Properties of Recombinant Chimeric Human Protein C and Activated Protein C Containing the γ-Carboxyglutamic Acid and Trailing Helical Stack Domains of Protein C Replaced by Those of Human Coagulation Factor IX†

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ABSTRACT: The properties of a recombinant (r) chimeric human protein C (PC) containing replacement of its γ-carboxyglutamic acid (Gla) and helical stack (HS) domains by those of human coagulation factor IX (fIX) have been examined. Titration with Ca^{2+} of the divalent cation-induced intrinsic fluorescence quenching of this chimera (r-GD_{IX}/PC) allowed determination of the [Ca^{2+}], of 1.8 mM, required to produce this alteration in 50% of the protein molecules. These values were 0.41 and 0.61 mM for wtr-PC and fIX, respectively. The chimera did not react with a Ca^{2+}-dependent, Gla domain-directed conformational monoclonal antibody (MAb) to r-PC but did interact with a similar MAb (H5B7) to fIX. The [Ca^{2+}] required to induced H5B7 binding to 50% of the r-GD_{IX}/PC molecules was 6.6 mM, while this same value for fIX was a nearly identical 7.2 mM. The [Ca^{2+}] needed for binding of 50% of r-GD_{IX}/PC to acidic phospholipid (PL) vesicles was 0.58 mM, while that for wtr-PC and fIX were 1.2 and 0.55 mM, respectively. The [protein] required for 50% binding of r-GD_{IX}/PC to PL at 20 mM Ca^{2+} was 0.29 μM. These same values for r-PC and fIX were 0.38 and 1.8 μM, respectively. The Ca^{2+}-mediated inhibition of the thrombin-catalyzed activation of r-GD_{IX}/PC was characterized by a K_i of 118 μM, a value similar to that of 125 μM obtained for this same inhibition of wtr-PC activation. The thrombin-catalyzed activation of both r-GD_{IX}/PC and wtr-PC was stimulated by soluble r-thrombomodulin. Similar to the case of wtr-PC, Ca^{2+} initially enhanced and, at higher concentrations, inhibited the activation of r-GD_{IX}/PC. The K_m and k_{cat} values for this latter activation at optimal [Ca^{2+}] (100 μM) were 4.1 μM and 2.5 s^{-1}, respectively. These same kinetic constants for activation of wtr-PC were 4.3 μM and 2.9 s^{-1}, respectively. These results show that many of the features needed for functional integrity of the Ca^{2+}-bound Gla/HS domains of PC are also present in those same modules of fIX, a finding that points to a generalized functional role for the Ca^{2+}-induced conformation of the structural unit consisting of the Gla and HS domains. The data also suggest that the Ca^{2+}-bound form of the Gla/HS region is an independently folded unit in PC and perhaps in fIX. Finally, it also appears that the functional Ca^{2+} site(s) outside of the Gla domain of PC and APC is independent of the specific nature of the native Gla/HS unit of PC.

PC[1] is the plasma protein zymogen of APC, a serine protease which acts as an anticoagulant. The major proteolytic activities of APC involve inactivation of fV/Va (Kisiel et al., 1977) and fVIII/fVIIIa (Vehar & Davie, 1980), resulting in loss of their cofactor activities in the prothrombinase and tenase complexes, respectively. These reactions of APC are stimulated by Ca^{2+}, PL, and a plasma cofactor, PS (Walker, 1981). Because of these APC-catalyzed cofactor inactivations, thrombin generation is retarded and coagulation is inhibited. Besides these enzymatic processes, another means of inhibition of coagulation by APC must occur, since an active-site mutation of Ser360Ala provides a variant r-APC that continues to function as an anticoagulant (Sun & Griffin, 1993). In addition to anticoagulation, APC maintains blood fluidity by stimulating fibrinolysis. The profibrinolytic role of APC, which is also enhanced by PS (de Fouw et al., 1993), is grounded in several documented effects, *e.g.*, through its involvement in release of plasminogen activators from vascular endothelial cells into blood (Comp & Esmon, 1981); though the ability of APC to neutralize plasminogen activator inhibitor-1, thus allowing plasminogen activators to function in plasminogen activation (Griffin et al., 1981; Sakata et al., 1985, 1986; Taylor & Lockhart, 1985); and through inhibition by APC of an antifibrinolytic component released as a consequence of prothrombin activation (Bajzar & Nesheim, 1991, 1993).

Human PC is synthesized as a single-chain protein containing 461 amino acids (Foster & Davie, 1984; Foster et

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[1] Abbreviations: PC, protein C; APC, activated protein C; fIX, coagulation factor IX; r-GD_{IX}/PC, a chimeric protein C in which residues 1–46 of protein C have been replaced by residues 1–47 of factor IX; r-GD_{IX}/APC, the activated form of r-GD_{IX}/PC; PS, protein S; TM, thrombomodulin; r-sTM, recombinant soluble thrombomodulin containing residues 1–497, a construct that lacks the transmembrane domain; fVIII, coagulation factor VIII; fVIIIa, activated coagulation factor VIII; Gla, γ-carboxyglutamic acid; Hya, β-hydroxyaspartic acid; EGF, epidermal growth factor; HS the helical stack region of protein C (amino acid residues 38–46) and/or factor IX (amino acid residues 39–47); wt, wild-type; r, recombinant; PL, 60%/40% (w/w) sonicated dispersion of chicken egg phosphatidylcholine (PhC)/bovine brain phosphatidylserine (PhS); MAb monoclonal antibody; APTT, activated partial thromboplastin time; S2222, benzoyl-L-Ile-L-Glu(OR)-Gly-L-Arg-*p*-nitroanilide; S2366, L-pyro-Glu-L-Pro-L-Arg-*p*-nitroanilide; $DodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; [$C_{50,Ca}$-FI], the total Ca^{2+} concentration required to alter the intrinsic fluorescence in half of the protein molecules; [$C_{50,Ca}$-MAb], the total Ca^{2+} concentration required to induce monoclonal antibody binding to 50% of the protein molecules; [$C_{50,Ca}$-PL], the total Ca^{2+} concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of protein; [$C_{50,P}$-PL], the total protein concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of Ca^{2+}.

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al., 1985). From consideration of the locations of the introns in the PC gene, as well as amino acid sequence homologies with other proteins, it has been proposed that PC contains several types of domains. Beginning at the amino terminus, these modules include a 23-residue signal peptide and a 19-residue propeptide, followed consecutively by the Gla domain, a short HS, two EGF-like structures, an activation peptide, and the trypsin-like catalytic region. In addition to cleavage of the signal peptide and propeptide (Foster et al., 1985), another proteolytic processing event occurs, liberating a dipeptide, Lys156-Arg157, prior to secretion of PC (Beckmann et al., 1985). These reactions provide mature PC, which exists in plasma as a light chain (residues 1–155), disulfide-linked to a heavy chain (residues 158–419). Other types of processking events occur prior to secretion. These include glycosylation of four Asn residues (Kisiel, 1979), β-hydroxylation of Asp71 (Drakenberg et al., 1983), and γ-carboxylation at nine precursor Glu residues, which exist at sequence positions 6, 7, 14, 16, 19, 20, 25, 26, and 29 of the light chain of PC (DiScipio & Davie, 1979). The catalytic triad responsible for the serine protease activity of APC is located in its heavy chain.

Activation of PC to APC is catalyzed by thrombin in solution in a reaction that is inhibited by Ca^{2+} (Amphlett et al., 1981). On cell surfaces, thrombin binds to TM to provide a potent PC activator complex (Esmon et al., 1982). A soluble mutant r-TM also associates with thrombin to activate PC in a process stimulated by low [Ca^{2+}] and inhibited by higher [Ca^{2+}] (Glaser et al., 1992).

