Functions of Individual γ-Carboxyglutamic Acid (Gla) Residues of Human Protein C. Determination of Functionally Nonessential Gla Residues and Correlations with Their Mode of Binding to Calcium†

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ABSTRACT: Previous studies from this laboratory have been directed toward elucidation of the roles of individual γ-carboxyglutamic acid (Gla) residues in Gla domain-related Ca^{2+}-directed properties of human protein C (PC) and activated protein C (APC). On the basis of results using recombinant variants of PC containing highly conservative (Asp) mutations of individual Gla residues, it was previously proposed that Gla^6, Gla^{14}, and Gla^{19} may not be essential for properties associated with the Ca^{2+}-dependent conformation of the Gla domain of these proteins. In this study, we have demonstrated that radical mutations to Val of Gla residues 14 and 19 resulted in 94% and 82%, respectively, of the Gla domain-related, Ca^{2+}- and phospholipid- (PL-) dependent anticoagulant (APTT) activity of wild-type recombinant (wtr) APC, while [Gla^6→Val]r-APC showed a complete loss of this same activity. The more conservative mutant [Gla^6→Gln]r-APC possessed 4% of the APTT activity of wtr-APC, whereas [Gla^6→Asp]r-APC was nearly fully active. As with wtr-PC, both [Gla^6→Val]r-PC and [Gla^6→Gln]r-PC displayed Ca^{2+}-dependent intrinsic fluorescence quenching, suggesting that they adopted a Ca^{2+}-induced conformation. However, Ca^{2+} titration data suggested that these conformations were not identical to that undergone by wtr-PC. In addition, the Ca^{2+}-mediated binding parameters of [Gla^6→Val]r-PC and [Gla^6→Gln]r-PC to acidic PL vesicles were found to be defective. These data were interpreted at the molecular level using a model for the Gla domain of PC based on the X-ray crystal structure of the Ca^{2+}/bovine prothrombin fragment 1 complex. We conclude that Gla^{14} and Gla^{19}, and the sole Ca^{2+} (Ca-7) that is coordinated to these two residues, are not essential for the Gla domain-related Ca^{2+}- and PL-dependent anticoagulant properties of PC and APC. A more complex situation exists with Gla^6. The model suggests that only a single carboxylate of this residue is employed in Ca^{2+} coordination, and this group interacts with two different Ca^{2+} ions, *viz.*, Ca-4 and Ca-5. The lone carboxylate of Asp may be able to substitute effectively for this same group in Gla^6, thus providing functional activity to [Gla^6→Asp]r-APC, and to a much lesser degree Gln may also be able to serve a coordination function. This suggests that Gla^6 is one of the functionally essential Ca^{2+} binding sites.

PC,[1] the zymogen form of the anticoagulant serine protease APC, is a member of the class of blood coagulation plasma proteins that requires vitamin K for the posttranslational processing events that result in γ-carboxylation of its first nine E residues. These latter amino acids are contained within a genomic exon identified as the Gla domain (amino acid residues 1−37). The principal activities of APC involve limited specific proteolysis of fV/Va (Kisiel et al., 1977) and fVIII/fVIIIa (Vehar & Davie, 1980) in reactions that are stimulated by Ca^{2+}, PL, and protein S (Walker, 1981). The action of APC on these proteins results in loss of their cofactor activities in the prothrombinase and tenase complexes, respectively. As a consequence, the rate of thrombin formation is diminished and coagulation is inhibited. Activation of PC is catalyzed in the fluid phase by thrombin, a reaction that is inhibited by Ca^{2+} (Amphlett et al., 1981). On cell surfaces, thrombin interacts with TM to provide a potent PC activator complex (Esmon et al., 1982).

Human PC is synthesized as a 461 amino acid single-chain protein (Foster & Davie, 1984; Foster et al., 1985). Analysis of the locations of the introns in the PC gene, as well as its amino acid sequence, reveals the presence of several types

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[1] Abbreviations: PC, protein C; APC, activated protein C; fVIII, coagulation factor VIII; fVIIIa, activated coagulation factor VIII; prothrombin fragment 1, a proteolytic fragment of bovine prothrombin containing amino acid residues 1−156; Gla, γ-carboxyglutamic acid; Gla domain (GD), the γ-carboxyglutamic acid-rich region of PC (amino acid residues 1−37); HS, the helical stack region of protein C (amino acid residues 38−46); 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], the total Ca^{2+} concentration required to alter the intrinsic fluorescence in one-half 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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of domains. These modules include a signal peptide and propeptide, followed consecutively by the GD, HS, two EGF-like structures, an activation peptide, and the trypsin-like catalytic region. A dipeptide, Lys^{156}-Arg^{157}, in PC is liberated from about 90% of the human PC molecules prior to their secretion from cells (Beckmann et al., 1985), yielding a mature PC which mainly exists in plasma as a light chain (residues 1−155), disulfide-linked to a heavy chain (residues 158−419). The catalytic triad responsible for the serine protease activity of APC is located in its heavy chain.

