Structure−Function Assessment of the Role of the Helical Stack Domain in the Properties of Human Recombinant Protein C and Activated Protein C[†]

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ABSTRACT: The role of the helical stack (HS) in defining the properties of human recombinant (r) protein C (PC) and activated protein C (APC) was assessed. To do so, several mutations were made in this region of the molecule and their effects on the proteins examined. Substitution of the entire HS of PC (residues 38−46) by that of human coagulation factor (f) IX (residues 39−47), yielding r-[HS_{IX}]PC, did not result in any substantial changes in the γ-carboxyglutamic acid domain (GD)-related Ca^{2+}-dependent properties of PC or APC, suggesting that the conformation of the HS may play a more dominant role in these Ca^{2+}-dependent properties than do the specific amino acids that differ between these two HS regions. On the other hand, the catalytic efficiency of activation of r-[HS_{IX}]PC by the thrombin/thrombomodulin complex was reduced to approximately one-third of that of wtr-PC, a result that demonstrates a specific role for the HS of PC in this activation process. Another mutation, [Ser^{42}→Pro], was generated in the HS region of r-PC, providing r-[$S^{42}P$]PC, a change that according to the empirical algorithm based on the Chou−Fasman secondary structure rules, would disrupt the α-helical conformation of the HS. The anticoagulant activity of the corresponding r-[$S^{42}P$]APC was found to be approximately 35% of that of wtr-APC. Because of the lack of any notable effects of this mutation on other GD-related Ca^{2+}-dependent properties of r-PC and r-APC, the basis of this anticoagulant activity loss may be due to its nonmaximal alignment with substrate on the PL surface. The results of this study indicate that the role of the HS of r-PC and r-APC is to provide a region of the protein that is needed to assure optimal alignment on the PL or cell surface of the active site of the enzyme with that of the cleavage sites of the substrates, perhaps by functioning as a scaffold for separation of the active site of APC from the PL surface.

Human protein C (PC)[1] is the plasma protein precursor of the serine protease APC. This latter enzyme possesses anticoagulant activity by virtue of its ability to catalyze specific peptide bond cleavages in two procoagulant cofactors, fV/fVa (Kisiel et al., 1977) and fVIII/fVIIIa (Vehar & Davie, 1980). As a result of these reactions, the modified proteins become ineffective cofactors for the prothrombinase and tenase complexes, thus inhibiting the generation of fIIa and fXa, respectively, and consequently retarding the formation of the fibrin clot. Activation of PC is accomplished by fIIa, in a process inhibited by Ca^{2+} (Amphlett et al., 1981). More efficient activation of PC occurs on cell surfaces, where it is catalyzed by fIIa bound to its cell surface receptor Tm (Esmon & Owen, 1981; Esmon et al., 1982). This event is stimulated by low concentrations of Ca^{2+}, and at higher cation concentrations this reaction undergoes inhibition (Glaser et al., 1992; Clarke et al., 1993).

The DNA sequence of PC contains eight exons separated by introns (Foster et al., 1985). The first exon extends from the 5′-untranslated sequence to the junction of the prepropeptide and propeptide, ending within the codon for Met^{-19}; exon 2 contains the entire GD, terminating at the boundary of amino acids Thr^{37}−Leu^{38}; the third exon is composed of a short α-helix, the HS, which stretches from Leu^{38} to within the codon for Asp^{46}; the fourth exon consists of the first EGF of PC (EGF1), which terminates in the codon for Glu^{92}, and also contains Hya^{71}; exon 5 similarly encodes EGF2, concluding within the codon for Val^{137}; the sixth exon, ending at Asn^{184}−Val^{185}, contains the PC activation peptide; exon 7, terminating in the codon for Gly^{224}, possesses the active site His^{211}; and the last codon, which extends into the 3′-untranslated region of the PC gene, contains the other two active site residues of the serine protease catalytic triad, Asp^{255} and Ser^{360}. This intron−exon relationship is very similar to that found in related vitamin K-dependent coagulation proteins, *viz.*, fVII (O'Hara et al., 1987), fIX (Yoshitake et al., 1985), and fX (Leytus et al., 1986), and suggests that these proteins may have evolved by exonic shuffling, with independent divergence to provide their distinct functions.

The importance of the specific exons of these proteins in governing their properties has been examined in several fashions, among which is through their artificial shuffling from one protein to another to generate chimeric molecules. With regard to PC, we have created and investigated the properties of a domain-substitution chimera wherein both EGF1 and EGF2 were replaced by those regions of human fIX (Yu et al., 1994), and another where the entire GD/HS of PC was replaced by these modules of fIX (Christiansen & Castellino, 1994). Substitutions of these regions affected the properties of the r-PC and r-APC chimeras, in the first

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case in functions relating to biological activity and activation capabilities, and in the second in properties associated with Ca^{2+} and PL binding.

The HS of these proteins appears to be important for the conferral of proper Ca^{2+} binding capabilities of the GD of PC (Colpitts & Castellino, 1993) and of the EGF1 of fX (Valcarce et al., 1993), and may be an important determinant in fVIIa for tissue factor binding (Petersen et al., 1994). These studies support an earlier conclusion that the HS may be needed for adoption of the proper Ca^{2+}-dependent conformation of bovine prothrombin (Soriano-Garcia et al., 1992). In order to more thoroughly examine the specific role of the HS in determining the properties of r-PC and r-APC, we generated strategic mutants in this region of the molecule which were designed to examine whether only the α-helical conformation is necessary for the HS to provide its functionality and/or whether specific amino acids in the HS are required for conferral of its specific properties. This paper provides a report of our findings.

MATERIALS AND METHODS

Proteins. r-PC and its mutants were expressed in human 293 cells and purified in two steps on FFQ columns at 4 °C as described previously (Zhang & Castellino, 1990). Each of the r-APC forms was prepared from the relevant zymogens by activation with the venom protease Protac C (American Diagnostica, New York, NY). Human r-fVIII was donated by the Genetics Institute (Cambridge, MA). Bovine fIXaβ was obtained by activation of bovine fIX (Amphlett et al., 1978) as published earlier (Amphlett et al., 1979). r-sTM (Solulin), lacking chondroitin sulfate (Glaser et al., 1992), was provided by Berlex Biosciences (Richmond, CA). Human fIX, fX, and fIIa were products of Enzyme Research Laboratories (South Bend, IN). r-y[Tyr^{63}]Hirudin (CGP 39393), an engineered form of hirudin expressed in yeast that lacks the sulfate group on Try^{63} (Meyback et al., 1987), was provided by Ciba-Geigy (Horsham, West Sussex, England).

