human factor IX (fIX), was constructed and the gene product was expressed in human 293 cells. A molecular subpopulation of the recombinant chimeric protein (r-[PC/ΔEGF-1,2/∇fIXEGF-1,2]) was purified that contained the full complement (9 residues/mol) of γ–carboxyglutamic acid (Gla). After conversion by thrombin to its activated form (r-[APC/ΔEGF-1,2/∇fIXEGF-1,2]), this latter enzyme was found to possess approximately 10% of the activity of wild-type recombinant APC (wtr-APC) in an APTT assay. In assay systems employing purified components, the activity of the mutant enzyme toward prothrombinase cofactor Va (fVa) and tenase cofactor VIII (fVIII) was approximately 30% and <10%, respectively, of that of wtr-APC. The chimeric protein displayed full reactivity with a Ca^{2+}-dependent monoclonal antibody to the Gla domain of PC, yielding a C_{50} for Ca^{2+} that was very similar to that obtained with wtr-PC (ca. 3.7 mM). Titrations of the dependency on Ca^{2+} of the intrinsic fluorescence of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] allowed calculation of a C_{50} value of 0.34 mM, again very similar to that of wtr-PC. As with wtr-PC, Ca^{2+} inhibited the thrombin-catalyzed activation of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] with a K_i of 148 μM, as compared to a K_i of 125 μM for wtr-PC. At a saturating level of Ca^{2+}, activation of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] by the thrombin/thrombomodulin (thrombin/TM) complex occurred at approximately 70% of the rate of that of wtr-PC. The results suggest that (1) despite the substitution of substantial domain regions of the light chain of PC with those of a functionally unrelated protein, the chimeric protein retains essential features of PC zymogen; (2) the ability of PC to adopt its Ca^{2+}-dependent conformation is not specifically dependent on its EGF-like regions; (3) the high-affinity Ca^{2+} sites responsible for inhibition of the thrombin-catalyzed activation of PC, and stimulation of this same activation by thrombin/TM, are not specifically dependent on the EGF-like domains of PC; and (4) determinants present in the EGF-like domains of APC play a role in its anticoagulant properties, perhaps by directing specific alignments with its physiological substrates on the phospholipid surface and/or through general subtle conformational properties of the enzyme that are dependent on the integrity of the EGF-like regions of PC. Additionally, the differences in activity of the mutant APC toward fVa and fVIII may be due to effects resulting from a specific interaction between the fIX EGF regions of [PC/ΔEGF-1,2/∇fIXEGF-1,2] and fVIII, a natural cofactor for fIXa.

Protein C (PC)[1] is the plasma protein zymogen of the anticoagulant and profibrinolytic serine protease APC. The anticoagulant activity of this enzyme is based on its ability to catalyze by limited proteolysis the inactivation of coagulation cofactors fV/fVa (Kisiel et al., 1977) and fVIII/fVIIIa (Vehar & Davie, 1980), thus inhibiting generation of thrombin and fXa, respectively. The presence of Ca^{2+} and PL (Kisiel et al., 1977), along with a cofactor, PS (Walker, 1980, 1981), results in stimulation of these reactions. The profibrinolytic activity of APC, which is also stimulated by PS (DeFouw et al., 1993), is a result of several complex effects. In this regard, it has been shown that APC causes release of plasminogen activators from vascular endothelial cells into blood (Comp & Esmon, 1981), thus promoting plasminogen activation. Further, it is believed that APC possesses the capability to interact with plasminogen activator inhibitor 1 (Taylor & Lockhart, 1985), thus diverting this inhibitor from inactivation of plasminogen activators and in this way promoting a fibrinolytic state. Finally, it has recently been shown that APC inhibits an antifibrinolytic component released as a consequence of prothrombin activation, thereby providing a fibrinolytic potential (Bajzar & Nesheim, 1991, 1993).

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[1] Abbreviations: PC, protein C; APC, activated protein C; EGF, epidermal growth factor; r-[PC/ΔEGF-1,2/∇fIXEGF-1,2], a recombinant chimera of protein C in which its two epidermal growth factor-like domains have been replaced by those same regions of human coagulation factor IX; r-[APC/ΔEGF-1,2/∇fIXEGF-1,2], the activated form of the protein C chimera described above; PS, protein S; TM, thrombomodulin; fV, coagulation factor V; fVa, activated coagulation factor V; i-fVa, activated coagulation factor V inactivated as a result of proteolysis with activated protein C; fVII, coagulation factor VII; fVIII, coagulation factor VIII; fVIIIa, activated coagulation factor VIII; i-fVIIIa; activated coagulation factor VIII inactivated as a result of proteolysis with activated protein C; fIX, coagulation factor IX; fIXaβ, activated coagulation factor IX; fX, coagulation factor X; fXa, activated coagulation factor X; Gla, γ-carboxyglutamic acid; Hya, β-hydroxyaspartic acid; Hyn, β-hydroxyasparagine; PCR, polymerase chain reaction; PL, 60%/40% (w/w) sonicated dispersion of chicken egg phosphatidylcholine/bovine brain phosphatidylserine; r, recombinant; wt, wild type; S2222, benzoyl-L-Ile-L-Glu(OR)-Gly-L-Arg-*p*-nitroanilide; S2366, L-pyro-Glu-L-Pro-L-Arg-*p*-nitroanilide; DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; APTT, activated partial thromboplastin time.

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PC is a member of a class of vitamin K-dependent proteins that are composed of a series of domain regions. The PC coding sequence is contained in eight exons in the genomic DNA (Foster et al., 1985). The first exon codes for a signal sequence, followed by an exon which encodes the propeptide region and progresses through the entire Gla domain. Next, a short hydrophobic helical stretch is found in the third exon, followed by two consecutive exons coding for EGF-like regions. The sixth exon translates into a stretch of amino acids containing the activation peptide of PC. This is then followed by two exons, the first of which encodes a polypeptide containing the histidine residue of the serine protease catalytic triad and another that encodes a peptide region that contains both the aspartic acid and serine residues of this same catalytic triad. Unprocessed PC is composed of a latent light chain of 155 amino acids, a latent heavy chain of 262 amino acids, and a dipeptide that connects the two chains. A considerable degree of processing occurs in the generation of the mature protein. This includes proteolytic cleavage of the leader polypeptide (Foster et al., 1985); glycosylation at four Asn residues (Kisiel, 1979); γ–carboxylation of nine Glu residues at sequence positions 6, 7, 14, 16, 19, 20, 25, 26, and 29, yielding Gla (DiScipio & Davie, 1979); β-hydroxylation at Asp71, providing Hya (Drakenberg et al., 1983); and cleavage of the dipeptide linker, Lys156-Arg157, between the latent heavy and light chains of PC (Foster & Davie, 1984).