The Gla domain of PC and other proteins of this class, perhaps in communication with the HS (Soriano-Garcia et al., 1992), appears to function by providing a series of Ca^{2+}-binding sites that allow these proteins to undergo alteration to the Ca^{2+}-dependent conformation required for binding to membranes (Borowski et al., 1986; Liebman et al., 1987; Liebman, 1993). The Ca^{2+}-induced conformation can be proved by intrinsic fluorescence changes (Nelsestuen, 1976; Prendergast & Mann, 1977; Strickland & Castellino, 1980; Astermark et al., 1991; Zhang & Castellino, 1992) and by interactions with conformation-specific antibodies (Keyt et al., 1982; Borowski et al., 1986; Wakabayashi et al., 1986; Liebman et al., 1987; Church et al., 1989; Zhang & Castellino, 1992; Liebman, 1993). This first group of sites is relatively nonspecific for the cation, with several divalent and trivalent cations serving in this capacity (Bajaj et al., 1976, 1977; Prendergast & Mann, 1977; Nelsestuen et al., 1981). A second class of four to five divalent cationic binding sites shows greater specificity for Ca^{2+} (Nelsestuen, 1976; Broowski et al., 1986; Liebman et al., 1987; Liebman, 1993) and is required for binding of the protein to acidic PL vesicles. These concepts are consistent with the conclusions from analysis of the X-ray structure of prothrombin fragment 1, which shows that three Ca^{2+} ions are buried within the folded structure of the Gla domain and four additional Ca^{2+} ions are surface exposed (Soriano-Garcia et al., 1992). Site directed mutagenesis based studies with human r-PC (Zhang & Castellino, 1990, 1992, 1993; Zhang, 1991; Zhang et al., 1992) and human r-prothrombin (Ratcliffe et al., 1993) have suggested roles for individual Gla residues in Ca^{2+} coordination and Ca^{2+}-dependent functional properties of the Gla domains of these proteins. Recent studies of the interactions of vitamin K dependent clotting proteins with acidic PL vesicles have strongly implicated a hydrophobic component to this binding energy (Atkins & Ganz, 1992; Zhang & Castellino, 1994).

The Gla domain may also contain recognition regions for binding of proteins of these types to cell receptors. There is support for the conclusion that the amino-terminal region of the Gla domain of fIX mediates its binding to an endothelial cell receptor (Cheung et al., 1992). Additionally, another observation suggests that the Gla domain of fVII may directly participate in its binding to TF (Ruf et al., 1991). However, some disagreement exists in this latter case (Wildgoose et al., 1991). It is as yet uncertain whether the Gla domain of PC is involved with binding to a recently reported endothelial cell receptor for this protein (Bangalore et al., 1993).

In order to investigate more completely the functions of PC and APC that specifically rely upon its Gla domain and to determine whether Gla domains contribute functional specificity to the proteins in which they reside, we have constructed a chimeric r-PC in which both the Gla and trailing HS domains of PC were replaced by these same regions of a functionally unrelated protein, fIX. The Ca^{2+}-dependent properties of this chimera were then investigated to determine whether the PC and APC functions that depend on Ca^{2+} binding were maintained. Since it has been suggested that interactions occur between the Gla domain and trailing HS (Soriano-Garcia et al., 1992) and that a combination of these regions may provide the minimal structure needed for intact Ca^{2+} and PL binding by this domain (Colpitts & Castellino, 1994), we decided to construct this chimeric r-PC by exchanging with r-PC both of these modules from fIX. The current manuscript is a summary of our investigations with this novel protein.

MATERIALS AND METHODS

Proteins. r-PC was expressed in human 293 cells and purified as described previously (Zhang & Castellino, 1990). wtr-APC and r-GD_{IX}/APC were prepared from wtr-PC and r-GD_{IX}/PC by activation with the *Agkistrodon contortix* venom activator of PC, Protac C (American Diagnostica, New York, NY). The progress of each activation reaction was monitored spectrophotometrically by the appearance of amidolytic activity of wtr-APC and r-GD_{IX}/APC toward the chromogenic substrate S2366 (Helena Laboratories, Beaumont, TX).

Human r-fVIII was provided by the Genetics Institute (Cambridge, MA). Bovine fX (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). r-sTM, lacing chondroitin sulfate (Solulin), was donated by Dr. J. Morser (Richmond, CA). Its properties have been described (Glaser et al., 1992). r-Hirudin was obtained from Dr. R. Wallis (Horsham, West Sussex, England). Human fIX, fX, and thrombin were products of Enzyme Research Laboratories (South Bend, IN).

The MAb to plasma PC JTC-1, which recognizes a Ca^{2+}-dependent epitope in the Gla domain, was provided by Drs. N. Aoki and K. Wakabayashi (Tokyo, Japan). The MAb H5B7, which recognizes a Ca^{2+}-dependent epitope in the Gla domain of human fIX (Velander et al., 1989), was obtained from Dr. D. Strickland (Rockville, MD). The antibodies were radiolabeled using the Iodobead (Pierce Chemical Company, Rockford, IL) procedure as described previously (Zhang & Castellino, 1992). The anti-human plasma PC MAb C3 (Heeb et al., 1988), which recognizes a Ca^{2+}-independent epitope in the EGF-regions of r-PC (Heeb et al., 1988; Yu et al., 1994), was provided by Dr. J. Griffin (La Jolla, CA). Restriction endonucleases were purchased from Promega (Madison, WI).

Lipids. PL vesicles containing 60/40 (w/w) PhC/PhS were prepared as previously described (Beals & Castellino, 1986).

The [PL] was determined as total organic phosphate (Lowry & Lopez, 1946) using a conversion factor of 25 (w/w) to obtain the weight concentrations of the PL vesicles.

Genes. The cDNAs coding for human PC and human fIX were provided by Dr. E. Davie (Seattle, WA). The cDNA for PC in pUC119 (Foster & Davie, 1984) was modified, providing plasmid p119[PC], as previously described (Zhang & Castellino, 1990).

The cDNA for fIX in pBR322 (Kurachi & Davie, 1982) was excised from this plasmid and inserted into pUC119, yielding plasmid p119[fIX], as described earlier (Yu et al., 1994).

Construction and Expression of r-GD$_{IX}$/PC. A unique restriction endonuclease site for *Bsp*EI was inserted after the second base of the codon for Ile^{-4} in PC by primer-directed mutagenesis, yielding plasmid p119[PC/*Bsp*EI]. The following synthetic oligonucleotide was employed for this step (the lower case letter represents the mutagenic base, and the restriction endonuclease cleavage site is indicated by *):

5′-CGG AT*C CGg AAA CGT GC

The Gla/HS domains of fIX (amino acid residues 1–47) were amplified from the cDNA from fIX using PCR technology. The primers used in amplification included introduction of a unique *Bsp*EI restriction site at the 5′-end and a unique *Bst*EII site at the 3′-end of the amplified product (a *Bst*EII site exists after the first base of the codon for Gly47 in wtr-PC). The synthetic oligonucleotide used for amplification of the 3′-end of the Gla/HS region of fIX was

5′-ACACTGgTC aC*C ATC AAC ATA CTG

and that used for the 5′-end of the Gla domain of fIX was

5′-CTGAATCt*C Cgg AAG AGG TAT AAT TCA

The cDNA encoding the Gla/HS domains of wtr-PC was excised from p119[PC] using a *Bsp*EI/*Bst*EII restriction digest. This resulted in p119[PC/ΔPC$_{-4-47}$]. Next, the PCR product containing the cDNA encoding these same domains of fIX was digested with a *Bst*EII/*Bsp*EI and ligated to the complementary sites in p119[PC/ΔPC$_{-4-47}$]. The restriction endonuclease digested PCR product possessed nucleotides needed to fill in the bases required for regeneration of the intact propeptide region of PC, as well as the first base of the codon for Gly47. This ligation resulted in p119[GD$_{IX}$/PC]. Screening of positive bacterial transformants was accomplished by loss of a *Sac*I site and introduction of an *Ear*I site, both of which occur consequent to replacement of the Gla/HS region of PC with that of fIX. The nucleotide sequence of the fIX-derived region of PC was found to be correct.

Expression and Purification of the Chimeric Protein. The chimeric cDNA encoding r-GD$_{IX}$/PC was excised from p119-[GD$_{IX}$/PC] employing a *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), providing pCIS2M[GD$_{IX}$/PC]. This plasmid was then transfected into human kidney 293 cells (ATCC CRL 1573). Positive clones were then selected by assay with the MAb C3.

Purification of r-GD$_{IX}$/PC was accomplished on FFQ columns at 4 °C in the same manner as that for wtr-PC (Zhang & Castellino, 1990).