The MAb to plasma PC, JTC3, which recognizes a Ca^{2+}-dependent epitope in the GD of PC, was provided by Dr. K. Wakabayashi (Tokyo, Japan). The antibody was radiolabeled using the Iodobead (Pierce Chemical Co., Rockford, IL) procedure. Our labeling protocol has been described (Zhang & Castellino, 1992). The anti-human plasma MAb, C3, possessing a Ca^{2+}-independent epitope within 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 Corp. (Madison, WI). Recombinant *Pfu* DNA polymerase was obtained from Stratagene (La Jolla, CA).

Lipids. PL vesicles were prepared from mixtures of PhC/PhS (60/40 w/w) as described (Beals & Castellino, 1986). The PL vesicle concentration 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. Full descriptions of the cDNAs encoding human PC (p119-[PC]) and human fIX (p119-[fIX]), both engineered into the plasmid pUC119, have been provided earlier (Zhang & Castellino, 1990; Yu et al., 1994).

Mutagenesis Strategy for Construction of the cDNA Encoding r-[HS_{IX}]PC. A PCR-based strategy involving gene splicing by overlap extension was used to construct the cDNA that codes for the chimera, r-[HS_{IX}]PC. The first two PCR primers (below) were used with our restriction site-modified p119-[PC] (Zhang & Castellino, 1990) as a template to provide fragment 1. This segment consisted of a stretch of cDNA beginning in the modified multiple cloning region at a location 45 nucleotides (base −171) upstream (the codon for the signal Met begins at nucleotide −126) of the cDNA of PC (forward primer 1) and ending at base 111 of PC (reverse primer 2). The modified p119-[PC] also contained a *Nhe*I restriction site between nucleotides −153 and −152. This was downstream of the material amplified with primer 1, below. In addition, fragment 1 possessed an additional 21 bases at its 3′-terminus containing nucleotides found in the HS region of the cDNA of fIX. The primers used were (the fIX nucleotides and amino acids are italicized) the following: (1) 5′-forward primer, 5′- ACT TGC AGT ATC TCC ACG; (2) 3′-reverse primer, 5′-*ATA CTG CTT CCA AAA TTC AGT* TGT GTC ATC CAC ATT TTT G.

Thus, the translated cDNA of fragment 1 contains the signal, propeptide, and GD regions of PC, through amino acid Thr^{37}. Downstream of Thr^{37} in this construct are the fIX amino acids contained in the HS of fIX, *viz.*, *Thr^{38}-Glu-Phe-Trp-Lys-Gln-Tyr^{44}* (the final two residues—Val^{45}-Asp^{46}—needed to complete the HS of fIX are identical in both PC and fIX and were therefore not altered).

The next stage of this construction was to generate fragment 2, which spans a cDNA segment of PC beginning at nucleotide 135 and terminating at base 531. Included in fragment 2, upstream of base 135, are the same 21 nucleotide sequence as in primer 2 that encoded sequences found in the HS region of the cDNA of fIX. The primers used for generation of fragment 2 were the following: (3) 5′-forward primer, 5′-*ACT GAA TTT TGG AAG CAG TAT* GTC GAC GGT GAC CAG TG; (4) 3′-reverse primer; 5′-CCT GGT CAT CTT CCC ATC.

In this case, the translated cDNA of fragment 2 begins at the HS of fIX (amino acid residues 38−44 of the chimeric

[1] Abbreviations: PC (APC), human protein C (activated human protein C); fIX (fIX$\alpha\beta$), human coagulation factor IX (activated factor IX); GD, γ-carboxyglutamic-rich domain of protein C (residues 1−37) or factor IX (residues 1−38); HS, helical stack region of protein C (residues 38−46) and/or factor IX (residues 39−47); EGF, epidermal growth factor homology regions of protein C (residues 47−137) or factor IX (residues 48−128); r-[HS_{IX}]PC (APC), chimeric recombinant protein C (activated protein C) in which residues 38−46 of human protein C have been replaced by residues 39−47 of human factor IX; r-[$W^{41}Y$]PC (APC), mutant recombinant protein C (activated protein C) in which residue Trp^{41} has been replaced with Tyr; r-[$S^{42}P$]PC (APC), mutant recombinant protein C (activated protein C) wherein Ser^{42} has been replaced with Pro; fIIa, thrombin; PS, protein S; Tm, thrombomodulin; r-sTm, recombinant soluble thrombomodulin containing residues 1−490 (this construct lacks the transmembrane domain); fVIII (fVIIIa), coagulation factor VIII (activated coagulation factor VIII); Gla, γ-carboxyglutamic acid; Hya, β-hydroxyaspartic acid; 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, S2366, L-pyro-Glu-L-Pro-L-Arg-*p*-nitroanilide; $NaDodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; [$C_{50,Ca}$-Fl], total Ca^{2+} concentration required to alter the intrinsic fluorescence in half of the protein molecules; [$C_{50,Ca}$-MAbJTC3], total Ca^{2+} concentration required to induce monoclonal antibody JTC3 binding to 50% of the protein molecules; [$C_{50,Ca}$-PL], total Ca^{2+} concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of protein; [$C_{50,PC}$-PL], total protein concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of Ca^{2+}.

construct) and PC sequences from amino acid residues 45−177.

After this point, another PCR extension reaction was conducted with fragments 1 and 2 as the templates, along with primers 1 and 4. These two PCR products were annealed through their overlapping sequences and amplified by PCR using primers 1 and 4. This provided a sufficient amount of a chimeric cDNA fragment (fragment 3) spanning nucleotides −171 to 531 of modified p119-[PC] (base 1 in this plasmid is defined as the first nucleotide encoding the amino-terminal residue of mature PC) with nucleotides 112−132 containing sequences from fIX.

Fragment 3 thus possesses a unique *Nhe*I restriction site in its 5′-region (between bases −153 and −152) and a unique *Sac*II restriction site in its 3′-region (between nucleotides 505 and 506). Digestion of fragment 3 with *Nhe*I/*Sac*II liberated cDNA fragment (fragment 4) containing bases −152 to 505. Fragment 4 was then inserted into the same restriction fragment of the modified p119-[PC], generating plasmid p119-[HS_{IX}]PC.

The cDNA encoding r-[HS_{IX}]PC was excised from p119-[HS_{IX}]PC by use of 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-[HS_{IX}]PC. This latter plasmid was then transfected into human kidney 293 cells (ATCC CRL 1573). Positive clones were then selected by assay with the MAb C3 (Zhang & Castellino, 1990).