The functional properties of the EGF regions of PC are not well understood, but EGF-like domains in other proteins are involved in protein–protein interactions. Examples of this are the involvement of the EGF-like domain of urokinase in binding to its cellular receptor (Appella et al., 1987); the binding of thrombin to EGF-like regions in TM (Suzuki et al., 1989); the interaction of fVIIIa with the carboxy-terminal EGF-like domain of fIX (Astermark & Stenflo, 1991); the participation of the first (amino-terminal) EGF-like domain of coagulation fVII in binding to tissue factor (Clarke et al., 1992); the interaction of EGF-like domains of PS with C4-binding protein (Dahlback et al., 1990) and PC (Ohlin et al., 1988a); and the interaction of the amino-terminal EGF-like domain of fIXa with fX (Astermark et al., 1992). Some of the EGF-like regions in a variety of proteins domains contain Hya and possess a high-affinity binding site for Ca^{2+} in these domains (Drakenberg et al., 1983). The amino–terminal EGF-like domains of bovine fVII (McMullen et al., 1983a; Hagen et al., 1986), human and bovine fIX (Morita et al., 1984; McCord et al., 1990), human and bovine fX (McMullen et al., 1983b; Persson et al., 1989, 1991; Selander-Sunnerhagen et al., 1992), and human and bovine PC (Johnson et al., 1983; Ohlin & Stenflo, 1987; Ohlin et al., 1988b) are relevant examples. Hyn exists in equivalent positions in EGF domains 2–4 of PS (Stenflo et al., 1987). These proteins show Ca^{2+}-dependent functional properties.

In order to probe more fully the properties of PC and APC that specifically depend upon its EGF-like domains, we have constructed a recombinant chimeric protein in which both EGF domains of PC were replaced by equivalent regions of a functionally unrelated protein, fIX. We believed that studies with this mutated r-PC would allow conclusions to be regarding the importance of the EGF-like domains in determining the structural and functional integrities of PC and APC.

MATERIALS AND METHODS

Proteins. Human wtr-PC was expressed in human kidney 293 cells and purified as described previously (Zhang & Castellino, 1990). Wild-type r-APC and r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] were prepared from wtr-PC and r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] by activation with the *Agikstrodon contortix contortix* venom activator of PC, Protac C (American Diagnostica, New York). The progress of each activation reaction was monitored spectrophotometrically by the appearance of amidolytic activity of wtr-APC and r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] toward the chromogenic substrate S2366 (Helena Laboratories, Beaumont, TX).

Human plasma fV was donated by Dr. Paula Tracy (Burlington, VT). This protein was converted to fVa by incubation in borosilicate glass tubes of fV (1 μM) with thrombin (final concentration 3 NIH units/mL) for 15 min at 37 °C. The final volume was 13 μL. The thrombin in the solution was inactivated by addition of DAPA (final concentration 10 μM) (Katzman et al., 1981). The final volume after activation was adjusted to 50 μL with a buffer of 20 mM Hepes-NaOH/150 mM NaCl/0.1% bovine serum albumin, pH 7.4.

Human r-fVIII was provided by Dr. Randall Kaufman (Cambridge, MA). Bovine fX (Bajaj & Mann, 1973), bovine prothrombin (Bajaj & Mann, 1973), and bovine fIX (Amphlett et al., 1978) were purified as described. Bovine fIX was converted to fIXaβ as published earlier (Amphlett et al., 1979). Rabbit TM was donated by Dr. Charles Esmon (Oklahoma City, OK). Antithrombin III, human fX, and human prothrombin were obtained from Enzyme Research Laboratories (South Bend, IN).

The Ca^{2+}-dependent monoclonal antibody to plasma PC, JTC-1, was provided by Drs. N. Aoki and K. Wakabayashi (Tokyo, Japan). This antibody was radiolabeled using the Iodobead (Pierce Chemical Co., Rockford, IL) procedure as described previously (Zhang & Castellino, 1992). Monoclonal anti-PC, C3 (Heeb et al., 1988), was provided by Dr. John Griffin (La Jolla, CA) and monoclonal antibody LI was generated in this laboratory (Zhang, 1991).

Restriction endonucleases were purchased from Fisher Scientific (Springfield, NJ) and Stratagene (La Jolla, CA) and were used according to the manufacturers' recommendations.

Genes. The cDNAs coding for human PC and human fIX were provided by Dr. Earl Davie (Seattle, WA). The cDNA for PC in pUC119 (Foster & Davie, 1984) was modified as previously described (Zhang & Castellino, 1990).

The cDNA for fIX in pBR322 was excised from this plasmid and inserted into pUC119 *via PstI* sites present outside of the fIX coding region (Kurachi & Davie, 1982).

Construction of the Chimeric PC Containing the Growth Factor-like Domains of fIX. For these manipulations, the starting materials were the cDNAs for the entire coding sequences of human PC (p119[PC]) and fIX (p119[fIX]), both in pUC119.

A unique restriction endonuclease site for *BclI* was inserted 3′ of base 267 in PC (base 1 begins the initiation codon for PC), yielding (p119[PC/BclI]) by annealing the single-strand cDNA of PC with the following synthetic oligonucleotides (the lower-case letters are the mutagenic bases and the restriction cleavage site is indicated by *), followed by routine extension:

5′-GAC GGT* GAt CAG TGC TTG

Another unique restriction site for *XmaIII* was inserted 3′ of

base 528 of PC in the same manner employing the oligonucleotide

5′-AG TGT CAC CC*g GCc GTG AAG TTC C

with the single-strand cDNA from p119[PC/*Bcl*I].

A *BCl*I/*Xma*III restriction digest resulted in excision of a fragment beginning at the first base of the codon for Asp48 of PC through the second base of the codon for Pro135. This eliminated the cDNA encoding both EGF domains of PC and provided plasmid p119[PC/ΔEGF1,2].

The cDNA coding for both EGF domains of fIX was amplified from p119[fIX] by PCR. The 5′-end of the EGF domains from fIX was amplified with the oligonucleotide

5′-T GGt* GAT CAG TGT GAG T

After cleavage of the PCR product with *Bcl*I, this oligonucleotide encoded the amino-terminal region of the EGF-1 domain of fIX in p119[PC/ΔEGF1-2] beginning at a location two amino acids downstream of the amino-terminal intron of EGF-1 of fIX. These two missing amino acids, *viz.*, Asp47 and Gly48 of fIX, which are identical to those of PC, *viz.*, Asp46 and Gly47 of PC, had been retained in their proper locations in construction of p119[PC/ΔEGF1,2].

