The Hydrophobic Nature of Residue-5 of Human Protein C Is a Major Determinant of Its Functional Interactions with Acidic Phospholipid Vesicles†

Louise R. Jalbert, Joyce C. Y. Chan, William T. Christiansen, and Francis J. Castellino*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

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ABSTRACT: We have previously proposed that a cluster of surface-exposed hydrophobic amino acids, *viz*., F^4, L^5, and L^8, present at the amino-terminus of the Ca^{2+}-bound form of γ-carboxyglutamic acid domain (GD) of human protein C (PC), contributes a substantial portion of the total functional binding energy of PC and its activated form, APC, to acidic phospholipid (PL) vesicles. A deeper understanding of the importance of the hydrophobic nature of sequence position 5, and the particular relevance of leucine at that location, was sought by examination of the properties of a series of mutant proteins containing A^5, V^5, I^5, and W^5 as replacements for L^5 in recombinant (r)-PC and APC. The Ca^{2+}- and PL-dependent plasma-based anticoagulant activities of [L^5A]r-APC, [L^5V]r-APC, [L^5I]r-APC, and [L^5W]r-APC were determined to be approximately 28%, 51%, 98%, and 105%, respectively, of that of wild-type r-APC. A similar trend in activities of the mutant enzymes was observed in *in vitro* factor V/Va and factor VIII/VIIIa inactivation assays. Apparently normal Ca^{2+}-dependent conformations were adopted by each of the mutant proteins, but the Ca^{2+}-bound form of [L^5A]r-PC was relatively the most defective of the mutants in its binding to PL. These results confirm the importance of the hydrophobic character at sequence position 5 as critical to the functional binding of PC to PL.

Protein C (PC),[1] the zymogen form of the anticoagulant enzyme APC, is a member of a group of vitamin K-dependent coagulation proteins that, along with coagulation factors VII, IX, and X, contain a very similar domain organization (Foster et al., 1985; Yoshitake et al., 1985; Leytus et al., 1986; O'Hara et al., 1987). One of the component modules, *viz*., the GD, is characterized by the presence of 9−12 Gla residues within the amino-terminal 40 amino acids of these and similar proteins. The integrity of this domain is required for proper Ca^{2+} binding to occur, a step that is requisite for effective functional interactions of PC and APC with PL. This concept has been affirmed by the complete loss of Ca^{2+}/PL-dependent biological activities of these types of proteins consequent to removal of the GD by limited proteolytic treatment of the protein (Esmon et al., 1983; Morita et al., 1984; Sugo et al., 1984; Morita & Jackson, 1986); by blockage of γ-carboxylation by administration of the vitamin K antagonist, warfarin (Sugo et al., 1985); and by mutagenesis of residues within GD_{PC} (Zhang & Castellino, 1990, 1991, 1994; Zhang et al., 1992; Christiansen et al., 1994).

The nature of the binding interactions of GD-containing coagulation proteins with PL vesicles has been intensely studied. The results from several such investigations suggest that at least two stages are required for this interaction to occur. First, upon occupancy of two to three cation binding sites within the GD, a conformational alteration take place, observable by intrinsic fluorescence changes (Nelsestuen, 1976; Prendergast & Mann, 1977; Strickland & Castellino, 1980; Zhang & Castellino, 1992) and by interactions with conformation-specific antibodies (Keyt et al., 1982; Borowski et al., 1986; Wakabayashi et al., 1986; Church et al., 1989; Zhang & Castellino, 1992). This first group of sites is relatively nonspecific for the type of multivalent cation used, with a variety of divalent and trivalent cations demonstrating binding capacity (Bajaj et al., 1976, 1977; Furie et al., 1976; Prendergast & Mann, 1977; Amphlett et al., 1978). A second group of approximately four to five divalent cation sites, with greater specificity for Ca^{2+} (Nelsestuen et al., 1976), is then required for interaction of the protein with acidic PL vesicles. More recent investigations also strongly suggest that hydrophobic interactions contribute substantially to the protein/PL binding stability (Atkins & Ganz, 1992) and that the

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* Correspondence to this author. Tel: (219) 631-6456. FAX: (219) 631-8149. E-mail: castellino.1@nd.edu.

