Hydrophobic Amino Acid Residues of Human Anticoagulation Protein C That Contribute to Its Functional Binding to Phospholipid Vesicles†

William T. Christiansen, Louise R. Jalbert, Rolanda M. Robertson, Ashish Jhingan, Mary Prorok, and Francis J. Castellino*

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

Received April 12, 1995; Revised Manuscript Received June 15, 1995®

ABSTRACT: The contributions to functional phospholipid (PL) binding of the cluster of amino acid side chains of human protein C (PC) comprising F^4, L^5, and L^8 have been assessed by construction of mutants of PC and activated protein C (APC) designed wherein a hydrophilic side chain replaced the wild-type hydrophobic groups at these positions. The PL-dependent plasma-based anticoagulant activities of [F^4Q]-r-APC and [L^8Q]r-APC were severely reduced to 5% and <2%, respectively, of wild-type r-APC. Activity losses of the mutants toward inactivation of coagulation factor VIII, measured in the complete *in vitro* tenase system, have also been observed. As evidenced through Ca^{2+}-induced intrinsic fluorescence changes, both [F^4Q]r-PC and [L^8Q]r-PC were able to adopt Ca^{2+}-dependent conformations that appeared similar to that of wtr-PC, ruling out shortcomings associated with such Ca^{2+}-induced transitions as the basis for their anticoagulant activity losses. However, despite this, [L^8Q]r-PC showed greatly defective macroscopic binding properties to PL vesicles, as did to a lesser extent [F^4Q]r-PC. These findings were similar to those reported previously for [L^5Q]r-PC/APC [Zhang, L., & Castellino, F. J. (1994) *J. Biol. Chem. 269,* 3590−3595]. We thus propose that the PL-dependent activity losses of these mutants are related to their suboptimal binding to PL or to their misorientation on the PL surface leading to poor alignment of the active sites of the r-APC mutants with the complementary cleavage sites on fVIII/fVIIIa and fV/fVa. These investigations confirm the importance of hydrophobic components of functional PL binding by PC and APC and implicate L^8, along with L^5, as the principal amino acid residue involved in these interactions. F^4 has a lesser direct involvement in this regard but may contribute to proper alignment of PC and APC on the PL surface.

PC[1] is a member of the family of vitamin K-dependent blood coagulation proteins an exists in plasma as a zymogen. Its activated form, APC, a serine protease, functions to maintain blood fluidity in one fashion by serving as an anticoagulant. Individuals heterozygous for PC deficiencies often display thrombotic complications, while those homozygous for such defects usually manifest life-threatening clotting events during infancy [for a review, see Reitsma et al. (1993)]. PC is activated at the endothelial cell surface as a result of a single peptide bond cleavage that is catalyzed by fIIa bound to a membrane-localized protein cofactor, Tm (Esmon et al., 1982). Slower activation of PC occurs in plasma, catalyzed by fIIa alone. This latter reaction is inhibited by Ca^{2+} (Amphlett et al., 1981). The anticoagulant activity of APC rests in its ability to inactivate by limited proteolysis the prothrombinase cofactors, fV/fVa (Kisiel et al., 1977), as well as the tenase cofactors, fVIII/fVIIIa (Vehar & Davie, 1980). Ca^{2+} and PL (Kisiel et al., 1977), along with a cofactor, protein S (Walker, 1980), stimulate these latter reactions.

The nucleotide sequence of the human PC gene has been determined. Its coding regions are contained in a series of exons which translate into a 42 amino acid residue leader sequence, followed by a 155 amino acid residue light chain that is disulfide-linked to a heavy chain composed of 262 amino acids (Foster et al., 1985). A dipeptide, K^{156}-R^{157}, is present in the translated protein that connects the heavy and light chains. Endoproteolytic removal of this latter dipeptide during normal processing is critical to subsequent release of the activation peptide of PC in the formation of APC (Foster et al., 1990). Among the processing steps that generate mature human plasma PC include cleavage of the leader polypeptide (Foster et al., 1985), γ-carboxylation of nine Gla-precursor E-residues that are present at amino acid sequence

† Supported by Grant HL-19982 from the National Institutes of Health, a graduate fellowship from the American Heart Association, Indiana Affiliate (to W.T.C.), a postdoctoral fellowship from the American Heart Association, Indiana Affiliate (to M.P.), and the Kleiderer/Pezold Family endowed professorship (to F.J.C.).