The GD of this general class of proteins functions by providing a number of Ca^{2+} binding sites, the occupation of which induces these proteins to undergo alteration to the GD-related Ca^{2+}-dependent conformation required for binding to PL (Borowski et al., 1986b; Liebman et al., 1987; Liebman, 1993). The HS, which comprises residues 37−46 in PC, probably influences the final conformation adopted by the GD and thereby influences binding of Ca^{2+} to this domain (Colpitts & Castellino, 1994). This Ca^{2+}-induced conformation can be probed 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., 1986b; Wakabayashi et al., 1986; Liebman et al., 1987; Church et al., 1989; Zhang & Castellino, 1992; Liebman, 1993). At least two different classes of Ca^{2+} sites have been found in the GD. The first group of tighter sites is nonspecific for the cation, with several divalent and trivalent cations demonstrating binding (Keyt et al., 1982; Borowski et al., 1986a; Wakabayashi et al., 1986; Liebman et al., 1987; Church et al., 1989, Liebman, 1993). An additional weaker class of four-to-five metal ion binding sites shows greater specificity for Ca^{2+} (Nelsestuen, 1976; Borowski et al., 1986b; Liebman et al., 1987; Liebman, 1993) and is required for binding of the protein to acidic PL vesicles. The X-ray crystal structure of Ca^{2+}/prothrombin fragment 1 with an ordered GD of prothrombin has been determined (Soriano-Garcia et al., 1992). This structure contains three buried and four additional solvent-exposed Ca^{2+} ions.

We have employed site-directed mutagenesis on human r-PC and r-APC (Zhang & Castellino, 1990, 1992, 1993; Zhang, 1991; Zhang et al., 1992) to investigate roles for individual Gla residues in Ca^{2+} coordination and GD-related Ca^{2+}-dependent functional properties of these proteins. With the use of Gla to Asp conservative mutations, we found that Gla residues 7, 16, 20, 25, 26, and 29 were of importance to all Ca^{2+}-dependent structural and functional properties ascribed to the GD of PC, while Gla residues 6, 14, and 19 provided the other extreme of appearing to be nonessential to most of these same properties. Since the mutations employed were so conservative, additional investigations with these latter three residues were in order, especially since the carboxylate group of Asp in these mutants could possibly substitute for that of a Gla in coordination to Ca^{2+}. Therefore, we investigate the properties of additional less conservative mutants at Gla^6, Gla^{14}, and Gla^{19} of r-PC and r-APC in this paper to expand and refine our knowledge on their roles in the functions of these proteins.

MATERIALS AND METHODS

Proteins. r-PC was expressed in human 293 cells and purified as described previously (Zhang & Castellino, 1990). Wild-type and mutant r-APCs were prepared from the corresponding r-PCs by activation with the venom protease, Protac C (American Diagnostica, New York, NY). The progress of each activation reaction was monitored spectrophotometrically by the appearance of amidolytic activity, using the chromogenic substrate S2366 (Helena Laboratories, Beaumont, TX).

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

cDNAs. A full description of the cDNA coding for human PC has been provided (Zhang & Castellino, 1990).

The cDNAs encoding r-PC mutants were constructed 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 to accomplish the changes were (the mutagenic bases are in lower case letters) (1) [Gla^6→Gln]r-PC, 5′-C TCC TTC CTG cAG GAa CTC CGT CAC A; (2) [Gla^6→Val]r-PC, 5′-AC TCC TTC CTG GtG GAa CTC CGT CAC AGC; (3) [Gla^{14}→Val]r-PC, 5′-AC AGC AGC CTG Gtt CGc GAG TGC ATA AGA G; and (4) [Gla^{19}→Val]r-PC, 5′-GG GAG TGC ATA GtG GAa ATC TGT GAC TTC.

Screening of the bacterial transformants for the colonies containing the desired mutations was accomplished by restriction endonuclease analysis. For mutants 1 and 2, a naturally occurring *Sac*I site was lost; in the case of mutant 3, a *Nru*I site was gained; and for mutant 4, a naturally occurring *Bgl*II site was lost. All cDNAs were subjected to nucleotide sequencing through the mutation sites.

Expression and Purification of the r-PCs. The cDNAs were expressed in 293 cells. Purification of subpopulations of the resulting proteins that were fully γ-carboxylated was accomplished by anion-exchange chromatography on FFQ resin (Pharmacia, Piscataway, NJ). These procedures have been described previously (Zhang, 1991; Zhang & Castellino, 1991).

APTT Assays. Small adjustments of stock solutions of wtr-APC and r-APC mutants were made so that each contained the same amidolytic activities per unit volume. The concentration range of the r-APCs in the assay solutions was approximately 0.025 μg/mL−0.6 μg/mL.

APTT assays were conducted at 37 °C with PC-deficient plasma using the APTT assay kit (Sigma Diagnostics, St. Louis, MO), essentially as described earlier (Zhang et al., 1992). Controls in this assay were performed in the absence of APC and with unactivated r-PCs in place of their respective APCs. Our standard clot times (*ca.* 47 s) were essentially the same as those of the manufacturer of the assay kit.

To calculate activities of the mutants relative to wtr-APC, double logarithmic plots of the clotting time *versus* the dilution of the stock solutions of the relevant APC were constructed for each r-APC sample. The displacement of the sample line from the wtr-APC sample was employed to calculate the relative activities of each sample. In the cases to be described herein, activities of the mutants were nearly all or none.

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

Interaction of PC 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-HCl/100 mM NaCl, pH 7.4. Plots of the molecular weight ratio of the protein (M_2) to PL (M_1) 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 [Ca^{2+}] of 20 mM. The resulting [$C_{50,P}$-PL] values were obtained in the same manner.

Western Analysis. Purified proteins, as well as those in the expression media, were first separated by 12% (w/v) gels, and the protein bands were transferred to Immobilon-P (Millipore, Bedford, MA). PC-containing bands were labeled by the murine MAb, C3 (Heeb et al., 1988). The presence of this antibody was detected enzymatically with a goat anti-mouse IgG/alkaline phosphatase complex, after addition of a chromogenic substrate for alkaline phosphatase. Our detailed method has been described earlier (Zhang & Castellino, 1990).