Activation of r-GD$_{IX}$/PC and Assay of r-GD$_{IX}$/APC. Activation kinetics of wtr-PC or r-GD$_{IX}$/PC were performed in a buffer consisting of 20 mM Tris-HCl/100 mM NaCl/0.1% gelatin, pH 7.4, at 37 °C. The activators were thrombin or thrombin/r-sTM, plus the desired levels of $CaCl_2$. Aliquots of the activation mixture were removed at various times, and the thrombin was neutralized by r-hirudin (3 units). The amount of wtr-APC or r-GD$_{IX}$/APC present at each time was determined by amidolytic assay of these enzymes with substrate S2366. The details of the assays have been described previously (Yu et al., 1994).

The overall anticoagulant activity of r-GD$_{IX}$/APC was measured by its ability to prolong the APTT of PC-deficient plasma. The APTT assay kit (Sigma Diagnostics, St. Louis, MO) was employed for these measurements, essentially as described earlier (Zhang et al., 1992). The reactivity of r-GD$_{IX}$/APC toward inactivation of coagulation fVIII was assessed by examination of the APC-catalyzed loss of cofactor activity of fVIII in the complete tenase system, using purified fX, fIXaβ, and fVIII, along with Ca^{2+} and PL. The rate of formation of fXa formed in this assay was determined with the aid of the fXa chromogenic substrate S2222. Details of this assay have been provided earlier (Yu et al., 1994). In both cases, we have expressed the activity of r-GD$_{IX}$/PC relative to an activity of 100% assigned to a wtr-APC solution of equal amidolytic activity of the mutant enzyme.

Intrinsic Fluorescence Titrations. The dependence on [Ca^{2+}] of adoption of the Ca^{2+}-induced conformation of r-GD$_{IX}$/PC was measured by titration of the intrinsic fluorescence quenching accompanying the binding of Ca^{2+} to r-GD$_{IX}$/PC. The experimental details have been provided earlier (Zhang & Castellino, 1992). These measurements were conducted at 20 °C using an SLM-Aminco 8000 recording spectrofluorometer (SLM-Aminco Instruments, Urbana, IL). The excitation and emission wavelengths were 283 and 340 nm, respectively, for r-PC and r-GD$_{IX}$/PC, and 283 and 360 nm, respectively, for fIX. Slit widths of 1 and 16 nm were used for excitation and emission, respectively.

MAb-H5B7 and -JTC-1 Binding to r-GD$_{IX}$/PC. Measurements of the dependencies on [Ca^{2+}] of the binding of the Ca^{2+}-dependent conformational MAb's [^{125}I]H5B7 and [^{125}I]-JTC-1 to r-GD$_{IX}$/PC, r-PC, and fIX and calculations of the [$C_{50,Ca}$-MAb] were accomplished as detailed earlier (Zhang & Castellino, 1994).

Interaction of r-PCs with Acidic PL. Protein/PL binding was measured at 20 °C using 90° relative light scattering techniques as previously described (Nelsestuen et al., 1976; Nelsestuen & Broderius, 1977; Nelsestuen & Lim, 1977). Excitation and emission wavelengths of 320 nm and slit widths of 4 nm were used. The buffer for these experiments was 20 mM Tris-Cl/100 mM NaCl, pH 7.4. Plots of the molecular weight ratio of the protein (M2) to PL (M1) against the [Ca^{2+}] (at constant [protein]) or the [protein] (at constant [Ca^{2+}]) were used to evaluate binding parameters (Nelsestuen & Lim, 1977). Our procedures for performing the titrations have been described (Zhang & Castellino, 1993). The total [Ca^{2+}] that resulted in 50% saturation of PL with protein, [$C_{50,Ca}$-PL], was obtained by iterative nonlinear least squares best fit of the data, allowing both [$C_{50,Ca}$-PL] and M_2/M_1 (max.) to float. In separate experiments, titrations with protein were conducted at a constant [Ca^{2+}] of 20 mM. The resulting [$C_{50,P}$-PL] was obtained in the same manner.

Analytical Methods. Amino-terminal amino acid sequence analysis was performed as described (Chibber et al., 1990), as were Gla (Zhang et al., 1992) and Hya (Yu et al., 1994) determinations. Our procedures for oligonucleotide synthetic, cDNA purification and sequencing, cell transfections, site-

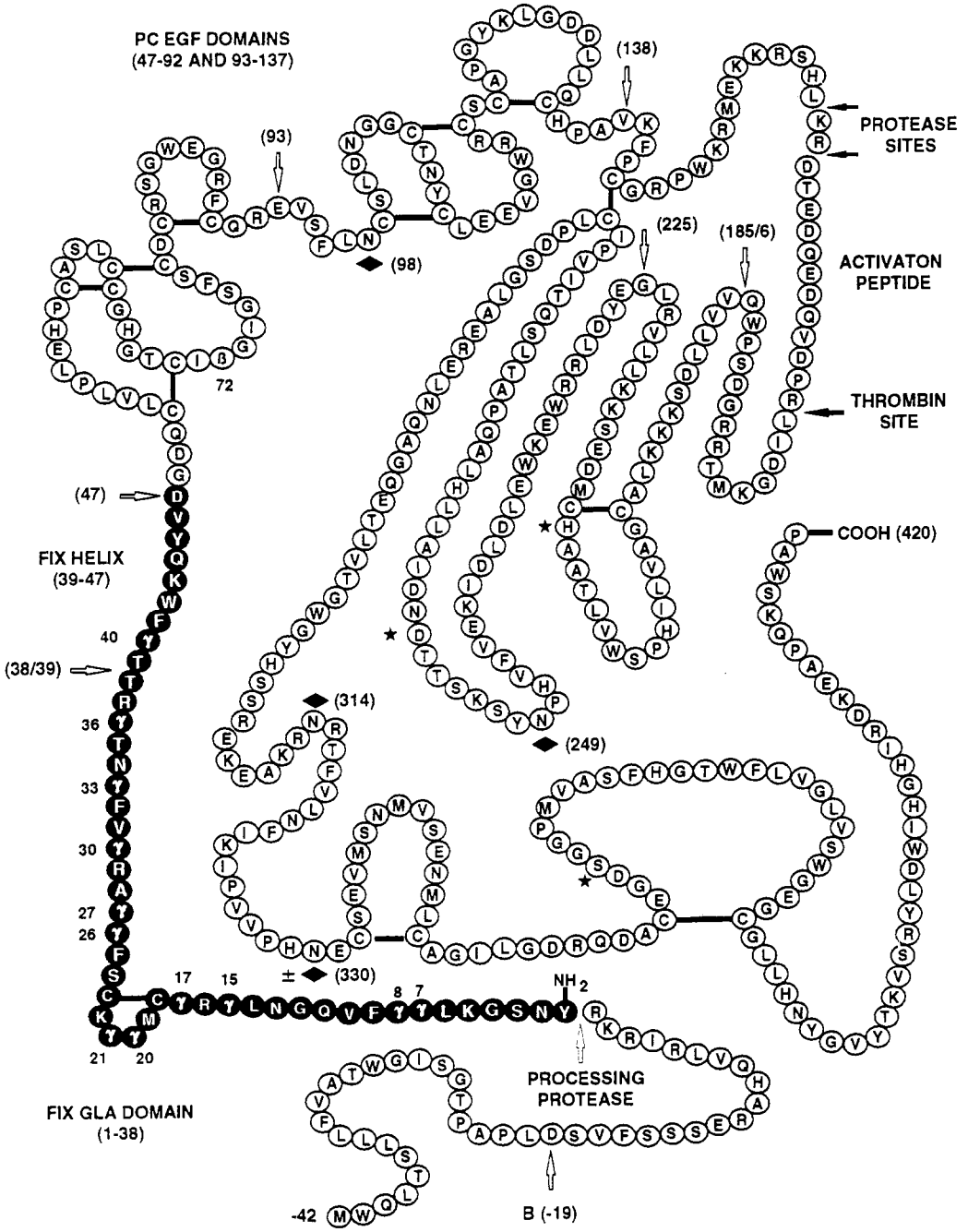

FIGURE 1: Primary structure of r-GD_{IX}/PC. The amino acids of fIX are indicated by color reversals. The unfilled arrows indicate the amino acid sequence positions of the introns in the gene, the filled diamonds are the locations of N-linked oligosaccharides, and the filled stars show the positions of the catalytic triad of amino acids in the activated form of this chimera.

specific mutagenesis, and Western analysis of the expressed PC molecules have been described earlier (Zhang & Castellino, 1990; Zhang et al., 1992), as has our protocol for PCR amplifications (De Serrano et al., 1992).