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[1] Abbreviations used: PC (APC), (activated) protein C; fIIa, thrombin; fV (fVa), (activated) coagulation factor V; fVIII (fVIIIa), (activated) coagulation factor VIII; prothrombin fragment 1, a proteolytic fragment of bovine prothrombin containing amino acid residues 1−156; MAb, monoclonal antibody; PS, protein S; Tm, thrombomodulin; Hya, β-hydroxyaspartic acid; Gla, γ-carboxyglutamic acid; GD, γ-carboxyglutamic acid-containing domain; GD_{PC}, the γ-carboxyglutamic acid-rich region of PC (amino acid residues 1−37); HS_{PC}, the helical stack region, residues 38−46, of PC; $EGF1_{PC}$, the epidermal growth factor homology region, residues 47−92, of PC; $EGF2_{PC}$, the epidermal growth factor homology region, residues 93−137, of PC; PL, 60%/40% (w/w) sonicated dispersion of chicken egg phosphatidylcholine (PhC)/bovine brain phosphatidylserine (PhS); $C_{50,Ca}$-Fl, the total Ca^{2+} concentration required to alter the intrinsic fluorescence in one-half of the protein molecules; $C_{50,Ca}$-JTC1, the total Ca^{2+} concentration required to induce binding to monoclonal antibody JTC1 of 50% of the protein molecules at constant protein concentration; $C_{50,Ca}$-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; $C_{50,PC}$-PL the total protein C or mutant protein C concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of Ca^{2+}; S2222, benzoyl-Ile-Glu(OR)-Gly-Arg-*p*-nitroanilide; S2366, L-pyro-Glu-Pro-Arg-*p*-nitroanilide; APTT, activated partial thromboplastin time; $DodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; FFQ, fast flow Q; wt, wild-type; r, recombinant.

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protein residues most likely involved in this interaction are the rigidly conserved L^5 (Zhang & Castellino, 1994), along with amino acid residues F^4 and L^8 (Christiansen et al., 1995b). According to models of GD_{PC} (Christiansen et al., 1994), based on the X-ray structure of the Ca^{2+}/bovine prothrombin fragment 1 complex (Soriano-Garcia et al., 1992), these three residues constitute an unusual cluster of solvent-exposed hydrophobic residues in the Ca^{2+}-bound form of GD_{PC}, as well as in other proteins of this class in which this pattern of residues exist. In order to probe more deeply into the absolute requirement of L^5 for PL-related functions and to determine whether altering hydrophobicity at this location would affect PL binding and PL-dependent functions of PC and APC in predictable manners, we constructed a series of mutant proteins altered at amino acid sequence position 5 and investigated their Ca^{2+}- and PL-dependent characteristics. The results of this investigation are presented herein.

MATERIALS AND METHODS

Proteins. wtr-PC and the mutant forms of this protein were expressed in human kidney 293 cells and isolated by FFQ chromatography as published previously (Zhang & Castellino, 1990). The corresponding r-APCs were prepared from the appropriate zymogens by activation with the venom protease, Protac C (American Diagnostica, New York, NY), as described earlier (Zhang & Castellino, 1990). Human r-fVIII was provided by the Genetics Institute (Cambridge, MA), and human plasma fVa was purchased from Hematologic Technologies (Burlington, VT). Human fIIa, fIX, and fX were donated by Enzyme Research Laboratories (South Bend, IN). Bovine fIXaβ was obtained by activation of bovine fIX (Amphlett et al., 1979). r-y[Tyr^{63}]hirudin (CGP 39393), a genetically engineered variant of hirudin expressed in yeast that lacks the sulfate group on Try^{63} (Meyback et al., 1987), was donated by Ciba-Geigy (Horsham, West Sussex, England). MAb-JTC1, which is directed to a Ca^{2+}-dependent epitope in GD_{PC} (Wakabayashi et al., 1986), was provided by Dr. K. Wakabayashi (Tokyo, Japan). The antibody was radiolabeled using the Iodobead (Pierce Chemical Company, Rockford, IL) procedure, as described previously (Zhang & Castellino, 1992). MAb-C3 (Heeb et al., 1988), specific for a Ca^{2+}-independent epitope within $EGF1_{PC}$ (Yu et al., 1994) and which was used for screening of PC antigen in transfected 293 cell colonies, was a gift of Dr. J. Griffin (La Jolla, CA). Restriction endonucleases were purchased from the Promega Corporation (Madison, WI).

Lipids. PL vesicles were prepared from mixtures (60/40 w/w) of PhC/PhS (Beals & Castellino, 1986). The PL vesicle concentration was determined as total organic phosphate (Lowry & Lopez, 1946) with use of a conversion factor of 25 (w/w) to obtain the weight concentrations of the PL vesicles.

cDNAs. The properties of the cDNA coding for wtr-PC (p119-[PC]) have been provided earlier (Zhang & Castellino, 1990).

Mutant cDNAs were constructed by primer-directed mutagenesis, using synthetic oligonucleotides on single-strand templates of p119[PC] (Kunkel et al., 1987). The mutagenic primers used for this purpose are listed below (the variant bases are indicated by lower case lettering). The wtr-PC sequence is shown for reference.

wt: 5'-GCC AAC TCC TTC CTG GAG GAG CTC CGT CAC AGC

r-[L^5A]PC: 5'-GCC AAC TCC TTC gcG GAG GAG CTg CGT CAC AGC

r-[L^5I]PC: 5'-GCC AAC TCC TTC aTc GAG GAG CTg CGT CAC AGC

r-[L^5V]PC: 5'-GCC AAC TCC TTC gTc GAG GAG CTg CGT CAC AGC

r-[L^5W]PC: 5'-GCC AAC TCC TTC tgG GAG GAG CTg CGT CAC AGC

Screenings of the bacterial transformants for colonies containing the desired mutations were accomplished by restriction endonuclease analyses. For each of the above variants, the mutations were accompanied by loss of a *Sac*I restriction endonuclease site (underlined in the wtr-PC sequece above).