* To whom to address correspondence.

® Abstract published in *Advance ACS Abstracts,* August 1, 1995.

[1] Abbreviations: PC, protein C; APC, activated protein C: fIIa, thrombin; fV, coagulation factor V; fVa, activated coagulation factor V; fVIII, coagulation factor VIII; fVIIIa, activated coagulation factor VIII; prothrombin fragment 1, a proteolytic fragment of bovine prothrombin containing amino acid residues 1−156; PS, protein S; Tm, thrombomodulin; MAb, monoclonal antibody; Hya, β-hydroxyaspartic acid; Gla, γ-carboxyglutamic acid; 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; $EGF1_{PC}$ and $EGF2_{PC}$, the first (residues 47−92) and second (residues 93−137) epidermal growth factor homology regions of PC, respectively; wt, wild type; r, recombinant; PL, 60%/40% (w/w) sonicated dispersion of chicken egg phosphatidylcholine (PhC)/bovine brain phosphatidylserine (PhS); S2366, L-pyro-E-P-R-*p*-nitroanilide; APTT, activated partial thromboplastin time; $NaDodSO_4$/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; $C_{50,PC}$-PL, the total protein C concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of Ca^{2+}.

0006-2960/95/0434-10376$09.00/0

positions 6, 7, 14, 16, 19, 20, 25, 26, and 29 of human PC (Beckmann et al., 1985), and β-hydroxylation of Asp^{71} (Drakenberg et al., 1983). Further processing events that take place include assembly of the usual types of N-linked oligosaccharides (Kisiel, 1979). On the basis of a consideration of the positions of the introns in the gene, as well as amino acid sequence homologies with other proteins, it is clear that PC contains several domains. These include the GD_{PC}, followed consecutively by the HS_{PC}, two consecutive regions homologous to human epidermal growth factor ($EGF1_{PC}$ and $EGF2_{PC}$), the activation dodecapeptide, and two exons that encode the serine protease chain. Fully processed human r-PC has been expressed in mammalian cell lines (Grinnell et al., 1987; Zhang & Castellino, 1990).

Binding to acidic PL is critical to the functioning of PC and APC, as well as to all Gla-containing coagulation proteins. Central to the interactions of these proteins with PL are their component GD modules, the Ca^{2+}-induced conformations of which interact with PL (Nelsestuen, 1976; Nelsestuen & Lim, 1977). In addition to electrostatic interactions that are known to be important for the association of the GD of the protein with PL, it has been suggested that hydroprobic components are significant ingredients of the total binding energy of this process (Atkins & Ganz, 1992; Zhang & Castellino, 1994). From a model of the Ca^{2+}/GD_{PC} complex (Christiansen et al., 1994), based on the X-ray crystal structure of the Ca^{2+}/GD complex of bovine prothrombin fragment 1 (Soriano-Garcia et al., 1992), we proposed that an unusual cluster of solvent-exposed hydrophobic residues, comprising F^4, L^5, and L^8, which are highly conserved in these proteins, may be critical to the hydrophobic component of the binding of PL to PC and to similar proteins (Zhang & Castellino, 1994). We have previously demonstrated the relevance of L^5 in this regard (Zhang & Castellino, 1994). In this paper, we have extended such analyses to F^4 and L^8 and present herein a summary of the findings of this investigation.

MATERIALS AND METHODS

Proteins and Peptides. wtr-PC and its mutants were expressed in human kidney 293 cells and isolated as reported previously (Zhang & Castellino, 1990). The corresponding r-APCs were prepared from the relevant zymogens by activation with the venom protease, Protac C (American Diagnostica, New York, NY), as described (Zhang & Castellino, 1990). Human r-fVIII was provided by the Genetics Institute (Cambridge, MA). Bovine fIXaβ was generated by activation of bovine fIX (Amphlett et al., 1979). Human fIIa, fIX, and fX were donated by Enzyme Research Laboratories (South Bend, IN). r-y[Tyr^{63}]hirudin (CGP 39393), an engineered form of hirudine expressed in yeast that lacks the sulfate group on Y^{63} (Meyback et al., 1987), was provided by Ciba-Geigy (Horsham, West Sussex, England).