Modeling of the Gla Domain and Trailing Helical Stack of Human PC. Since the amino acid sequences of the GD and the HS of bovine prothrombin and PC show about 70% conservation (Tulinsky et al., 1988b), the three-dimensional structure of these modules of PC was approximated from the same regions of the structure of Ca^{2+}/bovine prothrombin fragment 1 (Soriano-Garcia et al., 1992). The general method that was so successful for modeling of kringle structures was used (Tulinsky et al., 1988a). A Silicon Graphics interactive workstation driven by CHAIN was employed for the modeling. The sequence of the GD/HS of bovine prothrombin was first changed to correspond to the same number of amino acids PC by removing the extra Gly of bovine prothrombin at position 4. The peptide chain was reconnected and then followed by idealization. Subsequent modeling was performed by retaining the overall folding of the GD/HS of prothrombin and using the positions of like atoms of residues of prothrombin as guide coordinates when replacing side chains of the GD/HS region with those of PC. Only a few additional small free bond rotations were needed to relieve unusually close van der Waals contacts in the modeled PC structure, suggesting that the GD/HS of Ca^{2+}/bovine prothrombin fragment 1 was a good approximation of the resulting model of the folding of the GD/HS regions of PC. Finally, although the Gly^{47}-Asp^{48}-Gln^{49} insertion in PC at the end of the HS domain corresponds to helix formers, they were not modeled as such in the GD because there is some indication that these residues might be part of a β-strand of the amino-terminal EGF domain (Selander-Sunnerhagen et al., 1992).

RESULTS

Mutants of r-PC that contain substitutions of Val for Gla at amino acid sequence positions 6, 14, and 19 of the mature protein, and another with a $Gla^6 \rightarrow Gln$ replacement, have been constructed and expressed in human 293 cells. The location of these mutations within the primary structure of human PC is given in Figure 1.

The amounts of each of the proteins produced in the conditioned cell media were estimated by Western analysis and found to contain approximately 1−2 mg/L. The purification of all of these proteins was similarly accomplished with two steps on FFQ columns, employing elution with Ca^{2+} in the first and elution with NaCl in the second. An example of the chromatographic elution behavior of [$Gla^6 \rightarrow Gln$]r-PC is provided in Figure 2. All mutants appeared to be maximally γ-carboxylated as initially indicated by their elution positions from the first FFQ column of Figure 2A, which is representative of a protein containing at least seven Gla residues (Yan et al., 1990) and which we have found with many mutants to be a reliable indicator of γ-carboxylation of all available r-PC precursor Glu residues in variants containing single or double mutations at Gla residues (Zhang & Castellino, 1990, 1991; Zhang et al., 1992). Additional evidence for maximal γ-carboxylation of these four mutants has been discovered from direct Gla analysis, which yielded 7.8−8.2 residues of Gla/mol of each of the proteins and from amino acid sequence through 35 residues. In this latter case, each mutant protein contained less than 5% Glu at any location in which a Gla should have been present. Sequence results also showed that the desired mutation was present in the proper locations of these proteins. The final yields of fully γ-carboxylated mutant proteins ranged from approximately 30% to 75%, and it was not difficult to obtain 1−2 mg of each, an amount sufficient for all of the work reported herein.

Nonreduced and reduced $NaDodSO_4$/PAGE gel electrophoretograms of each of the mutant proteins were performed and were similar to those published previously for a large number of mutant r-PCs (Zhang et al., 1992). Each protein was mainly (>90%) present in its two-chain form, indicating that the processing dipeptide between the light and heavy chains of these zymogens had been effectively liberated. Further, the two protein components observed for each of the purified r-PC variants is also a property of plasma and wtr-PC, as well as all r-PC variants purified to date. The molecular basis of the doublet bands is due to variable glycosylation of r-PC (Yan et al., 1990).

Each mutant r-PC was fully activated to the corresponding r-APC, and its overall anticoagulant activity was measured using the standard APTT assay. Plots of the increase in clotting times against the concentration of r-APC are illustrated in Figure 3 for the mutants and compared to that of wtr-APC. In each case, final small adjustments of the stock solutions of the r-APC samples were made prior to the APTT assay in order that they possessed equal amidolytic activities. The data showed that the Ca^{2+}- and PL-dependent APTT activities of the $Gla^{14} \rightarrow Val$ and $Gla^{19} \rightarrow Val$ mutants were 94% and 82% of that of wtr-APC, similar to the