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

Intrinsic Fluorescence Titrations. The quantitative ability of Ca^{2+} to induce the Ca^{2+}-dependent conformations of r-PC mutants was evaluated by titrations of the effects of Ca^{2+} on the intrinsic fluorescence of these proteins (Zhang & Castellino, 1992). A SLM-Aminco 8100 recording spectrofluorimeter (SLM-Aminco Instruments, Urbana, IL) was used for this purpose.

MAb Binding to r-PC Mutants. Binding of the GD_{PC}-directed Ca^{2+}-dependent MAb, JTC1, to r-PC muteins was determined by solid state assay with [^{125}I]JTC1, as described earlier (Zhang & Castellino, 1992).

Interaction of r-PCs with Acidic PL. Binding of r-PC muteins to acidic PL was measured at 25 °C using 90° relative light-scattering methodology (Nelsestuen & Broderius, 1977; Nelsestuen & Lim, 1977). Our procedures have been described (Zhang & Castellino, 1993).

r-APC Assays. *Ex vivo* plasma-based APTT anticoagulant assays using PC-deficient human plasma, as well as *in vitro* fVa and fVIII inactivation assays in the complete prothrombinase and tenase systems, were used to determine the activities of the r-APC mutants. Our methodologies have been published (Zhang et al., 1992; Jhingan et al., 1994).

Analytical Methods. Methods for all DNA manipulations required for these studies have been described in previous publications (Menhart et al., 1991; De Serrano & Castellino, 1992; De Serrano et al., 1992). Protocols for Gla (Zhang & Castellino, 1990) and Hya (Yu et al., 1994) analyses, as well as for determinations for the amino-terminal amino acid sequences of the muteins (Chibber et al., 1990) and Western immunoblotting (Zhang & Castellino, 1990), have been described in the indicated publications.

RESULTS

Five separate mutations were engineered into the cDNA of r-PC, each of which would encode an alteration at amino acid L^5 of the translated product. This amino acid position was selected for strategic mutagenesis because of the importance that has been previously ascribed to L^5 in stabilizing functional binding of PC and APC to acidic PL vesicles (Zhang & Castellino, 1994). In order to further define the role of L^5 in these interactions, and to probe the essentiality of L at this location, a series of aliphatic mutations of this residue, *viz.*, L^5A, L^5V, and L^5I, were made and the properties of the resulting proteins were studied. An additional mutant, [L^5W]r-PC, was constructed in order that the importance of an expanded hydrophobic surface area present at sequence position 5 could be assessed.

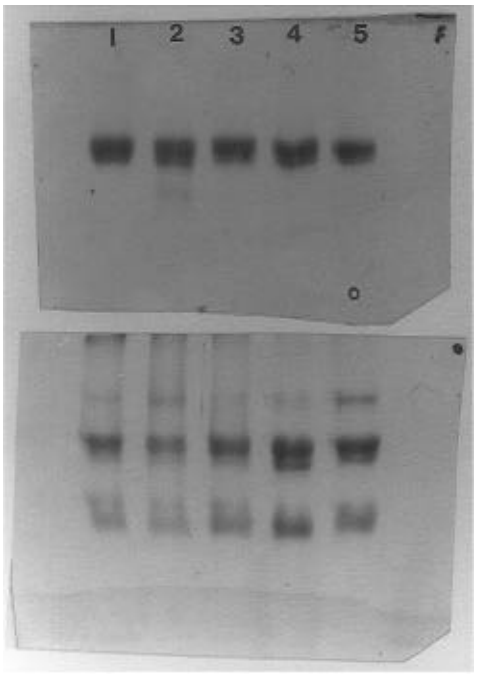

FIGURE 1: $DodSO_4$/PAGE of r-PC mutants. Top. Non-reduced gels. Lane 1, wtr-PC; lane 2, [L[5]A]r-PC; lane 3, [L[5]V]r-PC; lane 4, [L[5]I]r-PC; lane 5, [L[5]W]r-PC. Bottom. Reduced gels. Lane 6, wtr-PC; lane 7, [L[5]A]r-PC; lane 8, [L[5]V]r-PC; lane 9, [L[5]I]r-PC; lane 10, [L[5]W]r-PC.