The MAb, JTC1, which recognizes a Ca^{2+}-dependent epitope in GD_{PC} (Wakabayashi et al., 1986), was a gift of Dr. K. Wakabayashi (Tokyo, Japan). MAb-7D7B10, which is specific for the EDTA-dependent form of an epitope at the amino terminus of GD_{PC} (Orthner et al., 1989), was obtained from Dr. D. K. Strickland (Bethesda, MD). MAb-C3, which is directed to a Ca^{2+}-independent epitope within $EGF1_{PC}$ (Heeb et al., 1988; Yu et al., 1994), and which was used for detection of PC mutants in transfected 293 cell colonies, was obtained from Dr. J. Griffin (La Jolla, CA). The MAbs were radiolabeled using the Iodobead (Pierce Chemical Co., Rockford, IL) procedure as published earlier (Zhang & Castellino, 1992).

Restriction endonucleases were purchased from the Promega Corp. (Madison, WI).

Lipids. PL vesicles were prepared from mixtures of PhC/PhS (60/40 w/w) as described earlier (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.

cDNAs. A full description of the characteristics of the cDNA coding for human PC (p119[PC]) has been provided earlier (Zhang & Castellino, 1990).

The mutant cDNAs encoding [F^4Q]r-PC and [L^8Q]r-PC were constructed by primer-directed mutagenesis, using synthetic oligonucleotides on single-strand templates of p119-[PC] (Kunkel et al., 1987). The mutagenic primers used were (the mutagenic bases are in lower case letters)

[F^4Q]r-PC:

5′-CGT GCC AAC TCC cag CTG GAG GAG CTC

[L^8Q]r-PC: 5′-C GAG GAG Caa CGT CAC AGC AG

Screening of the bacterial transformants for the colonies containing the desired mutations was accomplished by restriction endonuclease analysis. For the [F^4Q]r-PC mutant, successful transformation with the mutagenesis primer resulted in simultaneous insertion of a *Pvu*II restriction site (underlined in the [F^4Q]r-PC sequence above), and in the case of [L^8Q]r-PC, successful bacterial transformation led to deletion of an endogenous *Sac*I site.

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

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

MAb Binding to r-PC Mutants. The binding of r-PC mutants of the GD_{PC}-specific, Ca^{2+}-dependent MAb, JTC1, and the GD_{PC}-directed, EDTA-dependent MAb, 7D7B10, was determined by either direct or displacement assays, essentially as described earlier (Zhang & Castellino, 1992).

Interaction of r-PCs with Acidic PL. The binding of r-PC mutants to acidic PL was measured at 25 °C using 90° relative light scattering methodology as previously described (Nelsestuen & Broderius, 1977; Nelsestuen & Lim, 1977). Our procedures for performing these titrations, as well as our data reduction methods, have been published (Zhang & Castellino, 1993).

r-APC Assays. Ex vivo plasma-based APTT anticoagulant assays with PC-deficient plasma and *in vitro* fV and fVIII inactivation assays in the prothrombinase and tenase complexes, respectively, were employed to determine the functional activities of the r-APC mutants. Our methodologies have been described (Jhingan et al., 1994).

Analytical Methods. The methods that we employed for DNA manipulations, which included oligonucleotide syn-

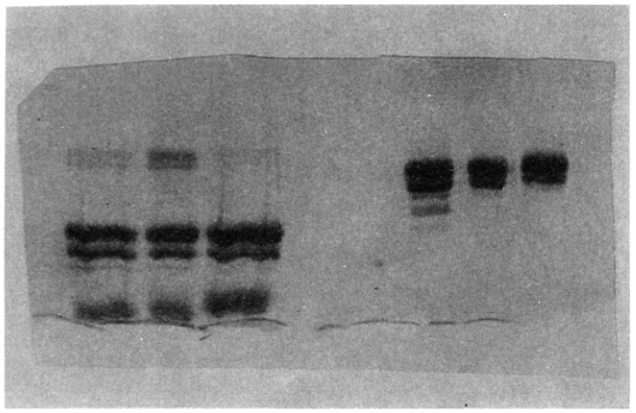

FIGURE 1: NaDodSO$_4$/PAGE analysis of r-PC and its mutants. Left to right: reduced wtr-PC; reduced [F^{4}Q]r-PC; reduced [L^{8}Q]r-PC; nonreduced wtr-PC; nonreduced [F^{4}Q]r-PC; nonreduced [L^{8}Q]-r-PC.

thesis, restriction site analysis, 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, have been 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) analyses, as well as determinations of the amino-terminal amino acid sequences of the mutant proteins (Chibber et al., 1990), were conducted as detailed in the indicated publications. Our protocols for Western immunoblotting with MAb-C3 have been published earlier (Zhang & Castellino, 1990).