RESULTS

A chimeric protein containing the Gla and trailing HS regions of r-fIX (residues 1–47) inserted in place of these same two domain regions of r-PC (residues 1–46) has been successfully expressed in human kidney 293 cells and purified. The primary structure of this chimeric protein is provided in Figure 1. The chimera, r-GD_{IX}/PC, was isolated in two successive steps on FFQ resin, as described earlier for wtr-PC (Zhang & Castellino, 1990). In the first step, a $CaCl_2$ gradient was applied, leading to the elution profile shown in Figure 2a. Two peaks were obtained and both were identified as r-GD_{IX}/PC-related antigen based on Western analysis with MAb C3 which reacts with the EGF-like domains of r-PC (Heeb et al., 1988; Yu et al., 1994). These fractions were pooled separately and subjected to another FFQ chromatography step, with elution by a NaCl gradient. In this latter step, a single peak was obtained for each of the fractions of Figure 2. The elution profile of peak 1 of Figure 2a is shown in Figure 2. Each purified component was subjected to amino-terminal amino acid sequencing. The first fraction of Figure 2a possessed the amino-terminal sequence predicted for the correctly processed chimeric protein and was used for further investigations herein. The second peak from Figure 2a possessed a heterogeneous amino terminus, containing amino acids clearly present in the propeptide region of the molecule. This protein fraction was apparently not correctly processed and was not employed for subsequent studies in this paper.

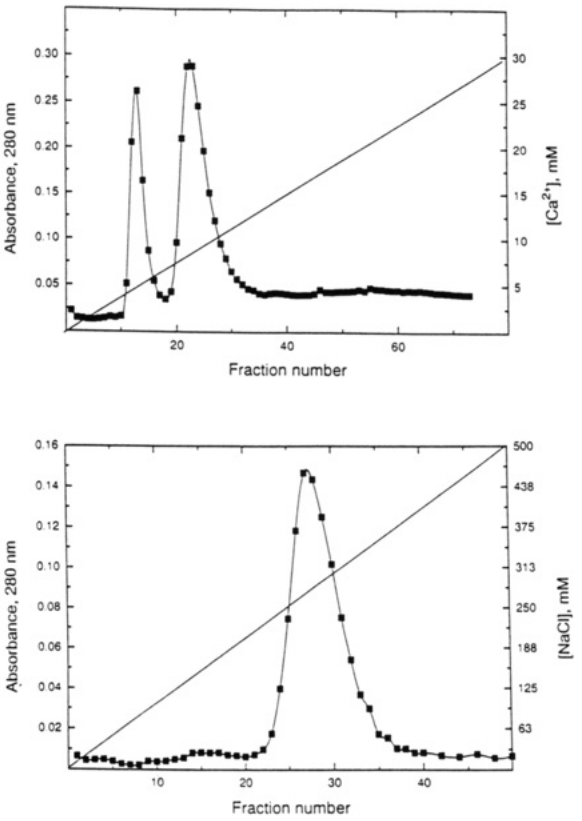

FIGURE 2: Chromatography of r-GD_{IX}/PC on FFQ anion-exchange chromatography at 4 °C. (A, top) A volume of 1 L of conditioned 293 culture medium was applied to a 5-mL column of FFQ, equilibrated with 20 mM Tris-HCl/150 mM NaCl/4 mM EDTA, pH 7.4, at 4 °C (TBS/EDTA). After washing the column with this 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. (B, bottom) Peak 1 of part A 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.

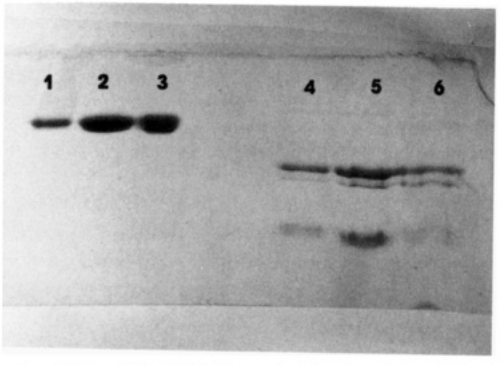

FIGURE 3: $NaDodSO_4$/PAGE analysis of r-GD_{IX}/PC: lane 1, nonreduced human plasma PC; lane 2, nonreduced wtr-PC expressed in kidney 293 cells; lane 3, nonreduced r-GD_{IX}/PC; lane 4, reduced human plasma PC; lane 5, reduced wtr-PC; lane 6, reduced r-GD_{IX}/APC.

$DodSO_4$/PAGE analysis of this chimeric protein (Figure 3) demonstrated that a two-chain protein was obtained, similar to the finding with wtr-PC. This result showed that, despite the substitutions in the chimeric r-fIX/PC, the PC processing reaction involving cleavage of the dipeptide Lys156-Arg157 occurred in the chimera, indicating that this late processing reaction (McClure et al., 1992) did not depend upon the presence of the Gla domain of PC or its trailing HS. The

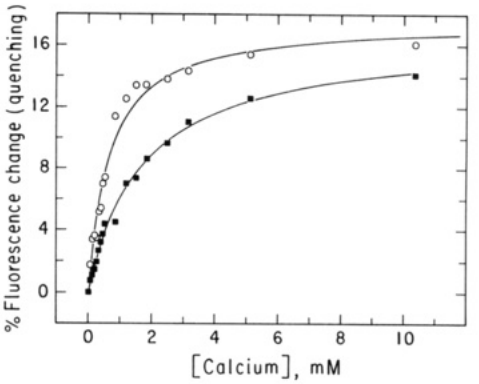

FIGURE 4: Titration of the effects of Ca^{2+} on the intrinsic fluorescence of r-GD_{IX}/PC and human plasma fIX. The protein (*ca.* 1 μM) was titrated with sequential additions of Ca^{2+} and the intrinsic fluorescence intensity measured. The buffer was 20 mM Tris-HCl/100 mM NaCl, pH 7.4, at 20 °C. 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 100 $[(F_0 - F)/F_0]$ and plotted as a function of the total $[Ca^{2+}]$. The $[C_{50,Ca}\text{-Fl}]$ and the ΔF_{max} (the total fluorescence change at saturation with Ca^{2+}) were calculated by nonlinear least squares iterative fitting of the data. The excitation and emission wavelengths used for titration of r-GD_{IX}/PC were 283 and 340 nm, respectively. Those for fIX were 283 and 360 nm, respectively: (○) fIX; (■) r-GD_{IX}/PC.

doublet band observed for r-GD_{IX}/PC is a common feature of all wtr-PC and all r-PC mutants purified to date and arises from molecular subpopulations of the protein that are differentially glycosylated.

Amino-terminal amino acid sequence analysis through 40 residues demonstrated that both chains of the chimera possessed the correct amino acid sequences. In addition, <5% Glu was found at each position that a Gla residue was expected, demonstrating that Gla residues have been introduced at each Glu-precursor residue in the Gla domain of the chimeric protein. This has been confirmed by Gla compositional analysis where 12.3 residues (expected, 12.0) of Gla/mol of protein have been found. Similarly, the chimeric protein contained 0.8 mol of Hya/mol of protein (expected, 1.0), further showing that correct processing has occurred in this portion of the protein molecules.