The nucleotide sequence of this r-PC chimera was obtained through the chimeric HS region.

Construction of the cDNAs Encoding r-[$W^{41}Y$]PC and r-[$S^{42}P$]PC. The cDNAs encoding these r-PC mutants were prepared by *in vitro* site-directed mutagenesis using synthetic oligonucleotides on single-strand templates of wtr-PC in pUC119 (Kunkel et al., 1987). The mutagenic primers used were (the mutagenic bases are in lower case letters):

r-[$W^{41}Y$]PC: 5′-CTG GCC TTC Tac TCC AAG CAC G

r-[$S^{42}P$]PC: 5′-G GCC TTC TGG cCC AAG
CAC GTg GAC GGT GAC

Screening of the bacterial transformants for the colonies containing the desired mutations was accomplished by restriction endonuclease analysis. For the [Trp^{42}→Tyr] mutant, successful transformation with the mutagenesis primer led to loss of an *Ava*II site in the wt DNA, and for the [Ser^{42}→Pro] mutant, the primer used resulted in loss of the unique *Sal*I site that is present in the wt gene. These characteristics were used to screen the bacterial transformants.

The nucleotide sequences of these r-PC mutants were determined around the altered regions.

Intrinsic Fluorescence Titrations. The dependency on the Ca^{2+} concentration of the adoption of the Ca^{2+}-induced conformation of r-PC mutants, as revealed through effects of Ca^{2+} on the intrinsic fluorescence of these proteins, was determined as described earlier (Zhang & Castellino, 1992). These measurements were conducted using a SLM-Aminco 8100 recording spectrofluorometer (SLM-Aminco Instruments, Urbana, IL).

MAb Binding to r-PC Mutants. The dependency on the Ca^{2+} concentration of the binding of the Ca^{2+}-dependent conformational MAb [^{125}I]-JTC3 to r-PC mutants was determined as detailed earlier (Zhang & Castellino, 1994).

Interaction of r-PCs with Acidic PL. The binding of r-PC mutants to acidic PL was measured at 20 °C using 90° relative light scattering techniques as previously described (Nelsestuen, 1976; Nelsestuen et al., 1976; Nelsestuen & Broderius, 1977; Nelsestuen & Lim, 1977). Our procedures for performing these titrations have been published (Zhang & Castellino, 1993).

Kinetic Analyses of the Activation of r-PC Mutants. The steady-state kinetic constants for activation of the r-PC mutants were performed with fIIa and fIIa/r-sTM as activators, plus the desired levels of Ca^{2+}. The concentrations of r-APC present at various times during the activations were determined by amidolytic assay of these enzymes with the chromogenic substrate S2366. The details of the assays have been described previously (Yu et al., 1994).

Functional Assays of r-APC Mutants. Plasma APTT anticoagulant assays as well as *in vitro* fVIII inactivation assays were employed to determine the functional activities of the r-APC mutants. Our methodologies have been thoroughly described earlier (Zhang et al., 1992; Jhingan et al., 1994; Yu et al., 1994).

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

RESULTS

Three r-PC mutants were constructed, expressed in human 293 cells, and purified, with the intent of evaluating the role of the HS region of PC and APC in directing its biochemical properties. One mutant, r-[HS_{IX}]PC, was investigated for the purpose of assessing whether amino acid specificity existed in the HS of PC, and/or whether only the helical conformation was required for its properties. Another mutant, r-[$S^{42}P$]PC, was studied since the mutation made would, according to Chou−Fasman calculations, disrupt the α-helical conformation in this region of the protein. Finally, our rationale for preparation of r-[$W^{41}Y$]PC was to determine the extent to which this particular Trp residue influenced the Ca^{2+}-dependent fluorescence alterations in r-PC. The locations of these changes in the primary structure of PC are diagrammed in Figure 1.

The FFQ chromatographic profiles observed during purification of r-[HS_{IX}]PC, r-[$W^{41}Y$]PC, and r-[$S^{42}P$]PC were similar to those published previously for wtr-PC, as well as a variety of other r-PC mutants (Zhang & Castellino, 1990, 1994; Yu et al., 1994). In these particular cases, highly homogeneous proteins were obtained, as shown by the