RESULTS

Two r-PC mutants, *viz.*, [F^{4}Q]r-PC and [L^{8}Q]r-PC, were constructed in order that the extent of the participation of these two amino acids in the putative hydrophobic component of the functional PL-dependent binding of PC and APC could be examined. Purification of the expressed mutants was accomplished in two consecutive steps on FFQ columns, one involving elution with $CaCl_2$ and the other with NaCl. The elution profiles from these columns were very similar to those for a number of other r-PC mutants that we have isolated and reported upon (Christiansen et al., 1994; Zhang & Castellino, 1990, 1991, 1994).

Processing similar to wtr-PC occurred in the mutant proteins, as verified by several observations. First, Figure 1 shows results of NaDodSO$_4$/PAGE gels of the r-PC mutants. It is seen therein that both variants exist in their two-chain forms. That the correct R^{157}–L^{158} peptide bond was cleaved in this latter processing step was demonstrated by automated amino-terminal amino acid sequencing of the mutants through at least 20 residues, the results of which demonstrated that the correct two chains were present. Additionally, both Gla and Hya contents were determined for each of the mutants. In the case of [F^{4}Q]r-PC, these values were 8.9 and 1.4 mol/mol, respectively, whereas for [L^{8}Q]r-PC, these same values were 9.1 and 1.0 mol/mol, respectively.

The abilities of the r-PC mutants to adopt the Ca^{2+}-dependent conformation of PC were examined by analyses of the effects of Ca^{2+} on the intrinsic fluorescence of the proteins. Addition of Ca^{2+} induced an intrinsic fluorescence quenching, which was readily titrated with this cation. The resulting data are illustrated in Figure 2 and summarized in Table 1. The $C_{50,Ca}$-Fl values obtained for [F^{4}Q]r-PC and [L^{8}Q]r-PC were 0.70 and 0.47 mM, respectively, similar to that of wtr-PC (0.40 mM).

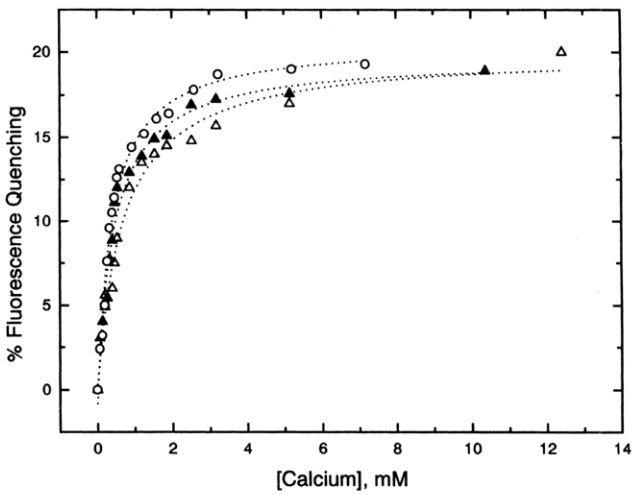

FIGURE 2: Effect of Ca^{2+} on the intrinsic fluorescence of r-PC. The relevant protein (*ca.* 1 μM) was titrated with Ca^{2+}, and the intrinsic fluorescence intensity changes were measured. The fluorescence of the protein obtained in the absence of Ca^{2+} (F_0) was adjusted to 1.0, and fluorescence values (F) were obtained after addition of Ca^{2+}. The present fluorescence quenching was determined 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. 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. Symbols: (○) wtr-PC; (△) [F^{4}Q]r-PC; (▲) [L^{8}Q]r-PC.