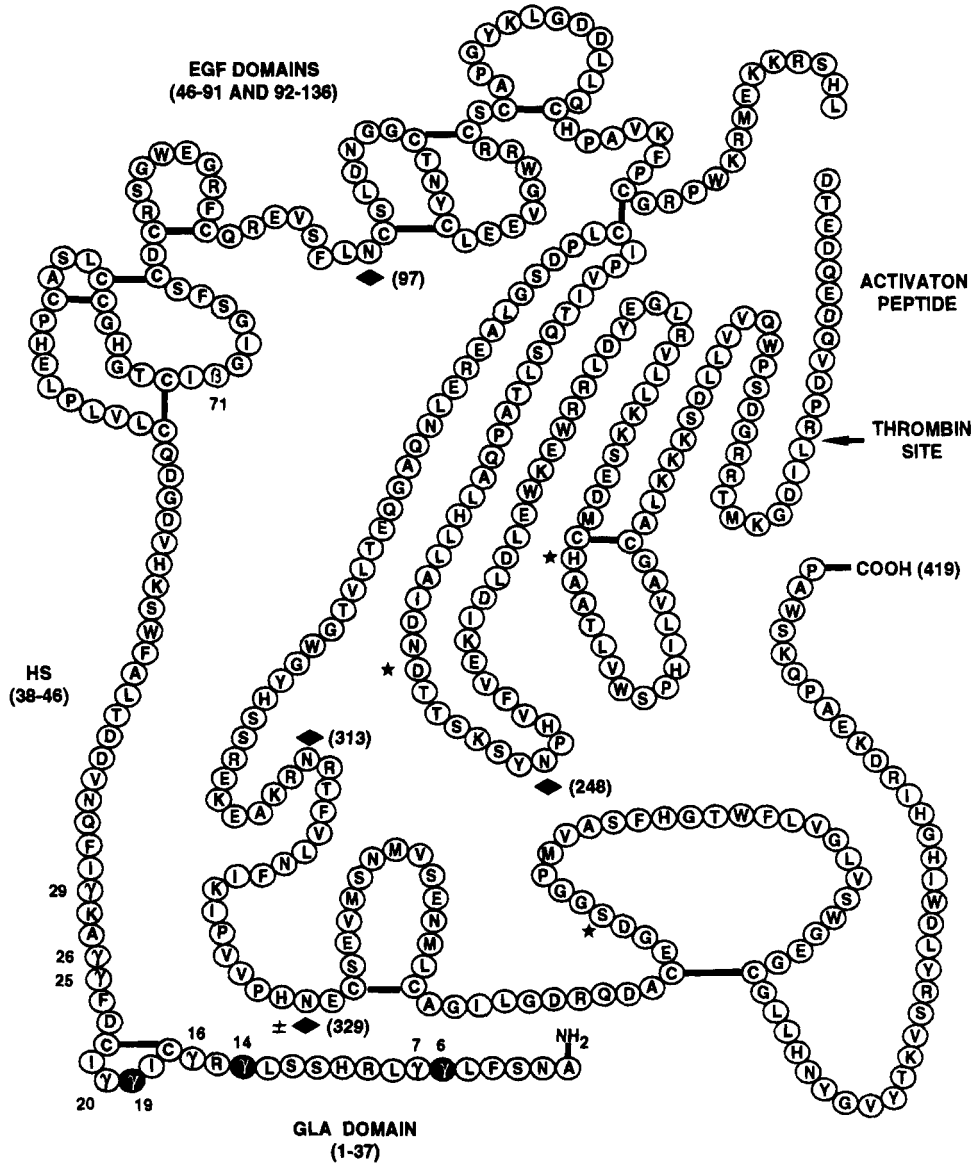

FIGURE 1: Primary structure of r-PC. The locations of the Gla residues that have undergone mutations in the current study are indicated by color reversals. The filled diamonds are the locations of N-linked oligosaccharides, and the filled stars show the positions of the catalytic triad of amino acids in the activated form of this protein.

activities found for [Gla^{14}→Asp]r-APC and [Gla^{19}→Asp]-r-APC (Zhang et al., 1992). These findings demonstrate that function was nearly fully preserved in both the conservative and radical mutants. On the other hand, since [Gla^{6}→Val]-r-APC was fully inactive, we tested in this assay the more conservative variant, [Gla^{6}→Gln]r-APC, and still found this variant to only possess a very small level of APTT activity, approximating a maximum of 4% of that of wtr-APC.

To assess whether proper Ca^{2+}-dependent conformational changes had occurred in the Gla^{6} mutants that showed greatly diminished activities in the anticoagulant assays, we examined the Ca^{2+} dependency of the intrinsic fluorescence quenching for these proteins. Such titrations for [Gla^{6}→Val]-r-PC and [Gla^{6}→Gln]r-PC are provided in Figure 4. Maximal fluorescence quenching of approximately 13.5% was found for each variant, with [$C_{50,Ca}$-Fl] values of 0.83 and 0.70 mM for [Gla^{6}→Val]r-PC and [Gla^{6}→Gln]r-PC, respectively. These values for wtr-PC and [Gla^{6}→Asp]r-PC were 19% and 0.4 mM and 17.6% and 0.66 mM, respectively (Zhang & Castellino, 1992).

To assess the Ca^{2+} dependency of binding of the Gla^{6} mutants to acidic PL, 90° light scattering was employed to measure the binding of [Gla^{6}→Val]r-PC and [Gla^{6}→Gln]-r-PC to PL vesicles containing 60% PhC/40% PhS. The results are illustrated in Figure 5. The [$C_{50,Ca}$-PL] obtained for [Gla^{6}→Val]r-PC was 5.2 mM, while that for [Gla^{6}→Gln]-r-PC was 7.1 mM. The shape of the titration curve for [Gla^{6}→Val]r-PC was also somewhat different from that of wtr-PC, with an indication that cooperative binding may have occurred. These same values for wtr-PC and [Gla^{6}→Asp]r-PC were 1.2 and 1.5 mM, respectively (Zhang & Castellino, 1992). Further, data in Figure 6 show that at 20 mM Ca^{2+} [Gla^{6}→Val]r-PC interacted with a [$C_{50,P}$-PL] of 0.31 μM, approximately the same as that of wtr-PC ([$C_{50,P}$-PL] = 0.38 μM) (Zhang & Castellino, 1993), while [Gla^{6}→Gln]r-PC displayed a [$C_{50,P}$-PL] of 1.8 μM under these same conditions.