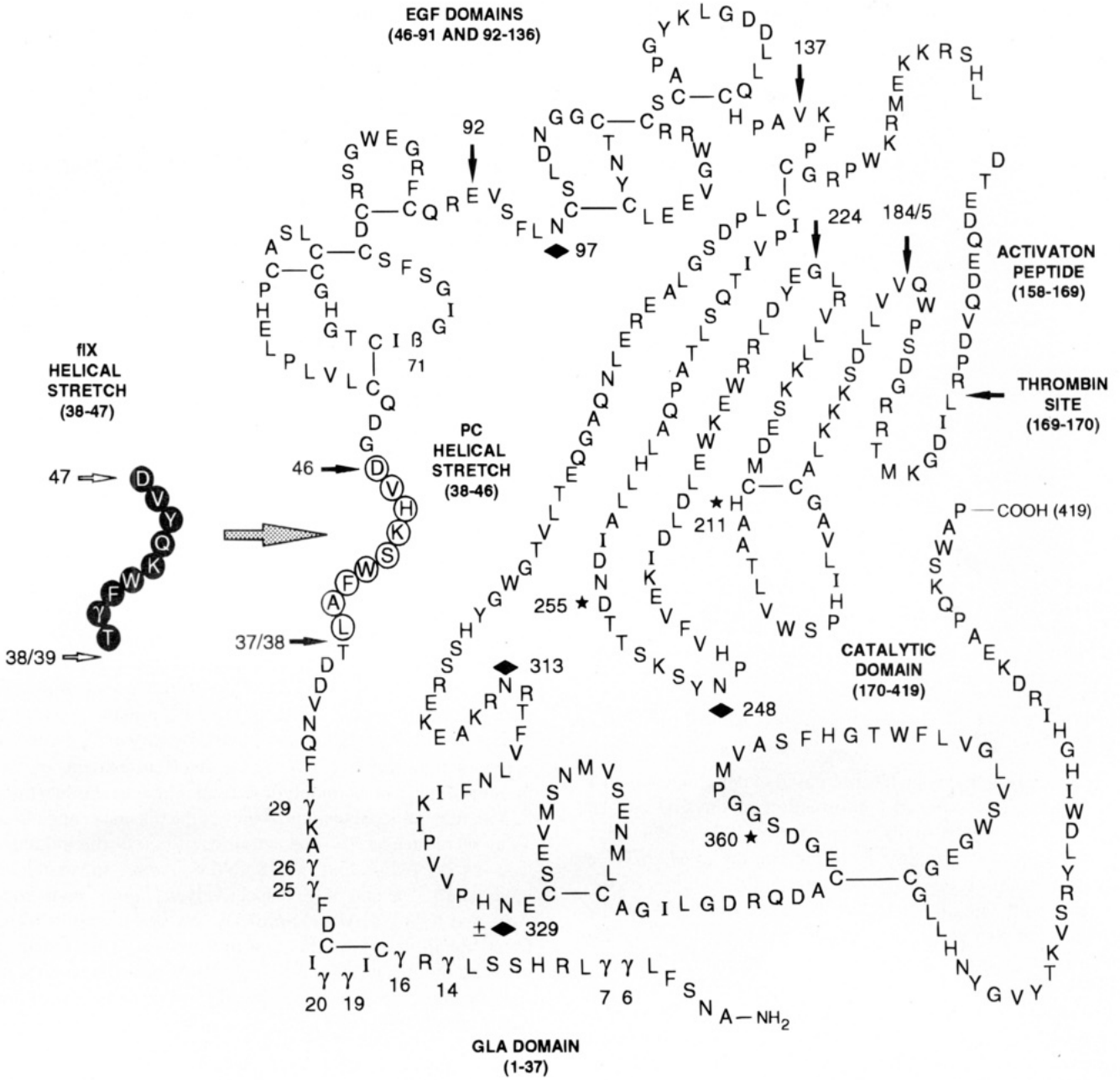

FIGURE 1: Primary structure of r-[HS_{IX}]PC. The amino acids of fIX are displayed using color reversals. The filled arrows indicate the amino acid sequence positions of the introns in the gene, the filled diamonds are the locations of N-linked oligosaccharides, and the filled stars show the positions of the catalytic triad of amino acids in the activated form of this chimera. γ refers to Gla residues in the mature protein, and β indicates the sequence position of Hya. The sequence gap between the carboxy terminus of the light chain and the amino terminus of the heavy chain results from liberation of the dipeptide Lys^{156}-Arg^{157} during maturation of PC, thus causing a sequence numbering discontinuity at this location.

$NaDodSO_4$/PAGE electrophoretograms of Figure 2. For the nonreduced gels, the doublet band pattern has been found in every mutant r-PC so far constructed, as well as in plasma PC, and is due to the existence of subpopulations of glycoforms of these proteins (Yan et al., 1990). Each of the bands is fully reactive on Western immunoblots with the anti-PC MAb, C3, demonstrated that they are in fact r-PC. In the reduced gels, the two chains of PC are observed, with very small amounts of single-chain forms of the proteins. This indicates that the Lys^{156}-Arg^{157} dipeptide has been effectively removed by endoproteolytic processing during maturation of r-PC, and the great majority of the r-PC populations of the mutants consist of two-chain, disulfide-linked protein. Other important processing events have also fully occurred in the mutant proteins—each contains its full complement of Gla and Hya residues (Table 1).

The Ca^{2+}-induced change in the intrinsic fluorescence of r-PC and its mutants has been employed as a measure of the ability of the proteins to adopt their Ca^{2+}-dependent conformations within the GD. Titrations of the change in this property with Ca^{2+} have been performed, and the data obtained are illustrated in Figure 3. The [$C_{50,Ca}$-Fl] values obtained (Table 1) do not demonstrate any substantial differences between the mutants and wtr-PC, suggesting that each undergoes the Ca^{2+}-dependent change at approximately the same concentrations of Ca^{2+}. The largest difference (2-fold) has been found between wtr-PC and r-[HS_{IX}]PC. This is not considered of importance to APTT function, but may have some relevance to the kinetics of activation of this r-PC mutant (*vide infra*).

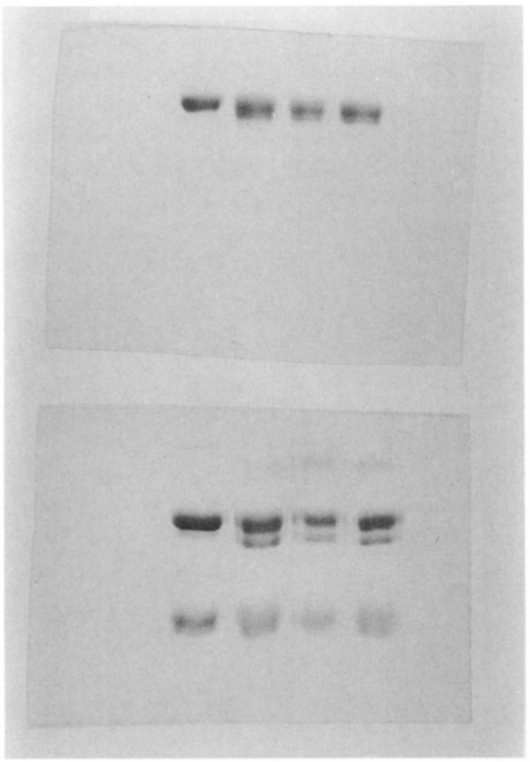

FIGURE 2: $NaDodSO_4$/PAGE analysis of proteins employed. (Top) Left-to-right: nonreduced wtr-PC; nonreduced r-[$W^{41}Y$]PC; nonreduced r-[$S^{42}P$]PC; nonreduced r-[HS_{IX}]PC. (Bottom) Left-to-right: reduced wtr-PC; reduced r-[$W^{41}Y$]PC; reduced r-[$S^{42}P$]PC; reduced r-[HS_{IX}]PC.

Another technique that has been used to determine whether the proper Ca^{2+}-induced conformation of the GD can be achieved is with use of appropriate MAbs. We have employed titrations with Ca^{2+} of the binding of MAb-JTC3 (Wakabayashi et al., 1986) for this purpose. Plots of the titration data are illustrated in Figure 4 for each of the proteins employed herein. While the [$C_{50,Ca}$-MAbJTC3] values for each of the proteins are essentially the same as for wtr-PC (Table 1), the plots of Figure 5 show that the curve for the Ca^{2+}/r-[HS_{IX}]PC complex is near saturation at an approximate 2-fold lower level than for the other mutants. That this is due to a slightly lower affinity (*ca.* 2-fold) of the Ca^{2+}/r-[HS_{IX}]PC complex for the MAb, as compared to the other mutants, and to wtr-PC, is confirmed by the fact that when the concentration of r-[HS_{IX}]/PC was doubled for this experiment the [$C_{50,Ca}$-MAbJTC3] was unchanged from that in Figure 4 and Table 1, while the saturation point was raised to approximately 95% of wtr-PC.