Table 1: Properties of r-PC Mutants

property	r-PC	[F^{4}Q]	[L^{5}Q][a]	[L^{8}Q]
PC[b]				
Gla (mol/mol)	8.9	8.9	9.1	9.1
Hya (mol/mol)	1.1	1.4	1.1	1.0
$C_{50,Ca}$-Fl (mM)[c]	0.40	0.70	0.24	0.47
$C_{50,Ca}$-PL (mM)[d]	1.2	1.7	>18	7.1
$C_{50,PC}$-PL (2 mM Ca^{2+}) (μM)[e]	0.40	0.50	6.2	6.5
$C_{50,PC}$-PL (20 mM Ca^{2+}) (μM)[e]	0.38	0.30	0.54	1.0
APC[f]				
APTT (% wild type)[g]	100	5	<2	<2
fV (% wild type)[h]	100	14	10	30
fVIII (% wild type)[h]	100	15	8	16

[a] The data for [L^{5}Q]r-PC and [L^{5}Q]r-APC are taken from Zhang and 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. [d] The Ca^{2+} concentration required to induce binding of 50% of the protein molecules to PL. [e] The protein concentration required to induce binding of 50% of the protein molecules to PL at Ca^{2+} concentrations of 2 or 20 mM. [f] The properties below are for the activated forms of the proteins. [g] 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. [h] The rate of inactivation of fV 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.

Two MAbs potentially revealing of the types of Ca^{2+}-dependent conformational alterations that occur in GD_{PC} were available for this study. The first, MAb-7D7B10, has been shown to react with amino the terminus of GD_{PC} in the presence of EDTA. The epitope for this MAb is lost in the presence of Ca^{2+} (Orthner et al., 1989). An assay of the

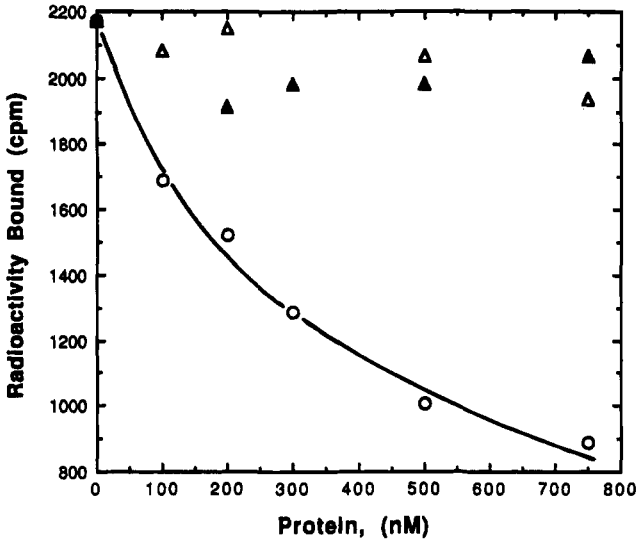

FIGURE 3: Ability of r-PC to displace MAb-7D7B10 from its epitope on wtr-PC. A solution of wtr-PC (80 μM) was employed to coat wells of a 96-well microtiter plate. To the plate were added a series of concentrations of the competitive proteins in the presence of a constant concentration (80 nM) of [^{125}I]7D7B10. The amount of MAb bound (cpm) to the insolubilized wtr-PC was then determined by γ counting of the excised wells. The 100% binding level was determined as the amount of [^{125}I]7DB10 bound in the absence of competing protein. The binding experiments were performed in a buffer of 20 mM Tris-HCl/100 mM NaCl/20 mM EDTA, pH 7.4, at room temperature. The proteins used were (O) wtr-PC, (△) [F^{4}Q]r-PC, and (▲) [L^{8}Q]r-PC.