Concomitantly, the following 3′-complementary strand oligonucleotide

3′-C AGG ACA CTT GGc CGg* CAC

was employed for amplification from p119[fIX] of the 3′-terminus of the EGF domains of fIX. After cleavage with *Xma*III, the remaining oligonucleotide coded for the carboxyl-terminal region of EGF-2 of fIX beginning at the third base of the codon for Lys122 of fIX, followed by the fIX residues Ser123, Cys124, and Glu125 and the first two bases for Pro126 of fIX. The third base of the codon for this identical Pro residue of PC, as well as the two remaining amino acids needed to complete the exon at the 3′-terminus of the EGF-2 domain, which are identical in fIX and PC, are present in p119[PC/ΔEGF1,2].

After ligation of the above *Bcl*I/*Xma*III-digested amplified EGF-1/EGF-2 domain of fIX into p119[PC/ΔEGF1,2], the resulting plasmid, p119[PC/ΔEGF-1,2/∇fIXEGF-1,2], contained exact replacement of the complete exons of the EGF-1/EGF-2 domain of PC by the complete exons of the EGF-1/EGF-2 domain of fIX. DNA sequence analysis of the insert demonstrated that the desired replacement occurred.

Expression and Purification of the Chimeric Protein. The chimeric cDNA, p119[PC/ΔEGF-1,2/∇fIXEGF-1,2], was excised from pUC119 employing an *Nhe*I/*Xho*I restriction digestion and inserted into these same restriction sites in the multiple cloning region of the mammalian cell expression vector pCIS2M (Zhang & Castellino, 1990), resulting in plasmid pCIS2M[PC/ΔEGF-1,2/∇fIXEGF-1,2]. This latter plasmid was transfected into human kidney 293 cells (ATCC CRL 1573), positive clones were selected by assay of the cell conditioned medium with goat anti-human PC polyclonal antibody (provided by Dr. Thomas Edgington, La Jolla, CA), and the clones secreting r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] were propagated as previously described (Zhang & Castellino, 1990).

Purification of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] was accomplished by FPLC on FFQ columns at 4 °C as described in the case of purification of wtr-PC (Zhang et al., 1992). Two separate column steps were required using this same resin, the first employing elution with a gradient of Ca^{2+}, and the second requiring elution with a NaCl gradient.

Fluorescence Titrations. The intrinsic fluorescence alteration accompanying the binding of Ca^{2+} to PC was titrated with Ca^{2+} as detailed previously (Zhang & Castellino, 1992). These measurements were carried out at 20 °C with use of a SLM-Aminco 8000 (SLM-Aminco Instruments, Urbana, IL) recording spectrofluorometer. The excitation and emission wavelengths were 283 and 340 nm, respectively.

Ca^{2+} Dependency of Monoclonal Antibody JTC-1 Binding to the Chimeric Protein. Quantitative measures of the dependencies on Ca^{2+} of the binding of the Ca^{2+}-dependent conformational antibody, [^{125}I]JTC-1, to wtr-APC and r-[PC/ΔEGF-1,2/∇fLXEGF-1,2] were measured by the microtiter plate assay described previously (Zhang & Castellino, 1992).

Activation Kinetics of the Chimeric Protein by Thrombin and the Thrombin/Thrombomodulin Complex. A solution of wtr-PC or r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] (2 μM final concentration), in a buffer consisting of 20 mM Tris-HCl/100 mM NaCl/0.1% gelatin, pH 7.4, at 37 °C was added to a solution containing the desired levels of $CaCl_2$ and thrombin (0.4 nM final concentration) ± TM (8 nM final concentration). Aliquots (30 μL) were removed at various times and the thrombin was neutralized by addition of 10 μL of a solution of antithrombin III (1 mg/mL)/heparin (50 μg/mL)/5 mM EDTA, pH 7.4. The amount of wtr-APC or r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] present at each time was determined by amidolytic assay of these enzymes with substrate S2366.

Amidolytic Assays of r-APC and the Chimera. Solutions were prepared with 20–400 μL of substrate S2366 (stock solution 4.2 mM in H_2O), 40 μL of Tris-HCl, pH 7.4 (stock solution 1 M) at 37 °C, and 80 μL of NaCl (stock solution 1 M). A quantity of H_2O was added to adjust final volumes to 0.79 mL. The amidolytic reaction was accelerated by addition of 10 μL of wtr-APC or r-[APC/ΔEGF-1,2/∇fIXEGF-1,2], and rates of *p*-nitroanilide release were monitored by the increase of absorbancy at 405 nm.

APTT Assays. These assays, designed to measure the overall anticoagulant activity of r-[APC/ΔEGF-1,2/∇fIX-EGF-1,2] relative to wtr-APC, were conducted at 37 °C with PC-deficient plasma using the APTT assay kit (Sigma Diagnostics, St. Louis, MO), essentially as described earlier (Zhang et al., 1992).

Factor VIII Inactivation Assays. For assays of the initial rates of activation of fX in a complete purified system, and the effect of r-APC and the chimeric r-APC on these rates, we proceeded in the following manner. In the first stage of the assay, the abilities of wtr-APC and the r-APC chimera to inactivate r-fVIII were examined. Here, an amount of fVIII (4.4 nM final concentration) was incubated with PL [40 μM (in phosphate) final concentration], the desired r-APC (*ca.* 0.5 nM final concentration, small adjustments were made such that equal amidolytic activites of both r-APCs were present), and $CaCl_2$ (2.5 mM final concentration). The final volume was 150 μL in a buffer of 25 mM Hepes-NaOH/150 mM NaCl, pH 7.4. The times of these incubations were varied from 0 to 10 min at 37 °C. For activation of the remaining fVIII prior to assay in the fX system, the complete volume of 150 μL of the above fVIII inactivation mixture was incubated with 10 μL of a solution of 01 μM fIXaβ [for protection of fVIIIa activity (Lollar et al., 1984)] and 10 μL of a solution of 2 units/mL thrombin at 37 °C for 1.5 min. The amount of fVIIIa present was then assessed by its ability to stimulate the initial activation rate of fX. For this stage, 170 μL of the above solution was added to another solution containing, in a spectrometer cuvette at 37 °C, final concentrations of 40 μM (in phosphate) PL, 2.5 mM $CaCl_2$, and

180 μM chromogenic substrate S2222 (Helena Laboratories, Beaumont, TX). The final volume was 790 μL. After the baseline was recorded for 1 min, substrate hydrolysis was accelerated by addition of 10 μL of a solution of 32 μM fX. The rate of amidolysis of S2222 by the generated fXa was recorded continually for 2–5 min at 405 nm.