The ability of r-GD_{IX}/PC to adopt a Ca^{2+}-dependent conformation in the Gla domain can be evaluated in one manner by the intrinsic fluorescence quenching induced by Ca^{2+}, as has been accomplished for other Gla-containing coagulation proteins (Prendergast & Mann, 1977; Strickland & Castellino, 1980; Johnson et al., 1983; Astermark et al., 1991). Data resulting from a titration with Ca^{2+} of the intrinsic fluorescence of r-GD_{IX}/PC and human plasma fIX are provided in Figure 4. In the case of the chimeric protein, examination of fluorescence excitation and emission spectra in the presence and absence of Ca^{2+} demonstrated that, like the case of PC (Zhang & Castellino, 1993), wavelengths of 283 and 340 nm, respectively, provided effective conditions for titrations with Ca^{2+}. However, in the case of fIX, use of an emission wavelength of 340 nm with an excitation wavelength of 283 nm did not result in a fluorescence change upon addition of Ca^{2+} at low slit widths. Difference intrinsic fluorescence emission spectral analysis (excitation at 283 nm and a wavelength scan of emission intensity) in the absence and presence of 10 mM Ca^{2+} demonstrated that the direction and extent of the Ca^{2+}-dependent intrinsic fluorescence change was a function of the emission wavelength and ranged from approximately 2% at 330 nm and >−16% at emission wavelengths of 350–360 nm. At the emission wavelength of

Table 1: Properties of r-GD$_{IX}$/PC and r-GD$_{IX}$/APC

property	r-PC	fIX	r-GD$_{IX}$/PC
	PC[a]		
[$C_{50,Ca}$-FI][b]	0.40 mM	0.61 mM	1.8 mM
[$C_{50,Ca}$-MAb(JTC-1)][c]	3.0 mM	N/A[d]	N/A[d]
[$C_{50,Ca}$-MAb(H5B7)][c]	N/A[d]	7.2 mM	6.6 mM
[$C_{50,Ca}$-PL][e]	1.2 mM	0.55 mM	0.58 mM
[$C_{50,P}$-PL][f]	0.38 μM	1.8 μM	0.29 μM
K_i(Ca^{2+}/thrombin)[g]	125 μM	N/A[d]	118 μM
K_m(thrombin/TM)[h]	4.3 μM	N/A[d]	4.1 μM
k_{cat}(thrombin/TM)[h]	2.9 s^{-1}	N/A[d]	2.5 s^{-1}
	APC[i]		
APTT[j]	100%	N/A[d]	100%
fVIII[k]	100%	N/A[d]	100%

[a] The properties below relate to the zymogen forms of the proteins. [b] The [Ca^{2+}] required to quench the fluorescence in 50% of the protein molecules. [c] The [Ca^{2+}] needed to induce MAb binding to 50% of the protein molecules. [d] The property described does not exist to any significant degree in this protein. [e] The [Ca^{2+}] required to induce binding of 50% of the protein molecules to PL. [f] The [protein] required to induce binding of 50% of the protein molecules to PL at a [Ca^{2+}] of 20 mM. [g] The inhibition (dissociation) constant of Ca^{2+} for the thrombin-catalyzed activation of r-GD$_{IX}$/PC or wtr-PC. [h] The steady-state kinetic constants for the activation of r-GD$_{IX}$/PC or wtr-PC by thrombin/TM at a [Ca^{2+}] of 100 μM. [i] The properties below relate to the activated forms of the proteins. [j] The anticoagulant activity of r-GD$_{IX}$/PC, relative to wtr-APC at the same amidolytic activity (set at 100%), as measured by the increase in the activated partial thromboplastin time of PC-deficient plasma. [k] The rate of inactivation of fVIII by r-GD$_{IX}$/APC, relative to wtr-APC at the same amidolytic activity (set at 100%), as measured in the complete tenase complex with purified components.

340 nm, which has been commonly employed to measure the Ca^{2+}-dependent intrinsic fluorescence alteration of PC, this same alteration for fIX was nearly zero. Thus, while intrinsic fluorescence titrations for fIX cannot be accomplished under the same conditions as those found for PC, use of a slightly higher emission wavelength allows such titrations to be readily performed.

The variation of the intrinsic fluorescence of r-GD$_{IX}$/PC and fIX on the [Ca^{2+}] is illustrated in Figure 4. The experimental points were best fit to a line possessing a [$C_{50,Ca}$-FI] of 1.8 mM, with a maximal fluorescence change of −16.8%. Those same values for human plasma fIX (Figure 4) were 0.61 mM and −17.9%, and those for wtr-PC were 0.40 mM and −19% (Zhang & Castellino, 1993), respectively. These data are summarized in Table 1. In all cases, addition of 40 mM EDTA at the conclusion of the titrations led to full reversal of the fluorescence quenching. Additionally, since titrations were accomplished by addition of 1-μL aliquots of solutions (up to 15 total) of $CaCl_2$ to 1.5 mL of protein, fluorescence corrections for volume changes were not necessary.

Another manner of assessing the chimeric protein for adoption and integrity of the Ca^{2+}-dependent conformation of PC and/or fIX is by examining the reactivity of r-GD$_{IX}$/PC with Ca^{2+}-dependent, Gla domain-directed MAb's to PC and fIX. One such MAb (JTC-1) recognizes an epitope in the Ca^{2+}-dependent conformation of the Gla domain of PC (Wakabayashi et al., 1986). From a titration with Ca^{2+} of the binding of this MAb to r-PC, a [$C_{50,Ca}$-MAb(JTC-1)] of 3.0 mM was obtained (Table 1), in agreement with a previous report (Zhang & Castellino, 1992). We found that this MAb did not interact with either fIX or r-GD$_{IX}$/PC at [Ca^{2+}] up to 40 mM. Another MAb (H5B7), directed toward the Ca^{2+}-dependent conformation of the Gla domain of fIX (Velander et al., 1989; Tharakan et al., 1990), was used to assess the integrity of the Ca^{2+}-induced conformation of the Gla domain of fIX in the chimeric protein. The [$C_{50,Ca}$-MAb(H5B7)] for

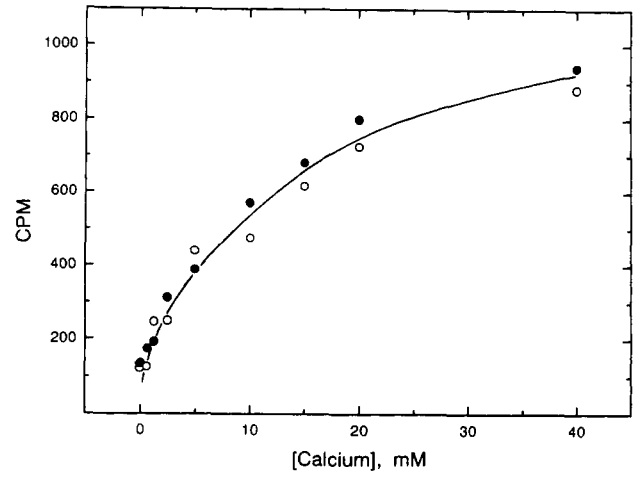

FIGURE 5: Effects on the [Ca^{2+}] of the binding of MAb H5B7 to r-GD$_{IX}$/PC (●) and human plasma fIX (○). Each of these proteins in the presence of the MAb [^{125}I]H5B7 was titrated in microtiter plates with sequential additions of Ca^{2+}, and the amount of antibody bound ([MAb]$_b$) was measured (in cpm). The [$C_{50,Ca}$-MAb(H5B7)] (the total [Ca^{2+}] at which 50% of the protein molecules were bound by the MAb H5B7) was calculated by nonlinear least squares minimization of the data allowing both [$C_{50,Ca}$-MAb(H5B7)] and $(cpm)_{max}$ to float during the iterations. For purposes of illustration a single line is drawn between data points for each protein, since the [$C_{50,Ca}$-MAb(H5B7)] values for each are very similar.

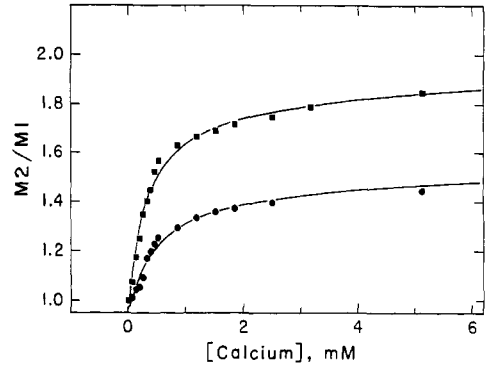

FIGURE 6: Effect of [Ca^{2+}] on the binding of r-GD$_{IX}$/PC (■) and fIX (●) to acidic PL. A Ca^{2+} titration of the binding of protein (1 μM) to PL vesicles (6 μg/mL). The molecular weights of the protein/PL complexes were determined by 90° relative light scattering after subtraction of the scattering of the nonbound protein. The [$C_{50,Ca}$-PL] (the total [Ca^{2+}] at which 50% of the protein molecules were bound to PL) was calculated by nonlinear least squares minimization of the data allowing both [$C_{50,Ca}$-PL] and the maximum attainable M_2/M_1 to float during the iterations.

this antibody to fIX was 7.2 mM, a value very similar to that of 6.6 mM found for r-GD$_{IX}$/PC (Figure 5 and Table 1). This MAb did not interact with r-PC, at least up to a [Ca^{2+}] of 40 mM.