ability of the mutant proteins to displace [^{125}I]7D7B10 from its epitope on wtr-PC was conducted. As seen in Figure 3, in the presence of 20 mM EDTA, neither [F^{4}Q]r-PC nor [L^{8}Q]r-PC, when employed in solution as the competing proteins, was able to inhibit binding of [^{125}I]7D7B10 to wtr-PC adsorbed to the microtiter plate wells. On the other hand, solution-phase wtr-PC effectively inhibited this same interaction (Figure 3). In a separate study, another MAb, JTC1, which has been shown to react with the amino-terminal region of the Ca^{2+}-bound form of GD_{PC} (Wakabayashi et al., 1986), was used for a similar purpose. We found (data not shown) that [F^{4}Q]r-PC, when adsorbed to microtiter plates, did not interact significantly with [^{125}I]JTC1, at least up to concentrations of 800 nM MAb and 40 mM Ca^{2+}. Under these same conditions, wtr-PC showed the proper Ca^{2+}-dependent interaction with [^{125}I]JTC1, being fully reactive at a MAb concentration of 40 nM in the presence of 40 mM Ca^{2+} (data not shown). In the case of [L^{8}Q]-r-PC, its reaction with JTC1 was weaker than that of wtr-PC, in that at a MAb concentration of 20 nM and a Ca^{2+} concentration of 40 mM, [^{125}I]JTC1 bound maximally to this mutant protein to a level of approximately 20% of that of wtr-PC. Upon titration of this latter interaction with Ca^{2+}, a $C_{50,Ca}$ for JTC1 of 6.5 mM was found. This value was very similar to that of the Ca^{2+} dependency of the wtr-PC interaction with JTC1 (4.5 mM), as well as for that of [L^{5}Q]r-PC binding to JTC1, of 6.0 mM (Zhang & Castellino, 1994). This further suggests that the Ca^{2+}-dependent conformation is adopted in [L^{8}Q]r-PC but that the Ca^{2+}/mutant protein complex is defective in its interaction with this MAb, most likely because L^8 is an important amino acid for stabilization of the interaction.

The Ca^{2+} dependency of the mutant proteins toward PL binding was examined by 90° relative light scatter techniques. The titration data, illustrated in Figure 4, show that whereas

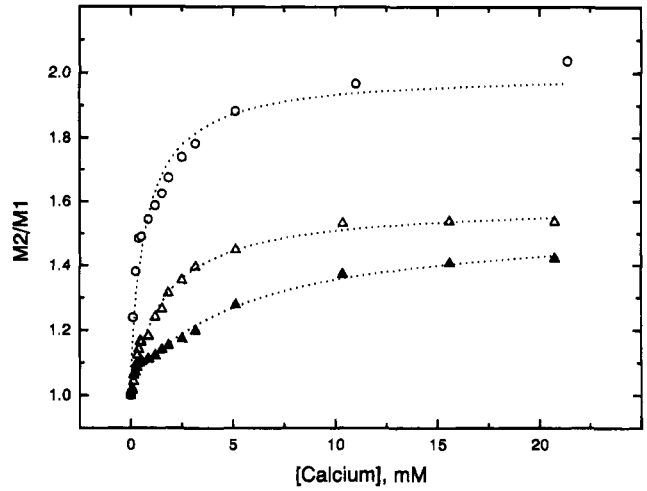

FIGURE 4: Effect of Ca^{2+} on the binding of r-PC to acidic PL [60%/40% (w/w) chicken egg phosphatidylcholine/bovine brain phosphatidylserine]. The molecular weights of the protein/PL complexes were determined by 90° relative light scattering. The $C_{50,Ca}$-PL was calculated by nonlinear least-squares 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 (6 μg/mL in phosphate) vesicle suspension. The buffer for these experiments was 20 mM Tris-HCl/100 mM NaCl, pH 7.4, at 20 °C. Excitation and emission wavelengths of 320 nm and slit widths of 4 nm were used. Symbols: (O) wtr-PC; (△) [F^{4}Q]r-PC; (▲) [L^{8}Q]r-PC.