All proteins were successfully purified using two FFQ column chromatographic steps, as previously detailed for a number of other r-PC mutant proteins (Zhang & Castellino, 1990, 1991, 1994; Zhang et al., 1992; Christiansen et al., 1994, 1995a; Yu et al., 1994). Reduced and nonreduced $DodSO_4$/PAGE gels for the purified proteins are provided in Figure 1. It is clear from these gels that, as in the case of wtr-PC, each mutant protein is present essentially in its two-chain form. The doublet band observed in wtr-PC and each of the r-PC mutant proteins, as well as in their component heavy chains, is due to the presence of r-PC glycoforms (Grinnell et al., 1987), and is a characteristic of all r-PC mutants isolated to date. That other processing events were complete in each of these mutant proteins is evidenced by the fact that the full complement of Hya (values of 0.9 mol/mol of r-PC to 1.4 mol/mol of r-PC were obtained for the mutants) and Gla residues (8.7 mol/mol of r-PC to 9.3 mol/mol of r-PC) were present in the proteins. In addition, amino acid sequence analysis through thirty amino acid residues showed that the mutations were present at their proper locations, that no other amino acid changes occurred in this region of the light and heavy chains, and that <7% of E appeared at any of the locations supposedly containing Gla residues.

In order to determine the extent to which the mutant proteins underwent their GD-related Ca^{2+}-dependent conformational transitions, the effects of Ca^{2+} on the protein intrinsic fluorescence were evaluated in each case. An example of the titration data obtained is provided in Figure 2 for [L[5]A]r-PC. Here, the maximum fluorescence (quenching) change upon saturation with Ca^{2+} was found to be 14%, with a $C_{50,Ca}$-Fl of 0.3 mM. For wtr-PC, these values were 15% and 0.4 mM, respectively. These same values for the rest of the proteins studied are summarized in Table 1.

The ability of these mutants to interact with a Ca^{2+}-dependent, GD-related MAb, JTC1, was next examined. The

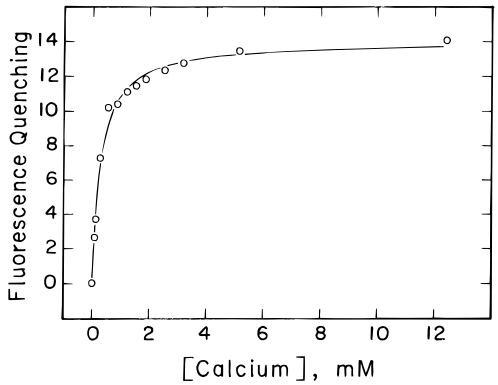

FIGURE 2: Effect of Ca^{2+} on the intrinsic fluorescence of PC. The protein (*ca.* 1 μM) was titrated with sequential additions of Ca^{2+}, and the intrinsic fluorescence intensity measured. The fluorescence (F_o) of the protein obtained in the absence of Ca^{2+} was adjusted to 1.0, and fluorescence values (F) were obtained after addition of Ca^{2+}. The percent fluorescence change (quenching) was calculated as $[(F_o - F)/F_o \times 100]$ 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 determined by nonlinear least-squares iterative fitting of the data. The excitation and emission wavelengths were 283 and 340 nm. Slit widths of 1 and 16 nm were used for excitation and emission, respectively. The buffer was 20 mM Tris-HCl/100 mM NaCl, pH 7.4 at 20 °C. The data shown are for the Ca^{2+}/[L[5]A]r-PC interaction.

data obtained from a Ca^{2+}-titration of JTC1 binding to each of the proteins are illustrated in Figure 3. While the antibody does not saturate at the same levels in the cases of the mutant proteins, and does not interact at all with [L[5]W]r-PC at the concentrations tested, the $C_{50,Ca}$-JTC1 values for those mutants that display reactivity are not greatly different from each other and range between 4.5 and 7.0 mM. Since this antibody is directed to an epitope within the amino-terminal twelve residues of wtr-PC, it is likely that the reduced saturation points are due to the comparative decreased abilities of the Ca^{2+}/r-PC complexes containing the mutant proteins to interact with MAb-JTC1. These maximal binding differences are seemingly more relevant to considerations of the importance of the specific amino acids on the integrity of the epitope, rather than on the ability of the r-PC to adopt a Ca^{2+}-dependent conformation in this region of the molecule.

Titrations of the effects of Ca^{2+} on the binding of the r-PC mutants to acidic PL were accomplished by 90° quasielastic light scatter. The data obtained are illustrated in Figure 4. Once again, while the saturation points of the mutants differ somewhat from each other, the $C_{50,Ca}$-PL values are within a factor of 2, suggesting that the Ca^{2+}-dependency of the interaction is similar in each case. The basis of the lowered saturation points cannot be stated with certainty. The most likely explanation is the diminished overall binding of the Ca^{2+}/protein complexes to PL at the protein concentrations used in the experiment of Figure 4. This is fortified by the observation that the C_{50} for binding of [L[5]W]r-PC to PL was found to be 0.51 μM at 2 mM Ca^{2+}, as determined by experiments similar to those of Figure 4 at varying protein concentrations, whereas that for wtr-PC under the same conditions was 0.34 μM. This slightly higher C_{50} for [L[5]W]r-PC could explain the slightly lower maximal binding observed for this mutant under the conditions of the experiments of Figure 4. However, other factors, such as fewer available binding sites for mutant proteins on PL and/or different binding orientations that affect the 90° light