DISCUSSION

Previous work from this laboratory has led to identification of specific Gla residues of PC that are involved in its various

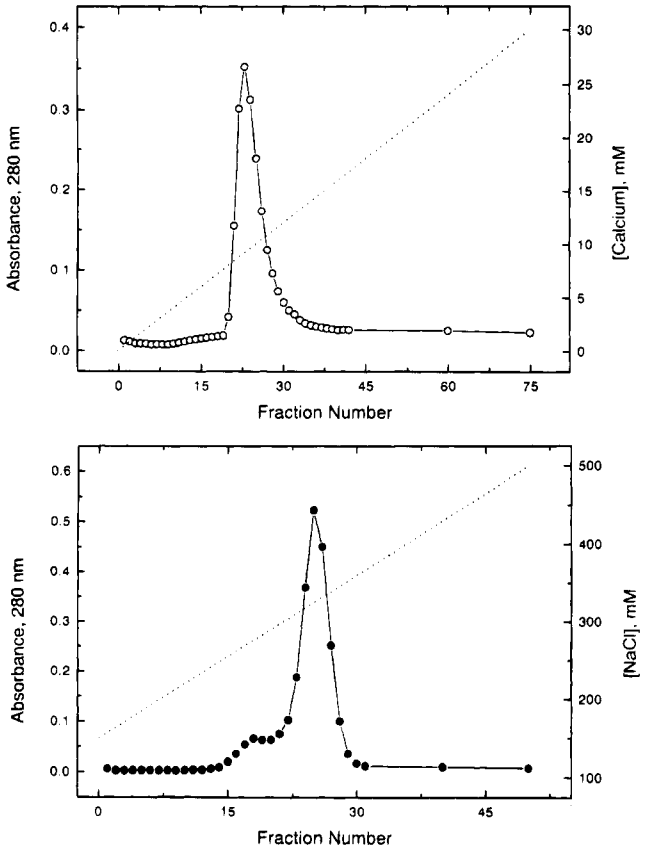

FIGURE 2: Chromatography of [Gla^6→Gln]r-PC on FFQ anion-exchange chromatography at 4 °C. (A, top) A volume of 800 mL of conditioned 293 cell culture medium was applied to a 5-mL column of FFQ, equilibrated with 20 mM Tris-HCl/150 mM NaCl/4 mM EDTA, pH 7.4 at 4 °C (TBS/EDTA). After the column was washed with this same buffer, followed by 20 mM Tris-HCl/150 mM NaCl, pH 7.4 (TBS), a linear $CaCl_2$ gradient was applied (120 mL, total volume). Fractions (1.6 mL) were collected at a flow rate of 0.4 mL/min. Those containing the major peak were pooled. (B, bottom) The major peak of (A) was dialyzed against TBS and reapplied to a 3-mL column of FFQ equilibrated in this same buffer. The indicated linear gradient of NaCl was applied (50 mL, total volume), and 1-mL fractions were collected. The flow rate was 0.25 mL/min. The fractions containing the major peak were pooled.

functions (Zhang, 1991; Zhang & Castellino, 1992, 1993; Zhang et al., 1992). Using very conservative mutations of Gla precursor Glu residues to Asp, we have shown that Gla^7, Gla^{16}, Gla^{20}, Gla^{25}, Gla^{26}, and Gla^{29} are important determinants of GD-related, Ca^{2+}- and PL-dependent properties of PC and APC. Under conditions of conservative mutagenesis, it did not appear that Gla^6, Gla^{14}, and Gla^{19} were of similar relevance. Therefore, in order to complete this phase of the investigations, it was necessary to construct and examine more radical mutations at these latter three residues.

Since radical alterations of Gla^{14} and Gla^{19} to Val did not influence the anticoagulant activities of the mutants, we conclude that essential Ca^{2+} and PL binding properties were maintained in these variants. Therefore, we did not pursue additional investigations with these mutants. A molecular interpretation of the results based on these two variants, along with previous studies with Asp mutations at these locations, is possible with assistance of the three-dimensional model of the Gla/HS domains of PC. Such a model based on the crystallographic coordinates of the Ca^{2+}/fragment 1 region of bovine prothrombin is provided (Soriano-Garcia et al., 1992). An overview of this structure is presented in Figure

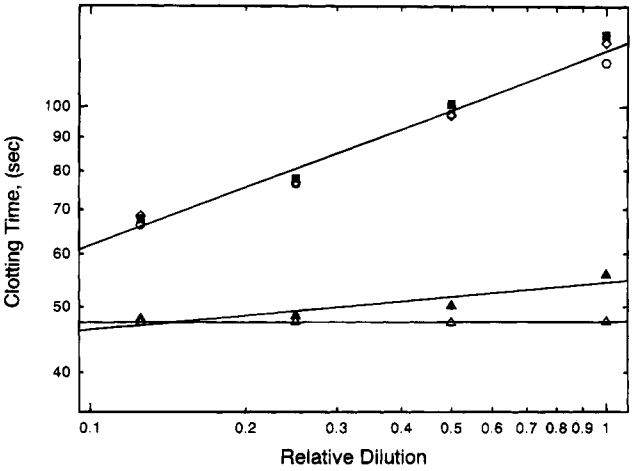

FIGURE 3: APTT assays of r-APC mutants. Stock solutions of all r-APCs were prepared and their amidolytic activities determined. Minor adjustments of stock concentrations were made such that all amidolytic activities were identical. A dose–response curve was obtained for each sample and plotted as the APTT times (clotting times) *versus* the relative dilution of the wtr-APC stock solution (stock solution of wtr-APC = 1.0, final concentration approximately 0.6 μg/mL). The displacement of the lines provided the activity of the r-APC sample relative to that of wtr-APC. Curves: (■) wtr-APC; (◇) [Gla^{14}→Val]r-PC; (○) [Gla^{19}→Val]-r-APC; (▲) [Gla^6→Gln]r-PC; (△) [Gla^6→Val]r-PC.