It is well established that the Ca^{2+} sites on the Gla domain of proteins of this type are important to binding of the protein to acidic PL vesicles. Thus, a comparison was undertaken of the Ca^{2+} dependency of binding to acidic PL (60% PhC/40% PhS) of r-GD$_{IX}$/PC and fIX under identical conditions. We have previously reported these data for r-PC under the same conditions (Zhang & Castellino, 1993). Examples of such titrations for the proteins under study here are illustrated in Figure 6. From these data, [$C_{50,Ca}$-PL] values of 0.58 mM for r-GD$_{IX}$/PC and 0.55 mM for fIX (Table 1) have been calculated by nonlinear least squares best fit minimizations of iterations that allowed the [$C_{50,Ca}$-PL] and the maximal M_2/M_1 to float. The [$C_{50,Ca}$-PL] for wtr-PC was previously found to be 1.2 mM (Zhang & Castellino, 1993). The binding

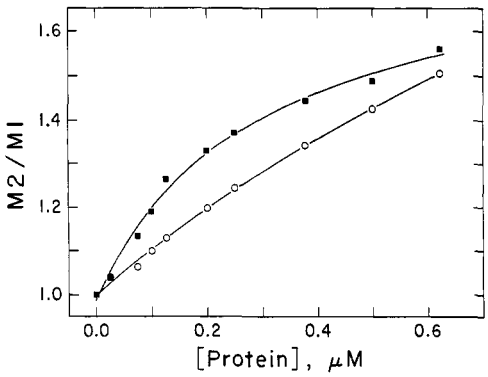

FIGURE 7: Measurement of the binding of r-GD_{IX}/PC to PL in the presence of Ca^{2+}. The dependence on [protein] of the interaction of r-GD_{IX}/PC (■) and fIX (○) with PL (6 μg/mL) in the presence of 20 mM $CaCl_2$. The molecular weights of the protein/PL complexes were determined by relative light scattering after subtraction of the light scattering of the nonbound protein. The [$C_{50,P}$-PL] (the total [protein] required for its half saturation with acidic PL) was calculated by nonlinear least squares minimization of the data allowing both [$C_{50,P}$-PL] and the maximum attainable M_2/M_1 to float during the iterations.

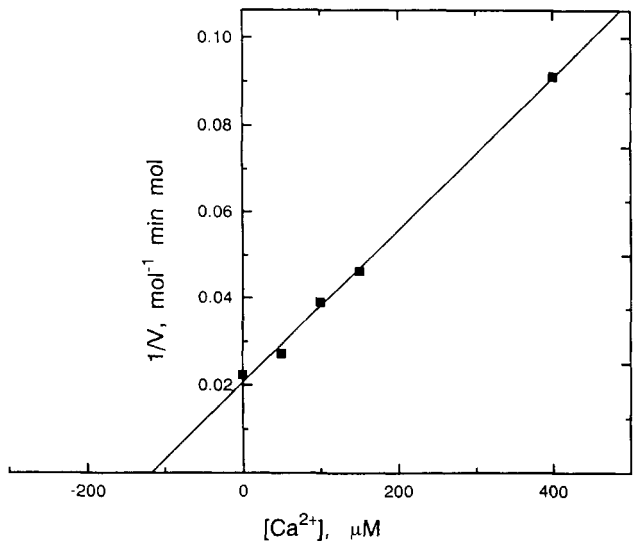

FIGURE 8: Steady-state kinetic analysis of the inhibition by Ca^{2+} of the thrombin-catalyzed activation of r-GD_{IX}/PC. To a solution of r-PC (1 μM, final concentration) was added various [Ca^{2+}], followed by thrombin (21 nM, final concentration). After incubation times of 4, 8, and 14 min, the thrombin was quenched by addition of hirudin (90 units/mL, final concentration), and the amount of r-GD_{IX}/APC formed was measured by its ability to hydrolyze the substrate S2366 (150 μM, final concentration). The plot illustrated is a measure of the reciprocal of the initial rate of formation of r-GD_{IX}/APC (the rate is expressed as mol of PC activated min^{-1} mol^{-1} of thrombin) as a function of the [Ca^{2+}]. The K_i for Ca^{2+} is the negative intercept of the abscissa. The buffer was 20 mM Tris-HCl/150 mM NaCl, pH 7.4, at 37 °C.

of each of these proteins to PL was nearly completely reversed (85%) after addition of 40 mM EDTA to the highest [Ca^{2+}] of the titration. Another set of PL-binding experiments was performed to obtain the [$C_{50,Ca}$-PL] for protein/PL binding at saturating [Ca^{2+}]. Titrations of r-GD_{IX}/PC and fIX into PL dispersions containing 20 mM Ca^{2+} are illustrated in Figure 7. The [$C_{50,P}$-PL] values were obtained from the data as described above for the [$C_{50,Ca}$-PL] calculations. The data show that the chimeric mutant binds more efficiently to these vesicles than does fIX, as revealed by its 6-fold lower [$C_{50,P}$-PL] (Table 1) when compared to that of fIX.

The ability of the Gla domain to influence of Gla domain-independent Ca^{2+} sites can be evaluated by examining the K_i

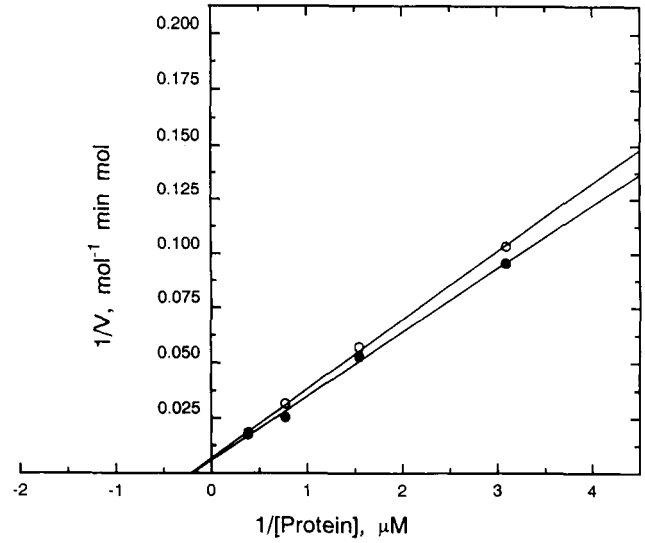

FIGURE 9: Steady-state kinetic analysis of the activation of r-GD_{IX}/PC by thrombin/r-sTM. A solution of the relevant r-PC in the presence of 100 μM $CaCl_2$ was incubated with a mixture of thrombin (1 nM)/TM (10 nM). After reaction times of 4, 8, and 14 min, the thrombin was quenched by hirudin (90 units/mL, final concentration), and the amount of the relevant r-APC present was measured by its ability to hydrolyze the substrate S2366 (150 μM, final concentration). The plot illustrated is a measure of the reciprocal of the initial rate of formation of r-GD_{IX}/APC (the rate is expressed as mol of PC activated min^{-1} mol^{-1} of thrombin) as a function of the reciprocal of the [protein]. The buffer was 20 mM Tris-HCl/0.1 mM $CaCl_2$/150 mM NaCl, pH 7.4, at 37 °C.

of Ca^{2+} toward inhibition of the steady-state activation of PC by thrombin (Amphlett et al., 1981). This K_i for Ca^{2+} was determined for both r-GD_{IX}/PC and wtr-PC. The kinetic data are illustrated in Figure 8 and are summarized in Table 1. The K_i value obtained for this mutant, of 118 μM, is nearly the same as that of 125 μM determined under identical conditions for wtr-PC (Zhang & Castellino, 1991). In addition, in order to assess the effect of the Gla domain switch with fIX on the steady-state kinetic properties of PC, we determined these kinetic properties for the chimera at 100 μM [Ca^{2+}], where separate studies (not shown) have demonstrated that maximal rates of activation occur for both wtr-PC and the chimeric mutant by the thrombin/r-sTM complex. Once again, the steady-state kinetic parameters that characterize the initial rate kinetics of this activation are approximately the same for the wild-type and chimeric proteins (Figure 9 and Table 1).