Table 1: Properties of r-PC and r-APC Mutants

property	r-PC	[L^{5}A]	[L^{5}V]	[L^{5}I]	[L^{5}Q][a]	[L^{5}W]
PC[b]						
$C_{50,Ca}$-Fl, mM (% max)[c]	0.4 (15%)	0.3 (14%)	0.5 (16%)	0.5 (15%)	0.3 (16%)	0.9 (13%)
$C_{50,Ca}$-PL, mM[d]	1.2	3.2	1.2	1.3	>18	1.2
APC[e]						
APTT, % wt[f]	100	28	51	90	<2	105
fVa, % wt[g]	100	25	40	85	10	120
fVIII, % wt[g]	100	29	50	98	8	115

[a] The data for [L^{5}Q]r-PC and [L^{5}Q]r-APC are taken from (Zhang & Castellino, 1994). [b] The properties below are for the zymogen forms of the proteins. [c] The Ca^{2+} concentration required to quench the fluorescence in 50% of the protein molecules at a constant concentration of Ca^{2+} (% maximal fluorescence change). [d] The Ca^{2+} concentration required to induce binding of 50% of the protein molecules to PL at constant protein concentration (1 μM). [e] The properties below are for the activated forms of the proteins. [f] The anticoagulant activity of the mutant r-APCs relative to wtr-APC (set at 100%) normalized to the same amidolytic activities, as measured by the increase in the activated partial thromboplastin time of PC-deficient plasma. [g] The rate of inactivation of fVa or fVIII by the mutant r-APCs relative to wtr-APC (set at 100%) normalized to the same amidolytic activities, as measured in the complete prothrombinase or tenase complexes, respectively, with purified components.

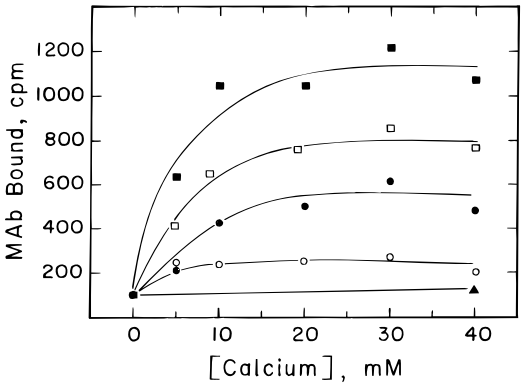

FIGURE 3: Effects of the Ca^{2+} on the binding of MAb-JTC1 to r-PC mutants at 25 °C. Incremental additions of Ca^{2+} were added the proteins, in the presence of [^{125}I]JTC1, and the amount of antibody (MAb) bound was measured. The MAb bound (as cpm) at each concentration of Ca^{2+} was plotted against the concentration of Ca^{2+}. The [$C_{50,Ca}$-JTC1] was calculated by nonlinear least-squares minimization of the data allowing both [$C_{50,Ca}$-JTC1] and cpm_{max} to float during the iterations. (■) wtr-PC; (○) [L^{5}A]r-PC; (●) [L^{5}V]r-PC; (□) [L^{5}I]r-PC; (▲) [L^{5}W]r-PC.

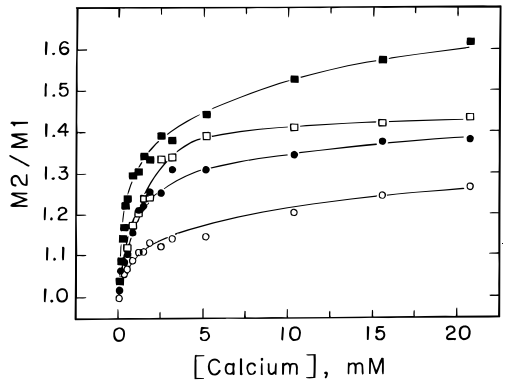

FIGURE 4: Effect of Ca^{2+} on the binding of PC mutants to acidic PL (60%/40% w/w: chicken egg phosphatidylcholine/bovine brain phosphatidylserine) at 20 °C. The molecular weights of the protein/PL complexes were determined by 90° relative light scattering. Excitation and emission wavelengths of 320 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 M_2/M_1 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; (○) [L^{5}A]r-PC; (●) [L^{5}V]r-PC; (□) [L^{5}I]r-PC. Because of overcrowding, the data for [L^{5}W]r-PC are not shown since they are very similar to the same data for [L^{5}I]r-PC.

scatter properties of the complexes that would alter the values at the saturation points cannot be excluded.