In order to determine whether the HS of r-PC communicates with the GD with regard to the ability of r-PC to interact with PL, we measured the binding of the mutant forms of r-PC to acidic PL vesicles, using 90° light scattering measurements. Both r-[HS_{IX}]PC and r-[$S^{42}P$]PC showed Ca^{2+} dependencies of PL binding (Figure 5 and Table 1) that were nearly identical to wtr-PC. The [$C_{50,Ca}$-PL] for r-[$W^{41}Y$]PC was approximately 2-fold greater those same values for r-[HS_{IX}]PC and r-[$S^{42}P$]PC. Additionally, the maximum M2/M1 value for the r-[$W^{41}Y$]PC mutant was approximately half of that for wtr-PC. Thus, this mutation does appear to influence its binding to PL. Binding of the Ca^{2+}/mutant complexes to the PL vesicles was measured by the same techniques at 2 mM Ca^{2+} (Figure 6) and at 20 mM Ca^{2+} (data not shown). The [$C_{50,PC}$-PL] values determined for the mutants were very similar to each other, and to wtr-PC (Table 1). The saturation point of r-[HS_{IX}]PC occurred at a higher M2/M1 value than for wtr-PC and the site-directed mutants, indicating either that its extent of binding to PL was greater for this mutant and/or that its conformation on the PL surface was different than the other proteins studied.

A Ca^{2+}-dependent property of the r-PC mutants that resides outside of the GD is the site(s) responsible for inhibition of the fIIa-catalyzed activation of this zymogen (Amphlett et al., 1981). Steady-state kinetic data for the influence of Ca^{2+} on the activation of each of the mutants, and for wtr-PC, are presented in Table 1. In each case, the K_i values for Ca^{2+} for this process were essentially the same, and ranged from 145 to 159 μM (Table 1).

The abilities of the r-PC variants to be activated by the fIIa/r-sTM complex were determined in the presence of an optimal concentration of Ca^{2+} for this reaction, which we found to be approximately 100 μM. Steady-state kinetic plots for the activation for each of the mutants were found to be of a routine nature, and values obtained for the steady-state parameters of this activation are listed in Table 1. Only the chimeric mutant, r-[HS_{IX}]PC, displayed activation properties significantly different from those of wtr-PC. In this latter case, the second-order efficiency (k_{cat}/K_m) of activation is approximately one-third of that of wtr-PC.

The activity of the mutant proteins toward biological substrates is reflected in their ability to prolong coagulation times in APTT assays. Each of the r-PC mutants was fully activated to its corresponding r-APC by Protac C, a venom activator not stimulated by Ca^{2+}. After adjustment of the proteins to the same amidolytic activities against the substrate S2366, their anticoagulant activities in the plasma coagulation assay were measured. The activities of two of the mutants, *viz.*, r-[$S^{42}P$]APC and r-[HS_{IX}]APC, were substantially lowered, to 35% and 68%, respectively, as compared to wtr-APC and r-[$W^{41}Y$]APC. Similarly lowered activities were found for these two mutants in *in vitro* assays designed to measure the inactivation rate of fVIII (Figure 7), one of the specific cofactors inactivated by APC. The activity data are summarized in Table 1.

DISCUSSION

The purpose of this investigation was to evaluate the role of the HS in defining the properties of r-PC. Because of the common modular nature of this and several other related proteins that possess different roles in blood coagulation, it is generally believed that they may have evolved from a common ancestor through exon shuffling, with divergence through site-specific gene alterations to different functional properties as organisms became more complex and required additional levels of physiologic control. Some confidence in such an interpretation would be gained if it can be proven that the different domains of these proteins possessed independent functional properties. This, in part, has been observed in the types of proteins addressed herein, with all of the propeptides having a responsibility for directing γ-carboxylation (Foster et al., 1987; Jorgensen et al., 1987; Huber et al., 1990; Gaussem et al., 1994); the GDs containing important Ca^{2+} and PL binding sites (Gitel et al., 1973; Nelsestuen et al., 1974; Nelsestuen, 1976; Colpitts & Castellino, 1994) which are important for their biological activities (Esmon et al., 1983; Sugo et al., 1984; Morita &

Table 1: Properties of r-[HS_{IX}]PC and r-[HS_{IX}]APC

parameter	mutant[a] wt[b]	[W41Y]PC[c]	[S42P]PC[d]	[HS_{IX}]PC[e]
	PC[f]			
Gla (calcd), mol/mol	8.9 (9.0)	9.0 (9.0)	9.1 (9.0)	9.6 (10.0)
Hya (calcd), mol/mol	1.1 (1.0)	1.1 (1.0)	1.0 (1.0)	1.0 (1.0)
[$C_{50,Ca}$-Fl], mM (% Fl_{max})	0.40 (19)	0.42 (13)	0.45 (19)	0.19 (25)
[$C_{50,Ca}$-MAbJTC3], mM	15	14	15	17
[$C_{50,Ca}$-PL], mM	1.2	2.8	1.1	1.1
[$C_{50,PC}$-PL] (2 mM Ca^{2+}), μM	0.38	0.36	0.40	0.39
[$C_{50,PC}$-PL] (20 mM Ca^{2+}), μM	0.38	0.42	0.42	0.52
K_i-Ca^{2+} (fIIa), μM	148	145	151	159
K_m (fIIa/r-sTm, 100 μM Ca^{2+}), μM	4.1	3.6	3.9	3.5
k_{cat} (fIIa/r-sTm, 100 μM Ca^{2+}), min^{-1}	32	28	25	8.2
k_{cat}/K_m (100 μM Ca^{2+}), min^{-1} μM^{-1}	7.4	7.8	6.4	2.3
	APC[g]			
APTT, % wt	100	81	35	82
fVIII (2.5 mM Ca^{2+}), % wt	100	95	44	94

[a] The values reported below are averaged from duplicate experiments that were reproducible to within 6% of each other. [b] Wild-type recombinant human PC or APC. [c] Recombinant human PC or APC wherein Trp^{41} is replaced by Y. [d] Recombinant human PC or APC wherein S^{42} is replaced by P. [e] Recombinant chimeric human PC or APC wherein the HS of PC/APC (residues 38−46) is replaced by the equivalent HS (residues 39−47) of human factor IX. [f] The properties below are for the zymogen forms of the proteins. [g] The properties below are for the activated forms of the proteins.