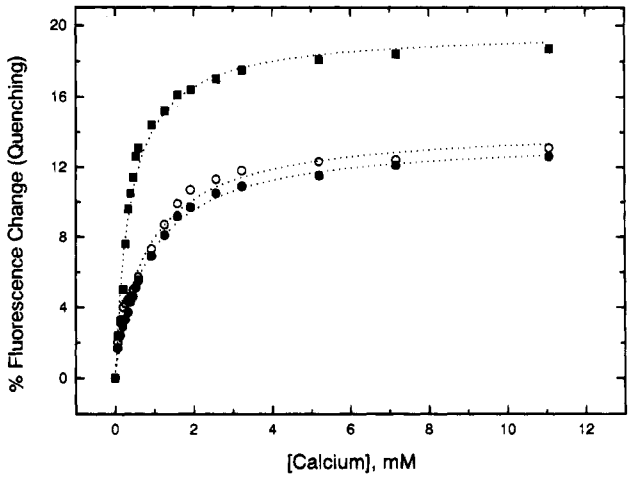

FIGURE 4: Titration of the effects of Ca^{2+} on the intrinsic fluorescence of mutant r-PCs. The protein (*ca.* 1 μM) was titrated with sequential additions of Ca^{2+} and the intrinsic fluorescence intensity measured. The buffer was 20 mM Tris-HCl/100 mM NaCl, pH 7.4 at 20 °C. The fluorescence (F_0) 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 quenching was calculated as $[(F_0 - F)/F_0 \times 100]$ 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 nonlinear least-squares iterative fitting of the data. The excitation and emission wavelengths used for the titrations were 283 and 340 nm, respectively. Curves: (■) wtr-PC; (○) [Gla^6→Gln]r-PC; (●) [Gla^6→Val]r-PC.

7, with a closer examination of the relevant amino acids for this study and their coordination loci to Ca^{2+} shown in Figure 8. As is seen in Figures 7 and 8, one Ca^{2+} ion, Ca-7, is coordinated only by the carboxylate groups of Gla residues located at positions 14 and 19. Mutation of Gla^{14} to Val would result in deletion of one of three coordination sites of Ca-7, and this would be expected to diminish the affinity of this particular Ca^{2+} ion for the protein. Since this particular alteration of Gla^{14} did not influence the APTT activity of

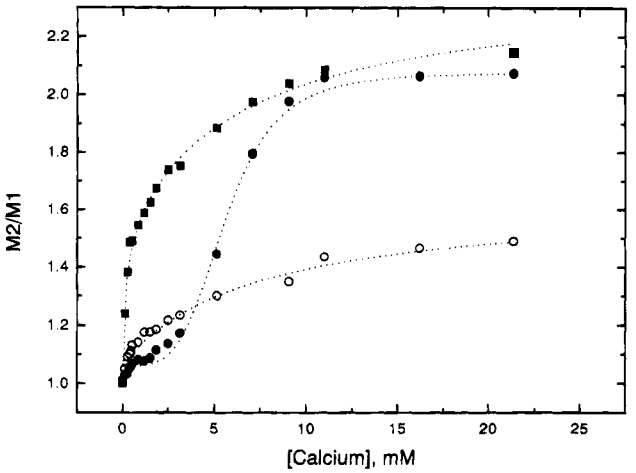

FIGURE 5: Effect of $[Ca^{2+}]$ on the binding of Gla^6 mutants to acidic PL. A Ca^{2+} titration of the binding of protein (1 μM) to PL vesicles (6 μg/mL). The relative molecular weights of the protein/PL complexes were determined by 90° relative light scattering after subtraction of the scattering 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. Curves: (■) wtr-PC; (○) [Gla^6→Gln]r-PC; (●) [Gla^6→Val]r-PC.

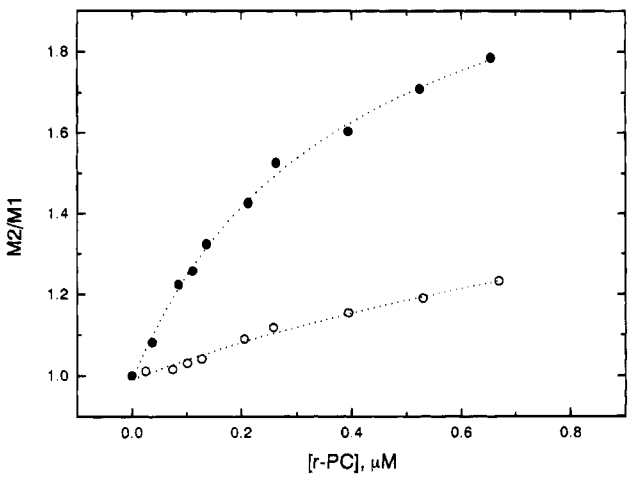

FIGURE 6: Measurement of the binding of r-PC mutants to PL in the presence of Ca^{2+}. The dependence on [protein] of the interaction of Gla^6 mutants with PL (6 μg/mL) in the presence of 20 mM $CaCl_2$. The molecular weights of the protein/PL complexes were determined by relative light scattering after 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. Curves: (○) [Gla^6→Gln]r-PLC; (●) [Gla^6→Val]r-PC.