Factor Va Inactivation Assays. Here, again, a two-stage assay was employed. In the first phase, fVa was inactivated with either wtr-APC or r-[APC/ΔEGF-1,2/∇fIXEGF-1,2]. These experiments were carried out at room temperature in borosilicate glass tubes by incubation of final concentrations of fVa (0.1 μM) with the particular r-APC (0.5 nM) for different time periods (usually 0–12 min) in a buffer consisting of 20 mM Hepes-NaOH/150 mM NaCl/2 mM $CaCl_2$ or 15 mM $CaCl_2$/20 μM PL, pH 7.4. The amount of fVa remaining was determined by fluorometric assay with purified components in the complete prothrombinase system (Solymoss et al., 1988). In this case, 9 μL of the above fVa/i-fVa mixture was added to the reaction mixture (final volume 1.0 mL) containing 1.39 μM prothrombin/2.5 mM $CaCl_2$/20 μM PL/3 μM DAPA. The activation reaction was accelerated by addition of 8 nM (final concentration) fXa. The fluorescence resulting from uptake of DAPA by the generated thrombin was monitored continuously at 25° with a SLM-Aminco (Urbana, IL) SPF-8000 spectrofluorometer. The excitation and emission wavelengths were 335 and 565 nm, respectively.

Data Analysis for the Effects of APC on the Prothrombinase and Tenase Assays. The data obtained in both of these assays were treated similarly, with the exception being that rate assays were employed in the case of fVIII inactivations to obtain the concentrations of fXa generated in the tenase assay, whereas in the fVa assays the amount of thrombin generated in the prothrombinase assay was determined directly by titration with DAPA.

Under the assay conditions described above, the initial rates of thrombin (fXa) production were linear and proportional to the concentration of fVa (fVIIIa) in the reaction mixture. The initial rates of thrombin (fXa) generation in the assay were calculated for different incubation times of fVa (fVIIIa) with the different r-APC mutants. From these rates, the percentage of fVa (fVIIIa) remaining was calculated. The plot of fVa (fVIIIa) remaining *versus* the incubation time of fVa (fVIIIa) with the particular r-APC was fit to a first-order exponential decay curve, from which the pseudo-first-order rate constant for the inactivation of fVa (fVIIIa) by the r-APC mutant was calculated. The ratios of these rate constants for the mutant r-APC to wtr-APC was used to determine the percent activity of the mutant r-APC in each of these assays.

Analytical Methods. Amino-terminal amino acid sequence analysis was performed as described (Chibber et al., 1990), as were Gla determinations (Zhang et al., 1992).

The Hya content was evaluated by standard ion-exchange amino acid analysis on HPLC using the column and lithium-based elution gradients that have been described previously (Grunau & Swiader, 1992), except that postcolumn *o*-phthaladehyde, rather than ninhydrin, detection was used. Protein hydrolysis was accomplished with 6 N HCl for 24 h at 110°. Both *erythro*- and *threo*-Hya were well resolved from each other and from all other amino acids.

Procedures for oligonucleotide synthesis, cDNA purification and sequencing, cell transfections, site-specific mutagenesis, and Western analysis of the expressed PC molecules have been described in detail in previous publications (Zhang & Castellino, 1990; Zhang et al., 1992).

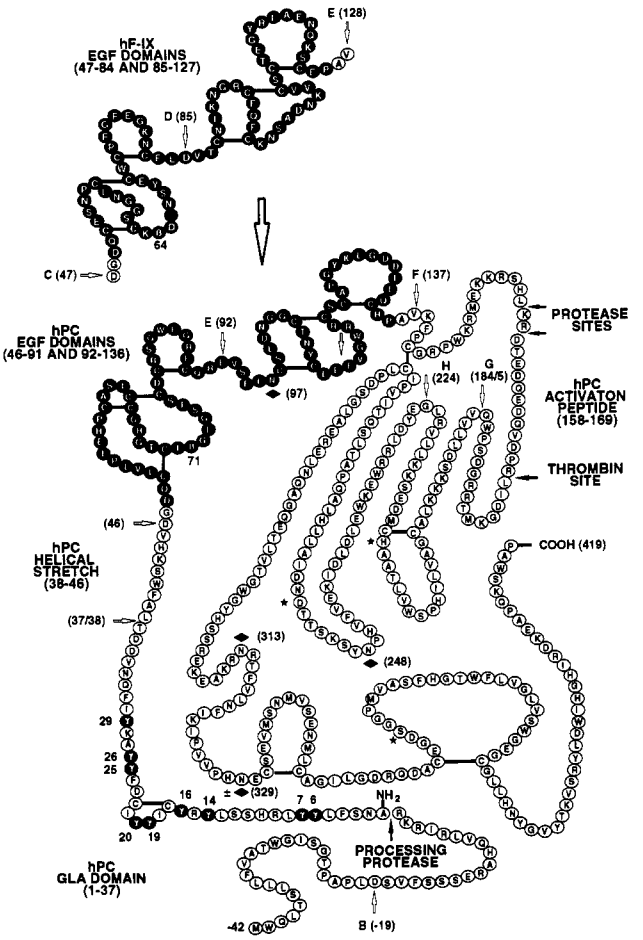

FIGURE 1: Primary structure of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2]. The amino acid sequence of the indicated EGF-like domain regions of human fIX (hF-IX), illustrated by color reversals, was amplified from the cDNA encoding this protein and used to replace the homologous locations (color reversals) of human PC.

RESULTS

A chimeric cDNA was constructed by exchange of the two EGF-like regions of human PC with those of human fIX. The replacements were made at the splice junctions that flanked the EGF-like regions in order that the entire two domains would be substituted, thus providing the best opportunity for the functional properties connected with these domains to be transported concomitantly. The primary structure of the chimeric protein that results from this construct is provided in Figure 1.