The plasma anticoagulant activities of the activated forms of these mutants were determined by assessment of the effects of the relevant APCs on the APTT times of PC-deficient plasma. Activations of the mutant PCs were accomplished by incubation with Protac C, a Ca^{2+}-independent activator. The clotting times were plotted against the APC concentrations, with all APC concentrations normalized to equal amidolytic activities with wtr-APC. An example of the data obtained is provided graphically for [L^{5}V]r-APC in Figure 5. The relative activities of the mutants calculated from all data obtained, summarized in Table 1, show that [L^{5}I]r-APC (95% of wtr-APC) is as active as wtr-APC, whereas [L^{5}A]r-APC (28% of wtr-APC) and [L^{5}V]r-APC (51% of wtr-APC) possess overall plasma anticoagulant activities that parallel the hydrophobicity of residue 5. Of interest, [L^{5}W]r-APC (*ca.*, 105% activity) is slightly up-regulated in terms of APC activity. A previous investigation has shown that introduction of a hydrophilic head group at this location in [L^{5}Q]r-APC led to a near total loss of plasma anticoagulant activity in the mutant (Zhang & Castellino, 1994). Similar trends in activities have been observed when these mutants were assayed using fVa (Figure 6) or fVIII (Figure 7) as the substrates for the relevant r-APC (Table 1). One interesting feature of these latter assays is the slightly, but consistently, higher activity of [L^{5}W]r-APC, as compared to wtr-APC.

DISCUSSION

The amino-terminal human vitamin K-dependent protein sequences below show the strict conservation of L at an amino acid sequence position corresponding to residue 5 of h (human) PC, which also is the case for its bovine (b) and mouse (m) counterparts.

hPC: NH_2-A N S F - **L** γ γ L

bPC: NH_2-A N S F - **L** γ γ L

mPC: NH_2-A N S F - **L** γ γ L

hfVII: NH_2-A N A F - **L** γ γ L

hfIX: NH_2-Y N S G K **L** γ γ F

hfX: NH_2-A N S F - **L** γ γ M

hfII: NH_2-A N T F - **L** γ γ V

hPS: NH_2-A N S L - **L** γ γ T

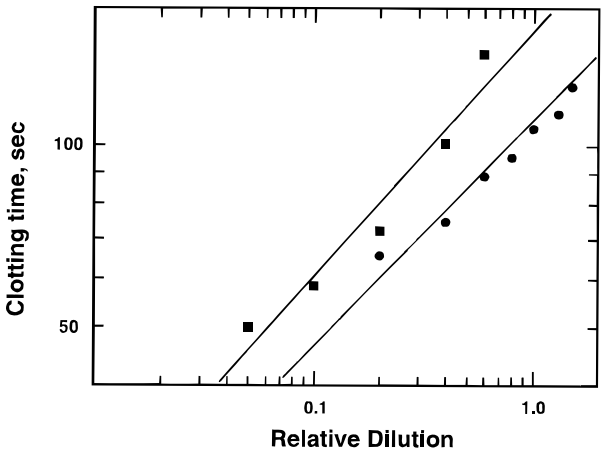

FIGURE 5: Anticoagulant activity of APC. Assay of the overall anticoagulant activity of (■) wtr-APC or (●) [L^{5}V]r-APC, each present at the same amidolytic activity, using APTT times with PC-deficient human plasma at 37 °C. Stock solutions of wtr-APC and [L^{5}V]r-APC were prepared, and their amidolytic activities were determined. Adjustments of the stock concentrations were made such that their 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 0.3 μg/mL). The displacement of the parallel lines provided the activity of the r-APC sample relative to that of wtr-APC.

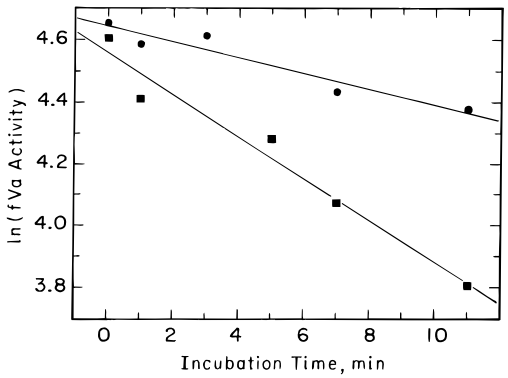

FIGURE 6: Inactivation of fVa by APC. Time course of inactivation of human fVa by (■) wtr-APC or (●) [L^{5}V]r-APC, each present at the same amidolytic activity. The buffer contained 20 mM Hepes-NaOH/150 mM NaCl, pH 7.4, 25 °C. After various times of incubation of (final concentrations) fVa (0.1 μM) with the APC (0.5 nM), in the presence of PL (20 μM) and Ca^{2+} (2.5 mM), an aliquot of the mixture was added to a cuvette containing (final concentrations) prothrombin (1.39 μM), $CaCl_2$ (2.5 mM), PL (20 μM), and DAPA (3 μM). Activation of prothrombin was accelerated by addition of fXa (8 nM). The fluorescence increase resulting from uptake of DAPA by the fIIa generated was monitored continuously by spectrofluorimetry. The initial rates of fIIa production were calculated from these experiments at each incubation time of APC/fVa, relative to wtr-APC which was assumed to be 100% active. The resulting first-order kinetic replots are illustrated. The ratio of the rate constant of [L^{5}V]r-APC to that of wtr-APC was employed to calculate the percentage activity of the mutant.