APC, it is likely that Ca-7 is also not required for this function. Similarly, one carboxylate of Gla^{19} provides two of the three coordination loci to Ca-7, and mutation of this residue to a Val would be expected to greatly diminish binding of Ca-7 to the protein. The fact that little anticoagulant activity was lost as a result of this change to a Val residue strongly supports the proposal that Ca-7 is not an important determinant for the functional activity of APC. The small activity loss that results from this mutation may be due to the involvement of one of the carboxylates of Gla^{19} in the coordination of Ca-6, and this particular cation may be needed for functional activity.

The APTT activity of APC requires productive PL binding, which in turn relies upon a prior Ca^{2+}-induced conformational change in the protein (Nelsestuen, 1976). The fact that both [Gla^{14}→Val]r-PC and [Gla^{19}→Val]r-PC possess nearly full APTT activity, as well as the observations (data not shown) that they both undergo Ca^{2+}-dependent intrinsic fluorescence quenching similarly to wtr-PC and that they react fully with the GD-directed, Ca^{2+}-dependent monoclonal antibody that is characteristic of the proper Ca^{2+}-dependent conformation of r-PC (Wakabayashi et al., 1986; Zhang & Castellino, 1992), suggests that neither Gla^{14}, Gla^{19}, nor Ca-7 are requirements for these functions.

A different picture emerges when the function of Gla^6 is considered. Here, only a single carboxylate is predicted to be involved in providing two coordination sites to Ca-5 and one to internally located Ca-4, which is also part of a μ-oxo bond (OE1, Figures 7 and 8) between these two Ca^{2+} ions. Since the role of Gla^6 in Ca^{2+} coordination is more involved than that of Gla^{14} or Gla^{19}, it is reasonably expected that its substitution by an amino acid side chain that cannot lead to Ca^{2+} coordination would diminish GD-related Ca^{2+}- and PL-dependent functions of PC and APC. The fact that [Gla^6→Asp]r-PC possesses nearly full functional properties of the wild-type proteins (Zhang & Castellino, 1992, 1993; Zhang et al., 1992) suggests that the carboxylate donated by the substituted Asp^6 residue replaces that of the single Gla carboxylate involved in coordination of Ca-4 and Ca-5. This can be accomplished with only minor positional readjustments in this region. Obviously, this same effect could not occur in the cases of [Gla^6→Val]r-PC/[Gla^6→Val]-r-APC, thus resulting in a loss of APTT activity, and also does not occur to any large extent in the mutants, [Gla^6→Gln]-r-PC/[Gla^6→Gln]r-APC, possibly because of the lack of charge and the impaired ability of Gln to form the μ-oxo bridge between Ca-4 and Ca-5. While the GD-related Ca^{2+}-dependent intrinsic fluorescence changes do occur with both of these mutants, some subtle abnormalities may be present in these cases, *viz.*, the smaller maximal fluorescence changes and the slightly higher $[C_{50,Ca}$-Fl] values. It is not likely that disruption of only one of the seven coordination sites for Ca-4 and two of the five such sites for Ca-5 would completely eliminate binding of these cations to the protein. Thus, the properties associated with Ca-4 and Ca-5 would probably be disrupted, but not eliminated, by the mutations designed herein.

An interesting observation was made in the $[C_{50,P}$-PL] value of [Gla^6→Val]r-PC, which suggests that it is at least as effective in PL binding at saturating $[Ca^{2+}]$ as is wtr-PC. On the basis of PL binding data for another mutant r-PC, *viz.*, [Leu^5→Gln]r-PC, we concluded that a hydrophobic effect was an important component of the total binding energy (Zhang & Castellino, 1994). This is consistent with the data for [Gla^6→Val]r-PC in the current study, a mutant which possesses another hydrophobic amino acid residue in the region of r-PC of importance to this component of its binding to PL (Figure 7). When a hydrophilic residue, Gln, is substituted for Gla at amino acid position 6, PL binding receives an assist neither from a hydrophobic component nor from a Ca^{2+} binding residue, and the $[C_{50,P}$-PL] is elevated to reflect to a 6-fold weaker affinity for the [Gla^6→Gln]-r-PC mutant. Additionally, while binding of [Gla→Val]-r-PC may be as effective as wtr-PC, its orientation on PL may not be optimal for specific fVa and fVIIIa cleavages, a