The chimeric cDNA was expressed in human 293 cells. Two separate FFQ steps were needed for the purification of the resulting mutated r-PC. In the first, a $CaCl_2$ gradient was employed to isolate a protein fraction that was fully γ-carboxylated. The elution profile from this column is illustrated in Figure 2 (top). The major peak obtained contained the r-PC-related antigen, as determined by Western blotting with monoclonal antibody LI, which has been characterized as reactive with the protease chain of PC (Zhang & Castellino, 1990). Interestingly, antibody C3 was not reactive with this chimeric protein, confirming that it is directed to an epitope within the EGF-like regions of PC. Final purification of the chimera was accomplished with a second FFQ step, in which elution was accomplished with a gradient of NaCl. The major fraction obtained from this column (Figure 2-bottom) was found to consist of highly purified r-[PC/ΔEGF-1,2/∇fIXEGF-1,2]. The purified protein was

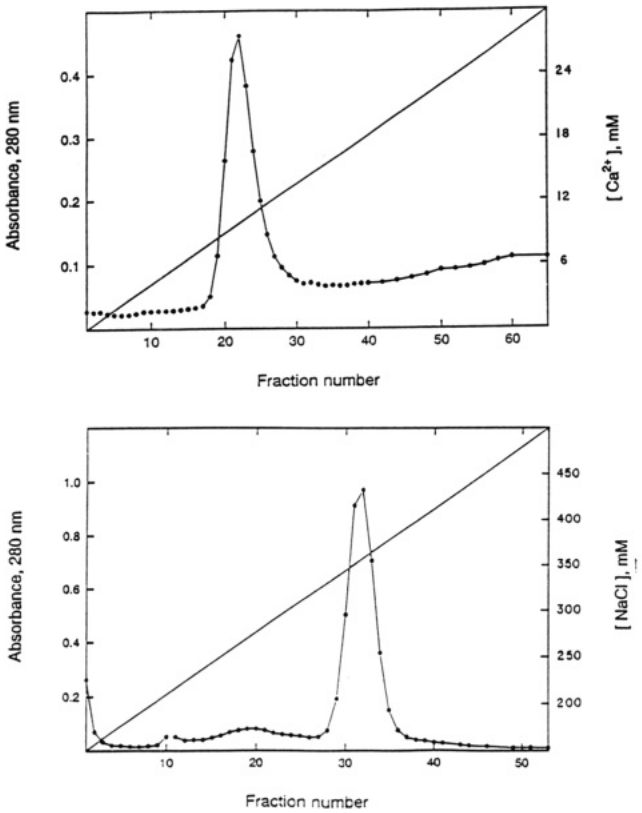

FIGURE 2: Chromatography of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] on FFQ anion-exchange chromatography at 4 °C. (Top) After batch elution with 30 mM $CaCl_2$ of the r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] contained in 2 L of conditioned human kidney 293 cell culture medium, the material was dialyzed against a buffer containing 20 mM Tris-HCl/150 mM NaCl/4 mM EDTA, pH 7.4. This sample was then applied to a column (5 mL) containing the same resin, equilibrated with a buffer of 20 mM Tris-HCl/150 mM NaCl/4 mM EDTA, pH 7.4, at 4 °C. After the column was washed with this same buffer, followed by 20 mM Tris-HCl/150 mM NaCl, pH 7.4, 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. (Bottom) The partially purified r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] isolated in the top panel was dialyzed against a buffer of 20 mM Tris-HCl/150 mM NaCl, pH 7.4 at 4 °C, 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.

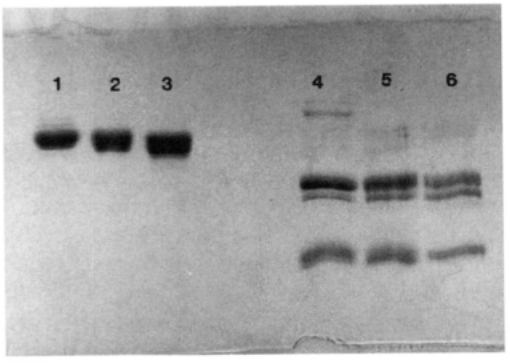

FIGURE 3: $NaDodSO_4$–PAGE analysis of r-[PC/ΔEGF-1,2/∇fIX-EGF-1,2]. (1) Nonreduced human plasma PC; (2) nonreduced wtr-PC expressed in kidney 293 cells; (3) nonreduced r-[PC/ΔEGF-1,2/∇fIXEGF-1,2]; (4) reduced human plasma PC; (5) reduced wtr-PC expressed in kidney 293 cells; (6) reduced r-[PC/ΔEGF-1,2/∇fIXEGF-1,2].

found to exist mainly in the two-chain form, as is the case for human plasma PC and wtr-PC, as revealed by reduced $NaDodSO_4$–PAGE (Figure 3). Additionally, Figure 3 shows that the chimeric r-PC contained the same doublet heavy chain band on $NaDodSO_4$–PAGE found for human plasma PC and wtr-PC, which has been attributed to glycoforms of r-PC (Yan et al., 1990). This material yielded 9.3 mol of Gla/mol of protein and 0.53 mol of Hya/mol of protein [approximately 0.9:0.1 (mol/mol) of *erythro:threo* Hya]. These results show that the chimera was fully γ-carboxylated and contained a similar amount of Hya as present in the EGF-1 region of fIX, which was incompletely hydroxylated (Fernlund & Stenflo, 1983). Amino-terminal amino acid sequence analysis through 40 residues confirmed that the Gla region of the chimera was not affected by the substitutions made. This sequence analysis also demonstrated that signal cleavage proceeded normally.

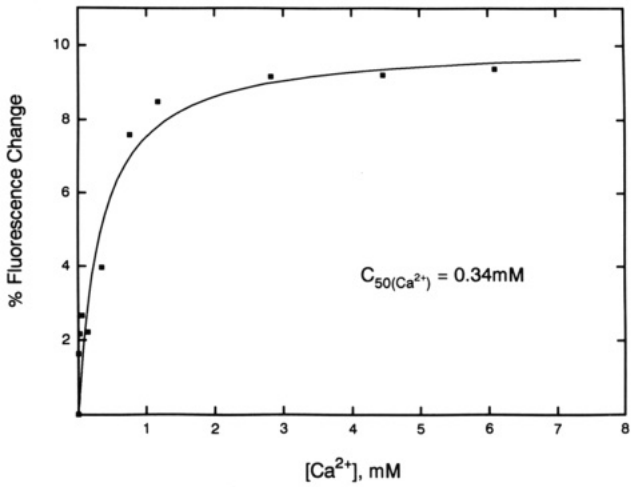

FIGURE 4: Titration of the variation with Ca^{2+} of the intrinsic fluorescence of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2]. The chimeric protein was titrated with sequential additions of Ca^{2+} and the intrinsic fluorescence intensity was measured. The fluorescence (F_0) of the protein obtained in the absence of Ca^{2+} was adjusted to 1.0, and fluorescence values (F) were obtained after addition of Ca^{2+}. The percent fluorescence change (quenching) was calculated as $[[(F_0-F)/F_0] \times 100]$ and plotted as a function of the total Ca^{2+} concentration. The C_{50} (the total Ca^{2+} concentration at which 50% of the r-PC molecules underwent the fluorescence change) and the ΔF_{max} (the total fluorescence change at saturation with Ca^{2+}) were calculated by nonlinear least-squares fitting of the data.