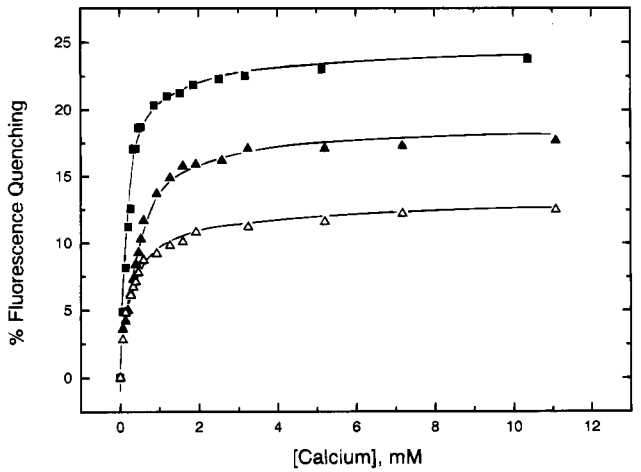

FIGURE 3: Titration of the effects of Ca^{2+} on the intrinsic fluorescence of r-PC and its mutants. The protein (*ca.* 1 μM) was titrated with sequential additions of Ca^{2+} and the intrinsic fluorescence intensity measured. The fluorescence (F_0) of the protein obtained in the absence of Ca^{2+} was adjusted to 1.0, and fluorescence values (F) were obtained after addition of Ca^{2+}. The percent fluorescent change (quenching) was calculated as $[(F_0 - F)/F_0] \times 100$ and plotted as a function of the total Ca^{2+} concentration. The [$C_{50,Ca}$−Fl] and the ΔFl_{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 were 283 and 340 nm, respectively. Slit widths of 1 and 16 nm were used for excitation and emission, respectively. The buffer was 20 mM Tris-HCl/100 mM NaCl, pH 7.4 at 20 °C. (△) r-[W41Y]PC; (▲) r-[S42P]PC; (■) r-[HS_{IX}]PC.

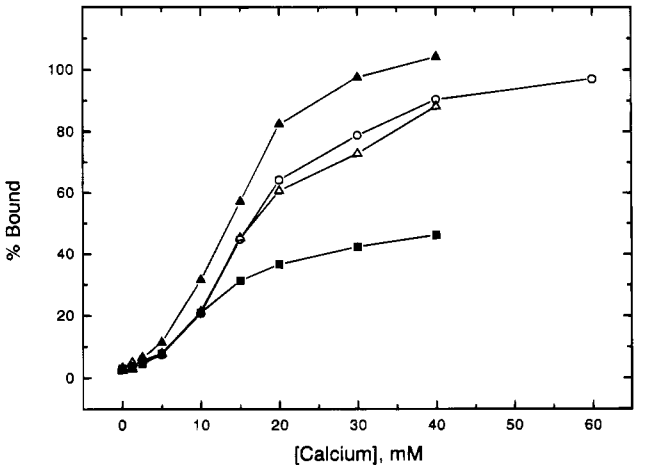

FIGURE 4: Effects of Ca^{2+} concentration on the binding of MAb-JTC3 to r-PC and its mutants at room temperature. The proteins, in the presence of [125I]-JTC3, were titrated in microtiter plates with Ca^{2+}, and the amount of antibody bound ([MAb]$_b$) was measured. The percent antibody bound at each concentration of Ca^{2+} was calculated as $(cpm_{bound}/cpm_0) \times 100$, where cpm_0 is the amount of MAb-JTC3 bound to wtr-PC at saturating concentrations of Ca^{2+}. The [$C_{50,Ca}$-MAbJTC3] was calculated by nonlinear least-squares minimization of the data allowing both [$C_{50,Ca}$-MAbJTC3] and cpm_{max} to float during the iterations. (○) wtr-PC; (△) r-[W41Y]-PC; (▲) r-[S42P]PC; (■) r-[HS_{IX}]PC. The maximum amounts of antibody bound under these conditions, normalized to wtr-PC, were 93% for r-[W41Y]PC and r-[S42P]PC, and 45% for r-[HS_{IX}]PC.

Kisiel, 1985; Sakai et al., 1990); the EGF regions also possessing important Ca^{2+} sites (Ohlin et al., 1988b; Hanford et al., 1990, 1991) and being responsible for protein−protein interactions (Ohlin et al., 1988a; Hogg et al., 1992); and the protease modules also containing a Ca^{2+} binding site (Bajaj et al., 1992), as well as possessing the catalytic machinery of the serine protease.

The role of the HS in structure−function relationships of HS-containing coagulation proteins has not been as intensely investigated, although it is clear that this module is required for both the GD (Colpitts & Castellino, 1994) and EGF1 (Valcarce et al., 1993) domains to fully express their Ca^{2+} binding sites. Another study is consistent with the complete exchangeability of the HS between r-fIX and r-fX, insofar as binding of r-fIX to endothelial cells is unaffected by this domain switch (Cheung et al., 1991). A further investigation of the role of the HS in r-fIX function has been performed using mutagenesis strategies, which provide additional evidence for the interaction between the HS and EGF1 regions of this protein (Hughes et al., 1994). These works also led to the implication that the highly conserved Phe^{41} (Phe^{40} of PC) was important to the interaction of r-fIX with acidic PL. However, these latter studies were not quantitative, and the mutant proteins were poorly characterized in

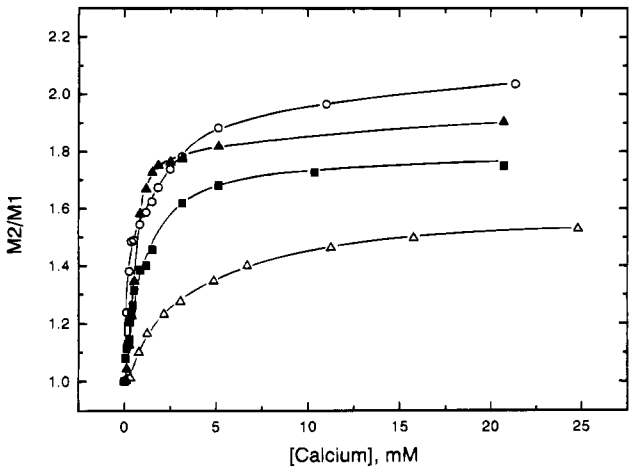

FIGURE 5: Effect of [Ca^{2+}] on the binding of r-PC mutants to acidic PL [60%/40% (w/w) chicken egg phosphatidylcholine/bovine brain phosphatidylserine] at 20 °C. The molecular weights of the protein/PL complexes were determined by 90° relative light scattering. Excitation and emission wavelengths of 320 nm and slit widths of 4 nm were used. The [$C_{50,Ca}$-PL] was calculated by nonlinear least-squares minimization of the data, allowing both [$C_{50,Ca}$-PL] and the maximum attainable M2/M1 to float during the iterations. Solutions of Ca^{2+} were titrated into a protein (1 μM)/PL vesicle (6 μg/mL in phosphate) suspension. The buffer for these experiments was 20 mM Tris-HCl/100 mM NaCl, pH 7.4. (○) wtr-PC; (△) r-[W^{41}Y]PC; (▲) r-[S^{42}P]PC; (■) r-[HS_{IX}]PC. The maximal M2/M1 values were 2.1 for wtr-PC, and 1.9, 1.8, and 1.6 for r-[S^{42}P]-PC, r-[HS_{IX}]PC, and r-[W^{41}Y]PC, respectively.