Finally, we have examined the activated form of the chimeric r-GD_{IX}/PC for its ability to adopt APC-specific enzymatic activities. First, we assessed the overall *in vitro* anticoagulant activity of the chimera, relative to that of wtr-APC, in a plasma anticoagulant assay, wherein the prolongation of the APTT time of PC-deficient plasma was the basis of the assay (Zhang et al., 1992). With wtr-APC and r-GD_{IX}/APC present at identical amidolytic activities, the APTT times with each enzyme were the same over a range of concentrations of each of the two enzymes. Next, an assay of the comparative abilities of these two enzymes to inactivate fVIII as a cofactor for the complete tenase reaction was examined with purified reaction components. The pseudo-first-order inactivation rates of fVIII for each of the two enzymes are indistinguishable from each other (Figure 10). We therefore assign the anticoagulant and fVIII inactivation activities of the chimeric mutant to be the same as those of wtr-APC.

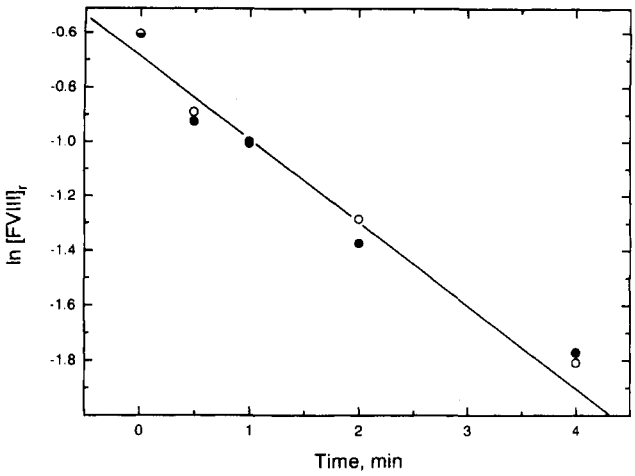

FIGURE 10: Time course of inactivation of human fVIII by wtr-APC and r-GD$_{IX}$/PC mutants at 37 °C, in a buffer containing 20 mM Hepes–NaOH/150 mM NaCl, pH 7.4. Each enzyme was present at the same amidolytic activity. A quantity of fVIII (6 nM, final concentration) was incubated with the particular 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, final concentration) and thrombin (0.12 units/mL) were added in order to activate the remaining fVIII. The amount of fVIIIa present was assayed in an otherwise complete fX activation assay. For this, the fVIIIa solution was added to a solution containing, in a spectrometer cuvette, final concentrations of 40 μM (in phosphate) PL, 2.5 mM $CaCl_2$, and 180 μM fXa chromogenic substrate S2222. The final volume was 790 μL. After recording the baseline 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 determined spectrophotometrically. The initial rates of fXa production were calculated from these experiments at each incubation time of APC with fVIII (relative to wtr-APC, which was assumed to be 100% active), and first-order kinetic replots are illustrated. The ratio of the rate constants for the mutants to that of wtr-APC was employed to calculate the percentage activity of each of the mutants. The symbols are as follows: (●) wtr-APC; (○) r-GD$_{IX}$/APC. The [fVIII] remaining is plotted as a function of the incubation times of fVIII and the relevant r-APC. A control incubation of fVIII with r-PC demonstrated that the loss of activity of fVIII was <10% over the time period illustrated on the graph.

DISCUSSION

A r-chimeric PC has been purified which contains the Gla domain and trailing HS of fIX as replacements for those same domains in PC. We constructed this protein in order to address issues as to the types of specificity conferred by these two domains in the properties of PC. Further, we believed that we could illuminate an important issue as to whether these domains could function independently in the intact protein. While Gla domains are highly homologous and likely bind Ca^{2+} in a similar manner, there are differences in the properties of the intact proteins in which they reside that have been assigned to their respective Gla domains. In addition to the need for specifically located Gla residues in this domain for proper functioning of r-PC and r-APC (Zhang, 1991; Zhang & Castellino, 1992, 1993b; Zhang et al., 1992), certain other highly conserved amino acid residues in the Gla domain of r-PC provide important functional characteristics. Specific examples involve Arg15 and Cys22, which are determinants for the anticoagulant activity of APC (Zhang and Castellino, 1992; Zhang et al., 1992), the basis of which in the former case appears to be a requirement for this residue for adoption of the Ca^{2+}-induced conformation of the Gla domain (Zhang & Castellino, 1992). We have also discovered that Leu5 is critical for the anticoagulant activity of r-APC because of its strong contribution to the hydrophobic binding energy of r-PC to PL (Zhang & Castellino, 1994).

The trailing HS exists as a separate exon in all vitamin K dependent proteins involved in blood coagulation or anticoagulation. From studies with synthetic peptides, we proposed that the HS is necessary for functional Ca^{2+} binding to the Gla domain of PC (Colpitts & Castellino, 1994), and another investigation has demonstrated the importance of this region for proper Ca^{2+} binding to the first EGF-like region of fX (Valcarce et al., 1993). The function of the HS has not been extensively investigated, but in one relevant study its participation in the binding of fIX to its endothelial cell receptor has been specifically excluded (Cheung et al., 1991).

On the basis of the above considerations, we addressed the cases of r-PC and APC in terms of identification of which of the specific properties of these proteins could tolerate substitution of the Gla and HS domains of a functionally unrelated protein. While it is clear from numerous other studies that the Gla modules of several proteins of these types are needed for many of their general Ca^{2+}/membrane-related functions, it has not been determined whether another intact Gla region could serve in an equivalent manner to an innate Gla domain to endow the proteins in which it resides with certain of its essential properties. PC and APC are particularly appropriate proteins to investigate in this regard because they contain Gla domains with the smallest number of Gla residues, which are strictly conserved in all other proteins of this type.

We have successfully purified the r-chimeric protein which contains the Gla domain and HS module of fIX coupled to the two EGF-like regions and protease domain of PC. The leader and propeptide sequences present were those from PC. This protein was not completely processed in 293 human kidney cells with regard to signal peptide release, since approximately half of the PC antigen-related chimeric protein contained amino acid sequences derived from the signal peptide region. We did not further investigate this population of molecules. Of the material isolated with the proper amino-terminal amino sequences, it appeared to be otherwise fully processed with regard to γ-carboxylation and β-hydroxylation, as well as cleavage of the dipeptide Lys156-Arg157 that provides mature two-chain PC. Thus, we conclude that the propeptide sequence of PC is not specific in directing γ-carboxylation exclusively to that protein, and despite the fact that the Gla domain of fIX possesses three additional Gla-precursor Glu residues, the propeptide of PC provides the recognition signal for this additional γ-carboxylation. Further, the fact that Asp72 in the chimeric protein is fully β-hydroxylated, as is the case in PC (Drakenberg et al., 1983; Yan et al., 1990; Zhang & Castellino, 1990), and not partially β-hydroxylated, as in fIX (Fernlung & Stenflo, 1983; Rabiet et al., 1987; Rees et al., 1988; Yu et al., 1994), suggests that neither the Gla domain nor the HS of PC or fIX contains recognition sequences for the β-hydroxylase that catalyzes this reaction, a conclusion in agreement with a separate observation on this matter (Yu et al., 1994). In addition, proteolytic processing of the Lys156-Arg157 dipeptide occured in the chimera to the same extent as in PC, suggesting that the Gla or HS domains of PC play no role, or at least no unique role, as recognition elements for this processing protease.

It was of interest to evaluate the nature of the Ca^{2+}-induced conformation of the chimeric fIX/PC molecule. One means of assessing this is through titration of the Ca^{2+} dependency of the change in intrinsic fluorescence of the chimeric protein, as compared to fIX and r-PC. The data obtained (Figure 4) show that approximately a 3-fold higher [Ca^{2+}] was required to induce the final Ca^{2+}-dependent conformation in r-GD$_{IX}$/APC, as compared to fIX, and a greater than 4-fold higher

[Ca^{2+}] was needed for this chimera when compared to r-PC. These results can be best explained by considering the possibility that the Gla/HS domains of fIX in the apo-Ca^{2+} form of the chimera undergo a different set of interactions with the remainder of the protein than is the case with interactions of the Gla/HS domains of fIX with other regions of fIX, and similarly of the Gla/HS domains of PC with other regions of PC. These altered interactions in the chimera might be more difficult to disrupt with Ca^{2+}, thereby leading to the Ca^{2+} effects observed. These properties may be related to the presence of additional Gla residues in the chimera. In this regard, it is of interest to note that when an extra Gla residue was mutated into r-PC at position 32 (r-PC[Gln32Gla]), the [$C_{50,Ca}$-FI] was similarly increased from 0.40 mM for wtr-PC to 1.48 mM for this r-PC mutant (Zhang & Castellino, 1992). In the chimeric r-GD_{IX}/APC, a Gla residue exists at the homologous sequence position 33, as well as well as at sequence positions 36 and 40 (Figure 1).