This rigid conservation of L^5 led us to investigate its role in the properties of r-PC (Zhang & Castellino, 1994). We concluded that this residue, as well as that of L^8 and, to a lesser extent, F^4, was critical amino acid side chains for contributing significant hydrophobic components to the total binding energy of these proteins to acidic PL vesicles (Christiansen et al., 1995b). This latter investigation was expanded herein and directed toward an evaluation of the

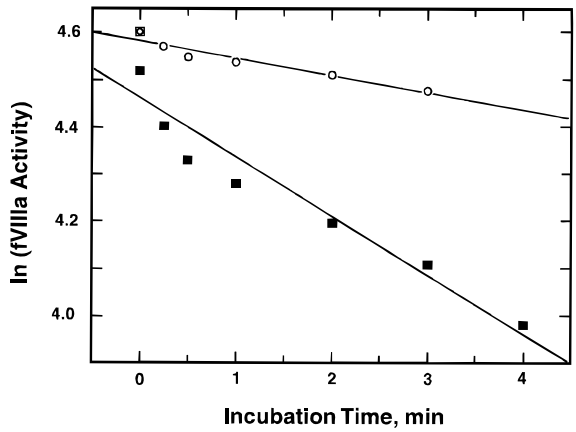

FIGURE 7: Inactivation of fVIII by r-APC. Time course of inactivation of human fVIII by (■) wtr-APC or (○) [L^{5}A]r-APC, each present at the same amidolytic activity. The buffer contained 20 mM Hepes-NaOH/150 mM NaCl, pH 7.4, 37 °C. A solution of fVIII (*ca.* 6 nM, final concentration) was incubated with the relevant APC (*ca.* 0.5 nM, final concentration), in the presence of (final concentrations) PL (40 μM) and Ca^{2+} (2.5 mM). Next, fIXaβ (*ca.* 6.5 nM) and fIIa (0.10 units/mL) were added in order to activate the remaining fVIII. The amount of fVIIIa present was then assayed in the complete tenase system. For this, fVIIIa was added to a solution containing (final concentrations) 40 μM (in phosphate) PL, 2.5 mM $CaCl_2$, and 180 μM of the fXa chromogenic substrate, S2222, in a final volume of 790 μL. The base line was recorded for 1 min, after which 10 μL of a solution of 32 μM fX was added to accelerate S2222 hydrolysis. The rate of amidolysis of this latter substrate by fXa was determined spectrophotometrically. The initial rates of fXa production were calculated from these experiments at each incubation time of APC/fVIII, relative to wtr-APC, which was assumed to be 100% active. The logarithm of the fVIII activity remaining (%) was plotted as a function of the incubation times, and first-order inactivation rate constants were calculated. The ratio of the rate constants of [L^{5}A]r-APC to that of wtr-APC was employed to calculate the percentage activity of each of the mutant. A control incubation of fVIII with r-PC demonstrated that the loss of activity of fVIII was <5% over the time period illustrated.

specific importance of L^5 in the functional PL binding properties of r-PC. The specific issue addressed was whether substitution of other aliphatic amino acids with differing hydrophonicities would confirm the predicted role of L^5 in PC/PL binding properties. This problem was approached by construction of mutant proteins with replacement of L^5 by A, V, or I, followed by investigation of those functional properties of the mutants that depended on the integrity of their Ca^{2+}-dependent binding properties to acidic PL vesicles. In addition, we believed that a change to W at this location would allow assessment of those same properties of a mutant possessing an expanded hydrophobic surface area at this location, as well as the extent to which this latter feature of the protein was the determinant in the provision of functional PL binding for APC.

Each r-PC mutant protein was successfully expressed and purified. Of great importance to studies of this type was our ability to isolate subfractions of the r-PC mutants that were fully processed with regard to removal of the K^{156}R^{157} dipeptide and with respect to the completeness of β-hydroxylation and γ-carboxylation. Further characterization of these mutant proteins by intrinsic fluorescence measurements demonstrated that their component GD_{PC} domains adopted Ca^{2+}-dependent conformations at approximately the same concentrations of this cation. Studies with a MAb (JTC1) that selectively interacted with the Ca^{2+}-dependent conformation of GD_{PC} also suggested that each mutant

protein was able to adopt the Ca^{2+}-dependent conformation similar to that of wtr-PC. Some ambiguity is present in this latter interpretation, however, since the saturation points of the plots, at the MAb concentrations used, were not identical for each of the r-PC mutants. This could indicate that similar Ca^{2+}-dependent conformations were not adopted by the mutants. Alternatively, these results are also consistent with past observations that suggested that L^5 is an essential amino acid for the epitope of the MAbs and that a substitution at that sequence position is not well tolerated (Zhang & Castellino, 1994; Christiansen et al., 1995b). We favor this latter interpretation since such a finding would be consistent with the location of the epitopes for each of the MAbs at the amino-terminus of the GD_{PC} (Wakabayashi et al., 1986; Orthner et al., 1989), and we have observed that increases in the MAb concentration led to higher saturation points in the binding isotherms, without alteration of the $C_{50,Ca}$-JTC1 value. Especially supportive of this conclusion are the data obtained for [L^5W]rPC, which, while not reacting with this particular MAb, possessed full activity in the APC assay systems. This could not have occurred if the Ca^{2+}-induced conformational change had not taken place.