With the purified hybrid protein in hand, we evaluated several of its Ca^{2+}-dependent properties. We first employed two methods, *viz.*, intrinsic fluorescence (Prendergast & Mann, 1977; Strickland & Castellino, 1980; Johnson et al., 1983; Astermark et al., 1991) and reaction with a Ca^{2+}-dependent monoclonal antibody (Keyt et al., 1982; Borowski et al., 1986; Wakabayashi et al., 1986; Church et al., 1989), that have been shown to monitor the adoption of the functional Ca^{2+}-dependent conformation of the Gla domain, since these characteristics might have been affected by exchange of the EGF-like regions of PC. A titration with Ca^{2+} of the intrinsic fluorescence quenching for r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] is illustrated in Figure 4. The C_{50} for Ca^{2+} determined from these data was found to be 0.34 mM, a value approximately the same as that found for wtr-PC, of 0.41 mM (Zhang & Castellino, 1992). This result suggests that Ca^{2+} induces similar conformational changes in the chimeric and wild-type proteins. Additional titrations with Ca^{2+} of the binding of wtr-PC and r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] to the Ca^{2+}-dependent antibody JTC-1 (Wakabayashi et al., 1986) are illustrated in Figure 5. The titration curves for these proteins are not distinguishable from each other and yield C_{50} values of approximately 3.7 mM.

The inhibition by Ca^{2+} of the thrombin-catalyzed activation rate of PC and the stimulation by Ca^{2+} of the thrombin/TM-catalyzed activation of PC (Esmon et al., 1983) are

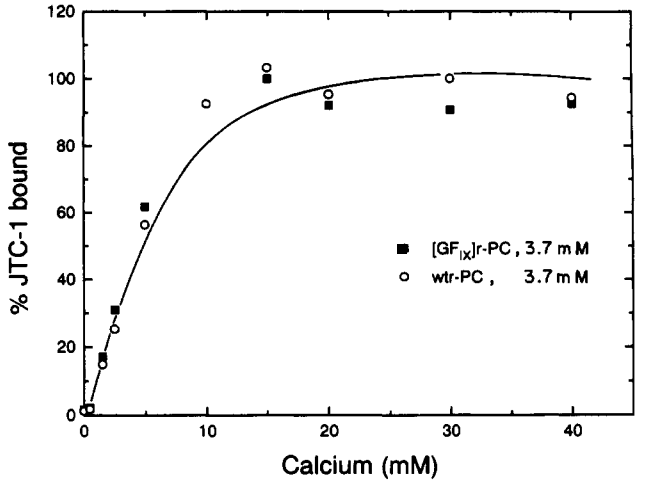

FIGURE 5: Dependence on the concentration of Ca^{2+} of monoclonal antibody binding to r-[PC/ΔEGF-1,2/∇fIXEGF-1,2]. The chimeric protein (■) or wtr-PC (○) was coated on wells of a microtiter plate. Monoclonal antibody [^{125}I]JTC-1 was added, followed by different concentrations of Ca^{2+} The amount of antibody bound ($[Ab]_b$) was measured. The maximum amount of antibody bound ($[Ab]_{max}$) to the protein adsorbed to the plate was obtained from an experiment at a saturating level of Ca^{2+}. The percent antibody bound at any Ca^{2+} concentration was calculated from $[Ab]_b/[Ab]_{max} \times 100$ and plotted as a function of the total Ca^{2+} concentration. The C_{50} (the total Ca^{2+} concentration at which 50% of the r-PC molecules were bound to antibody) as the total concentration of Ca^{2+} at 50% saturation with antibody.

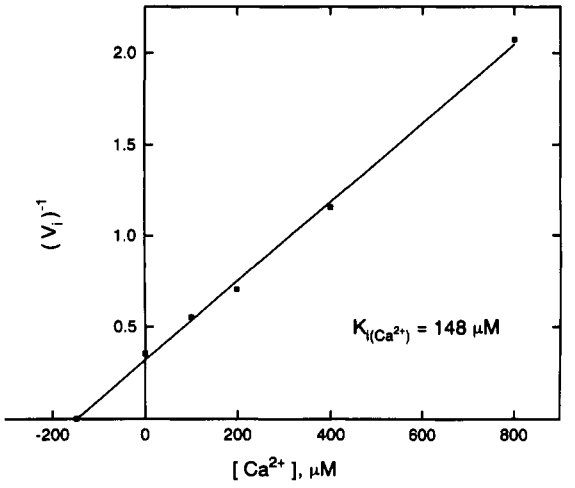

FIGURE 6: Inhibition by Ca^{2+} of the thrombin-catalyzed activation of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2]. A study-state kinetic plot illustrating the concentration dependence of Ca^{2+} on the inhibition of the initial rates of the thrombin-catalyzed activation of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] is shown. The amount of wtr-APC or r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] present at each time was determined by amidolytic assay of these enzymes with substrate S2366. The K_i for the reaction was determined as the negative intercept of the abscissa. The concentration of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] and thrombin were 2 μM and 0.4 nM, respectively. The buffer was 20 mM Tris-HCl/100 mM NaCl/0.1% gelatin, pH 7.4, at 37 °C.

believed to originate from Ca^{2+} sites outside of the Gla domain (Esmon et al., 1983). Thus, we investigated these Ca^{2+}-dependent properties of the chimeric r-PC prepared for this study. The data show that r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] was activated by thrombin at approximately the same rate as wtr-PC. As with the wt zymogen, the data of Figure 6 demonstrate that this same activation with the chimeric zymogen was inhibited by Ca^{2+}, providing a K_i for Ca^{2+} of approximately 148 μM. This value agrees well with that obtained for wtr-PC, 125 μM (Zhang & Castellino, 1990). It is further demonstrated that Ca^{2+} stimulates the activation by the thrombin/TM complex of r-[PC/ΔEGF-1,2/∇fIX-EGF-1,2]. At Ca^{2+} concentrations that saturate this effect, the initial activation rate of the chimeric protein was stimulated 82-fold over the activation rate with thrombin (at 2 mM Ca^{2+}), whereas the stimulation for wtr-PC under the same conditions was approximately 115-fold. Since Ca^{2+} inhibits the activation rate of both wtr-PC and r-[PC/ΔEGF-1,2/∇fIXEGF-1,2], it is also appropriate to compare the stimulations of these same initial activation rates obtained with thrombin/TM/Ca^{2+} and thrombin in the absence of Ca^{2+}. When this is done, the maximal stimulations are reduced to approximately 5.8-fold and 8-fold for the chimeric PC and wtr-PC, respectively. Thus, a large part of the stimulation of thrombin activation by TM is related to the reversal of the inhibition by Ca^{2+} of the thrombin-catalyzed activation of PC.