Investigations with Gla domain-directed, Ca^{2+}-dependent MAb's to PC and fIX were found to be very useful in evaluating the nature of the Ca^{2+}-bound form of these proteins. The MAb JTC-1, which we have successfully employed to measure adoption of the proper Ca^{2+}-induced conformation in Gla-domain mutants of r-PC (Zhang & Castellino, 1992), did not react with Ca^{2+}-induced conformations of either fIX or r-GD_{IX}/PC. In the former case, this demonstrates the high specificity of this MAb, and in the latter case the result shows that PC sequences outside of the Gla domain do not influence the nature of the epitope for this antibody on PC. Another MAb (H5B7) which possesses a similar specificity toward the Ca^{2+}-dependent conformation of the Gla domain of fIX has been tested in the same manner and shown not to possess the Ca^{2+}-dependent epitope in r-PC. However, this MAb recognizes equally (Figure 5) the Ca^{2+}-induced conformations of fIX and r-GD_{IX}/PC, showing that the Gla domain of fIX adopts the same Ca^{2+}-dependent structure whether this module (plus the HS) is present in fIX or r-PC.

The influence of Ca^{2+} on the interaction of these proteins with acidic PL has been assessed. Plots of the increase in scattering intensity of fIX/PL and r-GD_{IX}/PC-PL complexes as a function of [Ca^{2+}] are provided in Figure 6. The [$C_{50,Ca}$-PL] values (Table 1) calculated from these data show that the binding of fIX and the chimera to these PL vesicles are similarly dependent on [Ca^{2+}] and not greatly different from that of r-PC. The issue raised with interpretation of these data in the case of the chimera is why the [$Ca_{50,Ca}$-PL] is lower than the [$Ca_{50,Ca}$-FI] if the Ca^{2+}-induced conformation that is measured by intrinsic fluorescence quenching is indeed the same as that detected by divalent cation-specific MAb's, this latter conformation having been shown to be requisite to generation of additional Ca^{2+} sites needed for PL binding (Borowski et al., 1986; Liebman, 1993). It should be pointed out that a similar situation was found in the case of r-[PC/Gln32Gla], which, as stated above, possessed a higher [$C_{50,Ca}$-FI] than wtr-PC, but its [$C_{50,Ca}$-PL] was the same as that of wtr-PC (Zhang & Castellino, 1993). Thus, consideration of the presence of Gla33 in the chimeric construct with the remainder of the PC structure may be very important to the resolution of this issue. While there are a myriad of possible explanations involving altered conformations and domain interactions in the chimeric mutant that could be used to unravel these observations, these would be highly speculative, and only a few points will be made. Further, since the structural basis of the fluorescence quenching by Ca^{2+} is not completely understood, we would not at this time be able to fully resolve this matter. It is possible that the adoption of the Ca^{2+}-induced, PL-binding conformation occurs at a lower [Ca^{2+}] employing fluorescence-refractive Ca^{2+} sites within or outside of the Gla domain and that the fluorescence alteration monitors events that occur after this point. Use of this chimeric mutant may have led to distinction of these Ca^{2+} sites, whereas in the native molecules they may be sufficiently similar in Ca^{2+}-binding energies as to appear to be the same.

At saturating levels of Ca^{2+}, the [$C_{50,P}$-PL] values for r-PC and the chimeric fIX/r-PC (Figure 7) are nearly equal, suggesting that the specific nature of the Gla/HS domains is not critical to PL binding at saturating [Ca^{2+}]. Thus, the greater strength of binding of r-PC to PL, as compared to that of fIX (Table 1), is likely based in features of the protein outside of the Gla/HS domains that merely require the supporting features of a Gla/HS unit possessing the properties presented by the native PC or fIX Gla/HS domains. It is well-known that Gla-domainless proteins do not possess strongly PL-binding properties. Whether Gla domains and HS regions from other proteins could also function to conserve PL binding in PC is an important question and is being addressed by this laboratory.

The possible communication of the Gla/HS region with other Ca^{2+}-binding regions of PC has been examined by investigation of the effects of Ca^{2+} on the inhibition of the activation of r-GD_{IX}/PC by thrombin (Amphlett et al., 1981). This particular Ca^{2+} site exists outside of the Gla domain of PC (Johnson et al., 1983). In this paper, we show (Figure 8 and Table 1) that this particular Ca^{2+} site is essentially unaffected by the interchange of the native Gla/HS segment between fIX and PC. We also show that when the r-sTM/thrombin complex is used as the activator, Ca^{2+} first stimulates and, at higher concentrations, inhibits the activation of both r-PC and r-GD_{IX}/PC in the same manner as that described previously (Parkinson et al., 1990). This stimulatory Ca^{2+} site is also located outside of the Gla domain of PC and may be different from the Ca^{2+} site identified in inhibition of this activation with thrombin (Rezaie & Esmon, 1992). Thus, it appears either that, in the intact protein, the Gla/HS domains of PC do not specifically communicate with the high-affinity Ca^{2+} binding site(s) that exist outside of the Gla domain and/or that if such communication does exist, at least one other native Gla/HS region from a functionally unrelated protein also possesses this same information. Further, at the [Ca^{2+}] wherein maximal stimulation of activation by thrombin/r-sTM occurs for both wtr-PC and the chimera, the steady-state kinetic properties for both proteins are nearly identical, showing that the Gla/HS domains of either PC or fIX can function equally in PC to provide a fully functional zymogen. It is pointed out here that we have employed r-sTM as the cofactor, using a construction that contained a mutation in the amino acid responsible for anchoring the chondroitin sulfate moiety. Our observations are strictly limited to this type of molecule, and the behavior in this regard of cell-bound TM containing chondroitin sulfate is not known at this time.

After complete activation, both r-APC and r-GD_{IX}/APC possessed equivalent anticoagulant activity both in a PC-deficient plasma-based APTT assay and in a fVIII assay in an *in vitro* system with purified components of the tenase complex. This was quite a surprising result, since it was anticipated that, because of the lack of anticoagulant activity in a Gla-domainless form of APC (Esmon et al., 1983), specificity in the Gla/HS domains should reside at least in this most critical property. This once again argues that the presence of a native Gla domain/HS region may confer certain

properties to proteins of this type, by virtue of containing certain essential amino acids that function equivalently in such proteins and that allow similar Ca^{2+}-induced conformations to occur.

The studies presented herein argue strongly that the combination Gla/HS region, when present in a complex with Ca^{2+}, exists as a separate domain unit in PC, and probably also in fIX. The basis for this conclusion lies in the adoption and recognition of the Ca^{2+}-dependent conformation of the Gla domain of fIX when this domain, along with its HS, is present in PC. Also, the complete retention of many defining properties of PC and APC, when the Gla/HS region of these proteins is replaced by another such segment from a functionally unrelated protein, strongly suggests that the Ca^{2+}-induced folding information required for conferral of specific properties of the proteins in which these units reside is contained in the modules themselves. Likely, a Gla/HS region, with as yet not fully identified basic features, is sufficient to elucidate the properties of the protein in which it is present. Excluded from this conclusion at this juncture is the property of binding of these proteins to specific cell receptors, which may require specific amino acid residues of the innate Gla domain or Gla/HS unit of the relevant protein. This conclusion that the Gla/HS segment may exist as an independent domain in the presence of Ca^{2+} agrees with recent work that demonstrates that essential Ca^{2+} binding and Ca^{2+}-induced conformational features present in intact PC are also contained in a 48-mer synthetic peptide consisting of the entire Gla domain and HS module of PC. Thus, as a result of generation of appropriate chimeric proteins and synthetic polypeptides, we are now able to address questions concerning the functions of individual structural units in proteins of these types, which will lead to construction of new r-proteins containing combinations of predesigned properties.

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