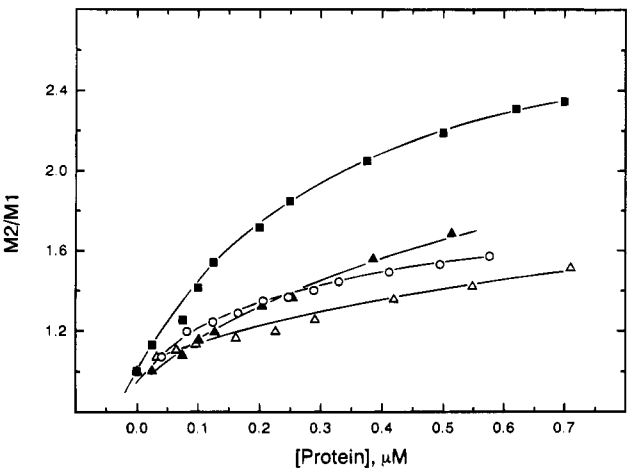

FIGURE 6: Measurement of the binding of r-PC mutants to acidic PL [60%/40% (w/w) chicken egg phosphatidylcholine/bovine brain phosphatidylserine] in the presence of Ca^{2+} at 20 °C. The dependence on protein concentrations of the interaction of r-PC mutants with PL (6 μg/mL) in the presence of 2 mM $CaCl_2$. The molecular weights of the protein/PL complexes were determined by relative light scattering after subtraction of the scattering of the nonbound protein. The [$C_{50,PC}$-PL] was calculated by nonlinear least-squares minimization of the data, allowing both [$C_{50,PC}$-PL] and the maximum attainable M2/M1 to float during the iterations. The maximal M2/M1 values were 1.6 for wtr-PC, and 2.4, 1.7, and 1.6 for r-[HS_{IX}]PC, r-[S^{42}P]PC, and r-[W^{41}Y]PC, respectively. (○) wtr-PC; (△) r-[W^{41}Y]PC; (▲) r-[S^{42}P]PC; (■) r-[HS_{IX}]PC.

terms of their extents of processing. Thus, the true importance of these findings cannot be assessed.

Our one chimeric and two site-directed mutants investigated herein have been highly purified, and are fully processed with regard to γ-carboxylation and β-hydroxylation (Table 1), as well as removal of the Lys^{156}-Arg^{157} dipeptide (Figure 2). This indicates that the HS is not involved in

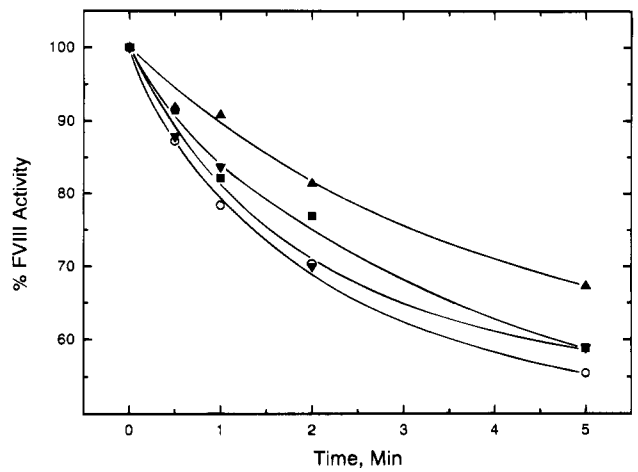

FIGURE 7: Inactivation of fVIII by r-APC mutants. Time course of inactivation of human fVIII by r-APC mutants at 37 °C, in a buffer containing 20 mM Hepes−NaOH/150 mM NaCl, pH 7.4. The r-PC mutants and wtr-APC were present at the same amidolytic activities. A solution of fVIII (6 nM final concentration) was incubated with the desired r-APC (0.5 nM final concentration), in the presence of (final concentrations) PL (40 μM) and Ca^{2+} (2.5 mM). Following this, fIXaβ (6.7 nM) and fIIa (0.12 unit/mL) were added in order to activate the remaining fVIII. The amount of fVIIIa present was assayed in the tenase system. For this, the fVIIIa solution was added to a solution containing final concentrations of 40 μM (in phosphate) PL, 2.5 mM $CaCl_2$, and a 180 μM aliquot of the fXa chromogenic substrate S2222. The final volume was 790 μL. After the base line 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 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). The fVIII concentration remaining is plotted as a function of the incubation times of fVIII, and the relevant r-APC and first-order inactivation rate constants were calculated. 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. 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. (○) wtr-APC; (△) r-[W^{41}Y]APC; (▲) r-[S^{42}P]APC; (■) r-[HS_{IX}]APC.

these processing events. Therefore, these mutants are highly suitable for the types of investigations conducted in the current study. Of interest is the fact that r-[HS_{IX}]PC contains a Glu (Glu^{39}) residue in the HS that is not present in PC. This residue is γ-carboxylated in the mature chimera, suggesting that the propeptide of PC does direct this extra γ-carboxylation step and that the vitamin K-dependent γ-carboxylase does not require the Gla residues present at residues 33 and 36 of fIX for Glu^{40} (Glu^{39} in r-[HS_{IX}]PC) to be processed in this manner. This is significant to investigations currently underway in several laboratories that are concerned with possible ordered γ-carboxylation of Glu residues.