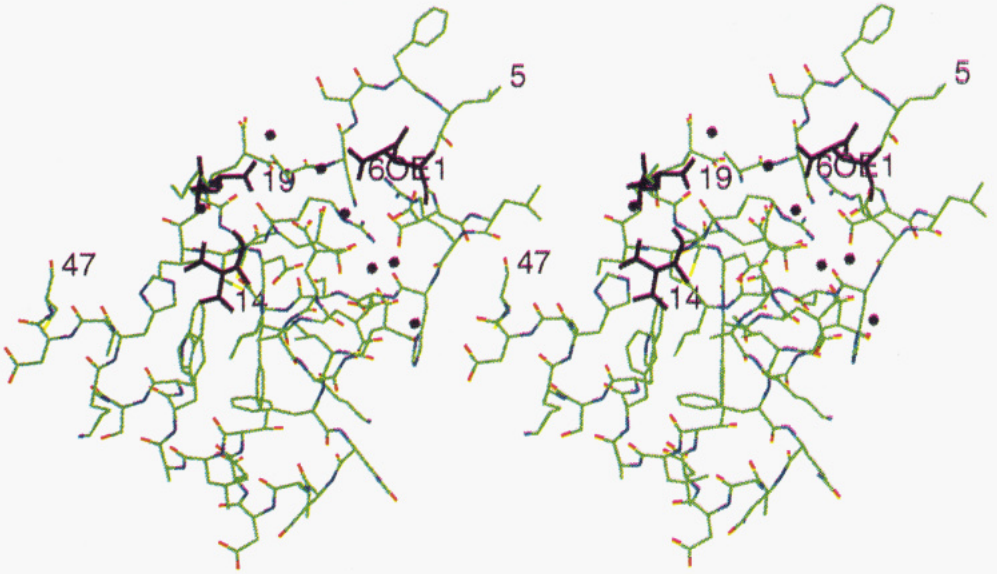

FIGURE 7: Stereoview of the modeled Gla domain and helical stack of PC. Gla residues 6, 14, and 19 are in black; the Ca^{2+} positions are in black squares and number 1−7 beginning with the rightmost square; the OE1 of Gla^6 involved in the μ-oxo bridge between Ca-4 and Ca-5 is indicated. The color coding is as follows: carbon, green; nitrogen, blue; oxygen, red; sulfur, yellow.

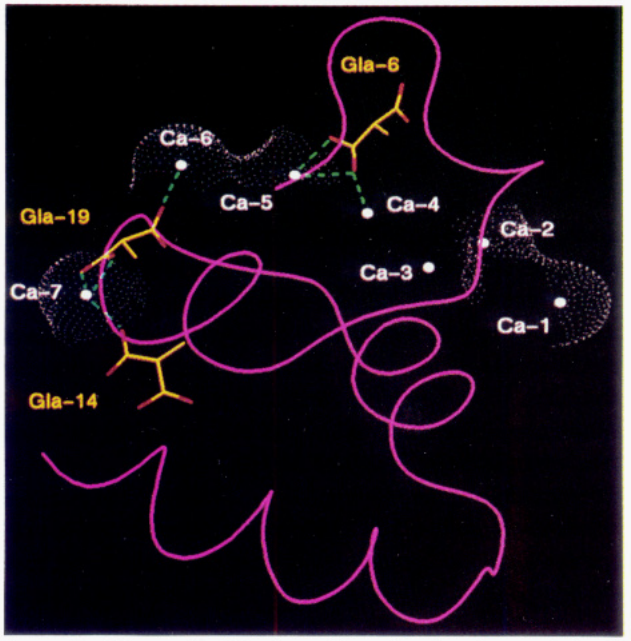

FIGURE 8: Schematic of the three-dimensional structure of the Gla domain and helical stack of PC based on the crystallographic coordinates of bovine prothrombin fragment 1. The locations of Gla^6, Gla^{14}, and Gla^{19} are emphasized, along with their proposed coordination sites to Ca^{2+} (green broken lines). Except for Ca-7, other Gla residues coordinate the remaining Ca^{2+} ions, but we only display those that originate from Gla^6, Gla^{14}, and Gla^{19}. The atoms involved in Ca^{2+}−O coordination are based on distance measurements in the model and have been assigned when Ca^{2+} and O atoms were located between 2.2 and 3 Å from each other. The backbone conformation is in magenta, Ca^{2+} is in white, and the calculated solvent exposure of each Ca^{2+} ion is depicted by white shadowing. Gla residues shown from the β-carbons are in yellow, with their oxygen atoms in red. The orientation of the molecule is similar to that displayed in Figure 7.

proposal based on the lack of the APTT activity of [Gla^6→Val]r-PC and the shape of the titration curve of the effect of Ca^{2+} on binding of this mutein to PL (Figure 5). Thus, not only is the strength of macroscopic PL binding of importance, but the proper orientation of the enzyme on the PL is also a significant factor.

The modeling of the GD of PC not only provided a rationale for the mutagenesis experiments but additionally revealed two new noteworthy structural aspects of the domain. The first is that of a stacked array of five aromatic residues in PC, consisting of the conserved triplet of the HS and the adjoining Phe^{24} and Phe^{31} (Figure 7). The other is the substitution of Leu^{38} in PC for Asp^{39} in bovine prothrombin and of Val^{45} in PC for Thr^{46} in bovine prothrombin. These substitutions in PC, along with Val^{35}, give the HS of PC pronounced amphipathic character. The full significance of these differences remains to be seen. Two other apparently drastic, but structurally innocuous, differences occur between Ca^{2+}/prothrombin fragment 1 and PC, *viz.*, Phe^{29} in prothrombin replaced by Lys^{28} in PC and Arg^{31} in bovine prothrombin substituted by Ile^{30} in PC. The former residue is on the surface, and the latter is buried in the X-ray crystal structure of Ca^{2+}/prothrombin fragment 1, both of which are contrary to expectations. The changes in PC are more consistent with the proposed model.

In conclusion, in this and in previous work, using variant recombinant molecules constructed by site-directed mutagenesis methods, we have clearly defined the functional roles of individual Gla residues in many of the GD-related Ca^{2+}- and PL-dependent functional properties of PC and APC. Only Gla residues at sequence positions 14 and 19, and the one Ca^{2+} (Ca-7) that is coordinated by these residues, appear to be nonessential to the *in vitro* functions examined to date of these proteins. We are now approaching an understanding of the molecular basis for the number and exact placement of Gla residues in PC and APC, and continuing work on these topics will further illuminate this important area of structure−function investigations on these highly relevant anticoagulant proteins.

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