Finally, the enzymatic activity of r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] toward its physiological substrates was assessed and compared to that of wtr-APC. In these experiments, both wtr-APC and r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] were employed at final concentrations which were slightly adjusted to provide equal amidolytic activities for both enzymes (the specific amidolytic activities of each enzyme were not greatly different from each other, *i.e.*, <10%). In the APTT assay, r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] possessed only approximately 10% of the activity of wtr-APC. In the assays designed to measure the rates of inactivation of fVa and fVIII (fVIII rather than fVIIIa was used because of the comparatively greater stability of fVIII) in the *in vitro* prothrombinase and tenase systems, respectively, the chimeric mutant possessed 30–32% of the activity of wtr-APC in the fVa assay and <10% of the activity wtr-APC in the fVIII assay. An example of the type data obtained is shown for fVa inactivation in Figure 7 and for fVIII inactivation in Figure 8.

DISCUSSION

In order to address the question of whether the EGF-like domains of PC were specifically required for the function of this protein, we prepared a recombinant chimeric PC derivative in which the EGF-like domains of r-PC were replaced with those from fIX. The Ca^{2+}-dependent properties of the resulting chimeric zymogen, r-[PC/ΔEGF-1,2/∇fIXEGF-1,2], and its activated product, r-[APC/ΔEGF-1,2/∇fIXEGF-1,2], were then assessed.

The first feature of the chimeric protein of interest is the fact that γ-carboxylation proceeded normally. Our purification procedure results in isolation of the subpopulation of protein that contains maximally γ-carboxylated material. Very little r-PC antigen was observed in any column fraction other than that collected and finally purified in yields approximating 80%, as estimated by ELISA. Thus, we believe that the EGF-like domains of r-PC are not specifically involved in γ-carboxylation of this protein. The Hya content of the chimeric protein was found to be approximately 0.5 mol/mol. In the case of wtr-PC, complete β-hydroxylation was observed at sequence position Asp71 (Zhang & Castellino, 1990), while the Hya precursor Asp residue present in the first EGF-like region of human fIX has been estimated to be approximately 30% β-hydroxylated (Fernlund & Stenflo, 1983). The chimeric protein was also incompletely β-hydroxylated, indicating that it is more similar to fIX than PC in this regard. Thus, the β-hydroxylase recognition sequence is likely present within the EGF domains and not extended to other regions of the protein, as has been suggested previously (Stenflo et al., 1987).

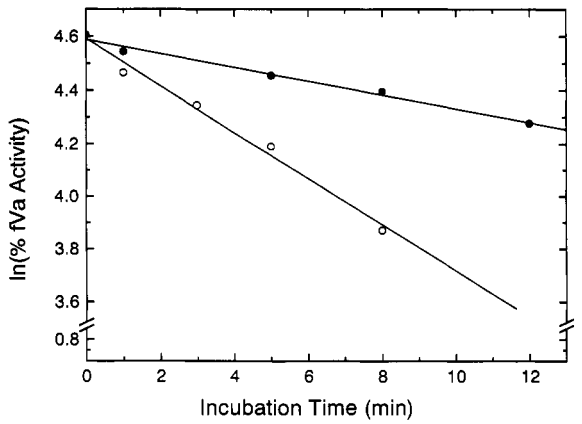

FIGURE 7: Time course of inactivation of human fVa by APC at 37 °C, pH 7.4. A quantity of fVa (0.1 μM final concentration) was incubated for different times with wtr-APC (○) or r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] (●) (0.5 nM final concentration), in the presence of (final concentrations) PL (20 μM) and Ca^{2+} (2.5 mM). In the second stage, the amount of fVa remaining was determined by fluorometric assay in the complete prothrombinase system. Here, 9 μL of the above fVa/ifVa mixture was added to the reaction mixture (final volume 1.0 mL) containing 1.39 μM prothrombin/2.5 mM $CaCl_2$/20 μM PL/3 μM DAPA. The activation reaction was accelerated by addition of 8 nM (final concentration) fXa. The fluorescence resulting from uptake of DAPA by the generated thrombin was monitored continuously at 25 °C with an SLM-Aminco (Urbana, IL) SPF-8000 spectrofluorometer. The excitation and emission wavelengths were 335 and 565 nm, respectively. The initial rates of thrombin production were calculated from these experiments at each incubation time of APC with fVa and are plotted on the graph relative to a value of 100% (calculated at zero time of incubation). The lines drawn through the data points are first-order kinetic fits of the experimental data. The ratios of the rate constants obtained provided the relative activity of the mutant. The concentration of each r-APC solution used was adjusted so that each provided the same amidolytic activity.

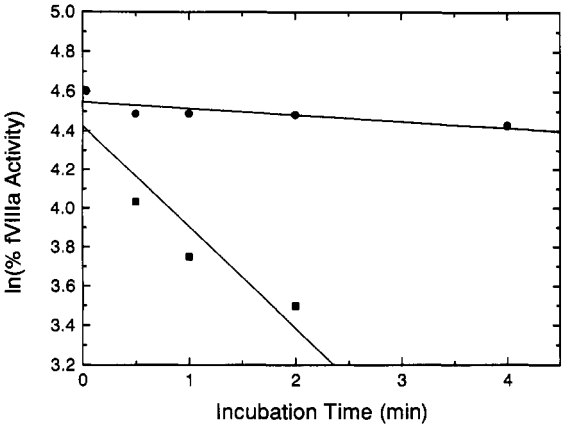

FIGURE 8: Time course of inactivation of human fVIII by APC at 37 °C, pH 7.4. A quantity of fVIII (4.4 nM final concentration) was incubated with wtr-APC (■) or r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] (●) (10.75 nM final concentration), in the presence of (final concentrations) PL (40 μM) and Ca^{2+} (2.5 mM). Next, fIXaβ (5.9 nM final concentration) and thrombin (0.12 unit/mL final concentration) were added in order to activate the remaining fVIII. The amount of fVIIIa present was assayed by a fX activation assay. Here, the fVIIIa solution was added to another solution containing final concentrations of 40 μM (in phosphate) PL, 2.5 mM $CaCl_2$, and 180 μM chromogenic substrate S2222 (final volume 790 μL). Substrate hydrolysis was accelerated by addition of 10 μL of a solution of 32 μM fX. The rate of amidolysis of S2222 by the generated fXa was determined spectrophotometrically. The initial rates of fXa production were calculated from these experiments at each incubation time of APC with fVIII and are plotted on the graph relative to a value of 100% (calculated at zero time of incubation). The line drawn through the data points are first-order kinetic fits of the experimental data. The ratios of the rate constants obtained provided the relative activity of the mutant. The concentration of each r-APC solution used was adjusted so that each provided the same amidolytic activity.