The three mutants underwent a Ca^{2+}-dependent conformational alteration that was measurable through intrinsic fluorescence changes (Figure 3 and Table 1), and then reacted with a MAb, JTC3 (Figure 4 and Table 1), that specifically revealed the integrity of the Ca^{2+}-dependent conformation. These findings suggested that despite the complete exchange of the HS (in r-[HS_{IX}]PC), or the disruption of the helical conformation (in r-[S^{42}P]PC), the Ca^{2+}-dependent changes in the GD nonetheless took place. Some small differences in this final conformation might be present in the mutants,

as revealed by the 2-fold lower Ca^{2+} concentration required to produce the maximal fluorescence change in r-[HS_{IX}]PC, the slightly higher maximal fluorescence of this mutant (Figure 3 and Table 1), and the approximate 2-fold lower affinity for the Ca^{2+}/r-[HS_{IX}]PC complex to MAb-JTC3 (Figure 4); such differences are not important to the functional APC activity of this mutant (Table 1). Of interest is the fact that the maximal intrinsic fluorescence change of the mutant, r-[$W^{41}Y$]PC, was still approximately 68% of that of wtr-PC. This showed that while Trp^{41} may contribute as one monitor of the Ca^{2+}-dependent fluorescence change in r-PC, this Trp residue was not the sole reporter of the change. This supports our earlier findings, employing a synthetic 47-mer peptide consisting of the GD and HS of PC, which although containing Trp^{41} did not show an intrinsic fluorescence change upon Ca^{2+} binding (Colpitts & Castellino, 1994). That not all of these types of proteins, despite their sequence homologies, behave identically in this regard is underscored by a similar investigation with a synthetic peptide containing the GD/HS of fIX, which demonstrated that the Ca^{2+}-induced fluorescence change did occur for this peptide (Jacobs et al., 1994).

The Ca^{2+} and protein dependencies of the binding of r-[HS_{IX}]PC and r-[$S^{42}P$]PC to acidic PL are not greatly different from each other and from that of wtr-PC (Figure 5 and Table 1). This suggests that neither the specific nature of the HS nor its helical conformation is essential for macroscopic PL binding. On the other hand, the PL binding characteristics of r-[$W^{41}Y$]PC show that a 2-fold greater concentration of Ca^{2+} is required for optimal binding. This is consistent with previous observations that indirectly showed that Trp^{41} (PC numbering) of prothrombin, fIX, and fX is needed for optimal PL binding (Pollock et al., 1988; Schwalbe et al., 1989). This may be due to Trp^{41} being required for the complete Ca^{2+}-dependent conformation of the GD to occur. In any event, the necessity of Trp^{41} is marginal, and it is clear that substitution of another aromatic residue in its place does not completely disrupt PL binding. At two different concentrations of Ca^{2+}, the [$C_{50,PC}$-PL] values for PL/PC binding are very similar to each other (Figure 6 and Table 1), confirming that PL binding of the Ca^{2+}−protein complexes is not affected by the mutations made. However, to the extent that M2/M1 is a measure of the stoichiometry of Ca^{2+}−protein/PL binding or of the final conformations of the Ca^{2+}−protein/PL complexes, it should be noted that some differences in the mutants are present therein. Since stoichiometries or final conformations cannot be quantified by this technique, any further conclusions on these points cannot be placed out of the realm of speculation.

Ca^{2+} serves as an inhibitor of the fIIa-catalyzed activation of PC, and the relevant Ca^{2+} site(s) exist(s) outside of the GD of PC (Amphlett et al., 1981; Esmon et al., 1983). In order to assess whether the integrity of this essential Ca^{2+} site has been disrupted by the mutations made in the HS of PC, we determined K_i values for Ca^{2+} on this process. The kinetic data (Table 1) indicated that the K_i was nearly identical for each of the mutants, thus demonstrating that these changes in the HS did not influence the kinetically-important Ca^{2+} binding site(s) involved in inhibition of PC activation by fIIa. Thus, either the HS of fIX serves equally to the HS of PC in this regard or the HS is not important for this functional property. The fact that the K_i is also not affected by the [Trp^{41}→Tyr] or [Ser^{42}→Pro] changes in the HS also suggests that the exact conformation of the HS may also not play a critical role in this function.

While the site-specific changes in the HS of PC do not influence the steady-state kinetic constants that characterize its activation with fIIa/r-sTM, these same parameters for the r-[HS_{IX}]PC activation at 100 μM Ca^{2+} were affected, with the major difference being an approximate 3-fold lowering of the k_{cat} value for this process (Table 1). Thus, the changes produced in the HS in this chimera play an inhibitory role in PC activation. Notably, an extra Gla residue is incorporated in r-[HS_{IX}]PC, and this may alter the specific Ca^{2+} binding properties that are needed for optimal activation by the fIIa/r-sTM complex. In the absence of a detailed kinetic study focused on this single property at a variety of Ca^{2+} concentrations, it is not possible to assess further the basis of this effect.

Finally, we show that the activities toward biologically-relevant substrates of the corresponding r-APC mutants were only significantly altered in the case of r-[$S^{42}P$]APC (Figure 7 and Table 1). It has been proposed that a precise alignment must occur of the active site of enzymes of this type on the PL or on cell surfaces, with the susceptible peptide bonds of substrates such as fV/fVa and fVIII/fVIIIa, that are also present on these same PL vesicles or cells, for maximal inactivation rates to occur (Lim et al., 1977; Isaacs et al., 1986; Mutucumarana et al., 1992). It is possible that in this mutant, which possesses a predicted conformationally-altered HS, despite the fact that its macroscopic binding to PL may not have been greatly affected, the orientation of the mutant r-APCs on the PL surface is no longer optimal with the substrate cleavage sites in this regard. This interpretation is consistent with our earlier similar observations with another mutant (Zhang & Castellino, 1994), and could serve as the basis for the reduced biological activities of these variant r-PCs. The same interpretation would also be consistent with a function of the HS as a scaffold for optimal separation of the active site of this enzyme from the PL surface, the latter of which is mainly occupied by the GD.

In conclusion, we demonstrate by generation of a select series of strategic mutations in the HS of PC that its specific nature is important to its activation by fIIa/r-sTM at maximal Ca^{2+} concentrations. This function is located outside of the GD of PC. Since the amino acids in the HS of PC that differ between PC and fIX are Leu^{38}, Ala^{39}, S^{42}, K^{43}, and H^{44}, any important amino acid that directly or indirectly influences this specific property of PC is reasonably expected to reside in this grouping. With regard to its being a determinant of Ca^{2+}-specific properties of the GD *via* indirect effects, it appears that the HS is interchangeable with at least one other module of this type from a protein with a completely different function. This suggests that the HS is needed for full expression of the Ca^{2+}-dependent properties of the GD of PC. The results of this investigation assist our understanding of the extent of the independent functional nature of modules of these types in intact proteins, and in particular furthers our understanding of the structure−function relationships of modules present in PC and APC.

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