After conversion with Protac C, a Ca^{2+}-independent activator, of each of these mutants to their respective r-APCs, the resulting anticoagulant activities were measured in the plasma-based APTT assay. Prior to assay, each r-APC concentration was adjusted so that equal amidolytic activities were present in the stock samples (no more than 10%−15% adjustments to the protein concentrations were necessary to accomplish this). We felt that the best manner of comparing these variants was to normalize any possible effects of the mutations on rate variations of the active sites and to focus on other effector-based regions of the mutant protein molecules. Thus, we decided on the approach of performing macromolecular activity assays using mutant enzymes normalized for small substrate hydrolysis rates. Since APTT assays do not reveal differences in mutant APC activities toward fV/fVIIIa or fVIII/fVIIIa, nor, as performed, do they uncover whether plasma-containing cofactor or inhibitors are also playing a roles in APC activity, we turned to activity assays using two major macromolecular substrates of APC, fVa and fVIII (fVIIIa is too unstable to be used productively in these assays), in *in vitro* systems containing purified components of the complete PL-dependent prothrombinase and tenase systems, respectively. The results of these investigations, the data of which are presented in Figures 5−7 and the results of which are summarized in Table 1, show that the mutants progressively lose activity through the following series: wtr-APC ≥ [L^5I]r-APC > [L^5V]r-APC > [L^5A]r-APC > [L^5Q]r-APC. Thus, it is clear that as the hydrophobic character at amino acid residue 5 is diminished through this series, the resulting APC activities of the mutants toward fVa and fVIII are similarly abated. This conclusion is fortified by inclusion of [L^5W]r-APC, which provides an expanded hydrophobic surface and which contains approximately 1.1−1.2-fold more activity than wtr-APC toward these macromolecular substrates. Since the influence of all of these mutations on activity has been demonstrated to be unrelated to active site defects, and is not likely to be due to overall Ca^{2+} binding deficiencies (Figures 2 and 3), the activity losses must be based in defects in exosites needed for proper assembly of the r-APC/Ca^{2+}/fVa (fVIII)/PL complex, as was shown previously in the case of the [L^5Q] mutant (Zhang & Castellino, 1994). These results also implicate directly as part of such a relevant exosite the hydrophobic character at L^5, a residue rigorously conserved in this class of proteins. Given the deficiencies in binding of the Ca^{2+}/protein complexes to PL that correlate with hydrophobicity at amino acid sequence position 5, the interpretations presented herein are consistent with previous conclusions that hydrophobic insertion of this region of the protein into the PL hydrocarbon (Zhang & Castellino, 1994; Christiansen et al., 1995b) and specific surface-oriented electrostatic forces provided by Ca^{2+}/PC-PL interactions (Zhang & Castellino, 1993) are both necessary for functional binding of PC and APC to acidic PL.

From the results of the current investigations, coupled to past studies from this laboratory (Zhang & Castellino, 1990, 1991, 1992, 1993, 1994; Zhang et al., 1992; Christiansen et al., 1994), we conclude that functional binding of the Ca^{2+}/APC complex to acidic PL vesicles, as observed by the ability of this enzyme to inactivate PL-bound fVa and fVIII, is a complex process that is not simply observed through macroscopic interactions of Ca^{2+}/PC(APC) with acidic PL, but must also take into account the integrity of the alignment on PL of the enzyme active site with the susceptible peptide bonds of the substrates that are cleaved in the inactivation process. We propose that Ca^{2+}/PC(APC) interactions *via* Gla residues, with the exception of Gla^{14} and Gla^{19}, that provide surface-exposed Ca^{2+}, *viz*., Ca-1, Ca-2, Ca-5, and Ca-6 (Christiansen et al., 1994), are required for electrostatic interactions of the protein with the acidic PL. Binding of Ca^{2+}, principally to Gla^{16} and Gla^{26}, provides non-surface-exposed Ca-3 and Ca-4 that mediate a conformational change in GD_{PC} resulting in the surface exposure and clustering of F^4, L^5, and L^8. These latter residues then become available for hydrophobic insertion into the PL hydrocarbon phase. Disruption of any of these critical binding forces *via* mutagenesis could observably diminish macroscopic binding and/or more subtly lead to reorientation of PC or APC on the PL surface, in either case leading to decreased activity of the enzyme toward its PL-bound substrates.

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