It is generally agreed that the fluorescence quenching observed upon addition of Ca^{2+} to proteins of this type is reflective of the weak classes of binding sites associated with the Gla domain. The C_{50} for Ca^{2+} for r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] calculated from such a titration (Figure 4) was essentially the same as that found for wtr-PC. This indicates that the EGF-like regions of r-PC are not specifically required for proper Ca^{2+}-binding to the Gla domain of r-PC. If the EGF-like regions influence this property at all, such effects may be of a more indirect nature. A similar conclusion is made from analysis of the binding of the Ca^{2+}-dependent conformational antibody JTC-1 to the chimeric protein. Titration with Ca^{2+} of the binding of this antibody to r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] (Figure 5) led to a C_{50} value for Ca^{2+} that was virtually identical to that found with wtr-PC. This confirms the nonessential nature of the specific EGF-like domains of r-PC in the ability of this protein to adopt its Ca^{2+}-dependent conformation in the Gla domain.

While the Ca^{2+} binding sites associated with the Gla domain of r-PC do not appear to be specifically influenced by its EGF-like domains, other functional Ca^{2+}-binding sites exist outside of the Gla domain. Current information on this topic suggests that a tight Ca^{2+}-binding site exists in the first EGF-domain of r-PC (Ohlin et al., 1988b) that is also present in this same region of fIX (Handford et al., 1990), with another tight Ca^{2+}-binding site existing outside of the Gla and EGF-like regions of PC (Rezaie et al., 1992). The former site appears as a tight site only in the intact protein and becomes weaker when the EGF domain is studied in isolation (Persson et al., 1989; Handford et al., 1991). The latter Ca^{2+} site of unknown location is influenced by Asp residues at P3 and P3′ of the scissible peptide bond of PC (Rezaie & Esmon, 1992). This site may be responsible for inhibition by Ca^{2+} of the activation of PC by thrombin (Rezaie & Esmon, 1992), in combination with residues present in the Gla domain (Zhang & Castellino, 1991). Further, while the high-affinity Ca^{2+} site responsible for stimulation of activation of PC by thrombin/TM is located outside of the first EGF domain of PC (Rezaie et al., 1992), it has been found that these Asp residues do not contribute in a positive manner to activation by this complex (Reziae & Esmon, 1992). We shown herein that these Ca^{2+} high-affinity binding sites appear to be present and functional in the chimeric mutant r-[PC/ΔEGF-1,2/∇fIXEGF-1,2]. Ca^{2+} inhibits the activation of this r-PC mutant by thrombin with a K_i approximately the same as that for wtr-PC (Figure 6). Ca^{2+} also stimulates the activation of this variant r-PC by the thrombin/TM complex to approximately 70% of the initial rate observed for similar activation of wtr-PC. These results suggest that substitution of the EGF-like regions of r-PC by those of fIX does not substantially affect these properties of r-PC zymogen. The effects of Ca^{2+} on activation of r-[PC/ΔEGF-1,2/∇fIXEGF-1,2] also indicate that the functionally relevant tight Ca^{2+}-binding sites are present in this chimeric protein. The fact that the chimeric protein is incompletely β-hydroxylated in its first EGF region is unlikely to influence these conclusions since β-hydroxylation of the appropriate Asp residue is not of importance to the ability of the EGF domain to bind Ca^{2+} (Morita & Kisiel, 1985; Handford et al., 1990).

Despite the fact that the Ca^{2+}-dependent properties of the chimeric zymogen did not appear to be greatly influenced by this particular exchange of the EGF-like domains, the Ca^{2+}-dependent physiological activities of r-[APC/ΔEGF-1,2/

∇fIXEGF-1,2] toward fVa and fVIII were greatly reduced from those of wtr-APC. The reduced activity of r-[APC/ΔEGF-1,2/∇fIXEGF-1,2] toward fVa and fVIII may be due to indirect effects. We have postulated in past publications that alignment of the sensitive peptide bonds of the substrates fVa and fVIII with the active site of APC on the PL surface is crucial to the activity of APC toward these substrates (Zhang & Castellino, 1992, 1993; Zhang et al., 1992). It is certainly conceivable that substitution of the EGF-like regions of r-APC with those of fIX could affect the orientation of the enzyme on the PL surface, thus affecting the proper alignment of the enzyme active site with the cleavage sites of the protein substrates, especially since six additional amino acids have been inserted into the chimeric r-PC *via* the EGF domain replacements with those of fIX. Alternatively, exchange of the EGF-like regions of these proteins could have subtly altered the gross folding of the chimeric enzyme, thus reducing activity. Such misfolding, however, could not be extreme since as shown herein the Ca^{2+}-dependent properties of the zymogen form were not substantially different from those of wtr-PC. In addition, it is possible that more direct effects of the inserted EGF-like regions from fIX into the chimeric r-APC play a partial role in the activity of the chimeric enzyme. In this regard, since activity differences toward fVIII and fVa have been obtained with the chimeric enzyme that have not been observed to occur with wtr-APC, it is possible that preferential interactions of the EGF-like regions of fIX (or fIXa) incorporated into the chimeric APC with the cofactor for fIXa, *viz.*, fVIII (Lin et al., 1990; Astermark & Stenflo, 1991), that are unlikely to also occur with fVa, have an influence in its activity toward these physiological substrates. Possibly, due to specific interactions of the EGF-like regions of the chimeric APC with fVIII, a less productive enzyme–substrate complex has been generated, not optimal for the inactivation of fVIII, and/or competition of the chimeric r-APC with fIXa occurred in the assay, thus inhibiting fX activation.

In conclusion, the results of this study suggest that (1) despite the substitution of functionally important domain regions of the light chain of PC with those of a protein with an entirely different activity, the chimeric protein retains essential features of PC zymogen; (2) the ability of PC to adopt its Ca^{2+}-dependent conformation is not specifically dependent on its EGF-like regions since EGF-like domains of at least one other protein can be exchanged with those of PC without adverse effects on this property; (3) the high-affinity Ca^{2+} sites responsible for inhibition of the thrombin-catalyzed activation of PC, and stimulation of this same activation by thrombin/TM, are not specifically dependent on the EGF-like domains of PC, again since EGF-like regions from at least one other protein can replace those of PC and yet the resulting product does maintain essential features of PC zymogen; and (4) determinants present in the EGF-like domains of APC play a role in its anticoagulant properties, perhaps by being necessary for specific alignments with its physiological substrates on the PL surface, through general and subtle conformational properties of the enzyme that are dependent on the integrity of the EGF-like regions, and/or by effects on substrates resulting from direct interactions with the chimeric enzyme through the newly inserted EGF domains of fIX.

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