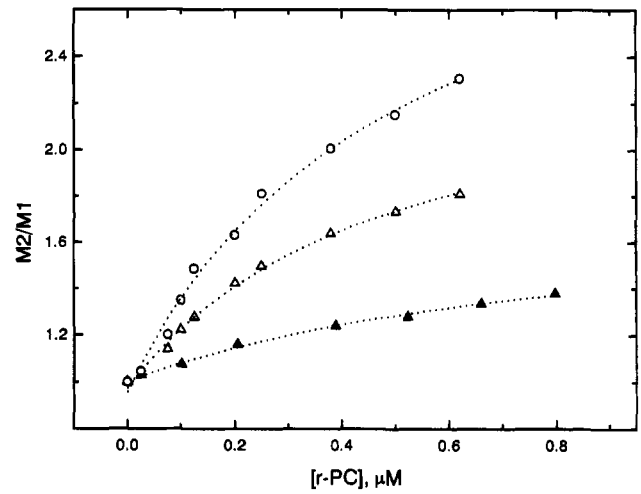

FIGURE 5: Measurement of the binding of r-PC to acidic PL [60%/40% (w/w) chicken egg phosphatidylcholine/bovine brain phosphatidylserine] in the presence of Ca^{2+}. The dependence on protein concentrations of the interaction of r-PC mutants with PL (6 μg/mL) in the presence of 2 mM $CaCl_2$. The molecular weights of the protein/PL complexes were determined by 90° 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 M_2/M_1 to float during the iterations. The buffer for these experiments was 20 mM Tris-HCl/100 mM NaCl/2 mM $CaCl_2$, pH 7.4, at 20 °C. Symbols: (O) wtr-PC; (△) [F^{4}Q]r-PC; (▲) [L^{8}Q]r-PC.

[F^{4}Q]r-PC displayed a $C_{50,Ca}$-PL that was nearly the same as that of wtr-PC, this same value for [L^{8}Q]r-PC was nearly 6-fold greater. In both cases, it also appeared that the extent of maximum binding of the Ca^{2+}/mutant protein complexes to the PL vesicles was less than that of wtr-PC. Similar titrations with protein at two different concentrations of Ca^{2+}, *viz.*, 2 and 20 mM, were also conducted, and an example of the data obtained at 2 mM Ca^{2+} is illustrated in Figure 5. The $C_{50,PC}$-PL values found, summarized in Table 1, demonstrated that the binding of [L^{8}Q]r-PC to PL was greatly

strengthened at high concentrations of Ca^{2+}, similar to previous results for [L^5Q]r-PC (Zhang & Castellino, 1994). However, for wtr-PC and [F^4Q]r-PC, this same binding interaction was not affected in this manner by high Ca^{2+} concentrations, and the $C_{50,PC}$-PL values were nearly the same for these latter two proteins at each of the two Ca^{2+} concentrations.

Finally, PL-dependent assays of the corresponding r-APC mutants were conducted. The results are summarized in Table 1. In the cases of plasma-based prolongation of the APTT activity of PC-deficient plasma, we found that very low anticoagulant activities were present in [F^4Q]r-APC and [L^8Q]r-APC. The activities improved somewhat when assays were conducted in purified systems with either fV or fVIIIa (Figure 6) as the substrates. However, the highest activity noted in these cases was no greater than 30% of that of wtr-APC. These results demonstrated that highly deleterious effects on the Ca^{2+}/PL-dependent activity of r-APC occurred as a result of alteration to hydrophilic side chains of two hydrophobic amino acid residues, *viz.*, F^4 and L^8. These amino acids are well conserved in their hydrophobic character in proteins of this type and are present in the hydrophobic cluster situated at the amino terminus of GD_{PC}. A similar observation has been previously reported for L^5, a rigidly conserved amino acid in this same location of PC (Zhang & Castellino, 1994).

DISCUSSION

The modeled structure of the Ca^{2+}/GD_{PC} complex (Figure 7) clearly shows the presence of two separate clusters of hydrophobic amino acid residues, one consisting of F^4, L^5, and L^8, all of which are exposed to solvent. Another more internalized group exists that contains most of the remaining hydrophobic amino acids of GD_{PC} and HS_{PC}. The placement and grouping of the former three amino acids suggest that they are functionally important, and the most logical role for these residues, especially considering their high degree of solvent exposure, appeared to be one of involvement in binding to substrates, inhibitors, or macromolecular effectors. As a consequence of an investigation with [L^5Q]r-PC, a mutant protein in which a hydrophilic residue was substituted for one that is hydrophobic, we proposed that the function of this amino-terminal cluster of residues was to contribute to hydrophobic binding energies of these proteins in the functional PC/PL interaction (Zhang & Castellino, 1994), in a manner exemplified by the model depicted in Figure 7. In order to test this hypothesis more rigorously, we constructed mutations at the two remaining amino acids of this cluster that were not previously examined, F^4 and L^8. Once again, we believed that the most effective conclusions could be drawn were the mutations as simple as possible, but would also allow replacement of a hydrophobic with a hydrophilic amino acid side chain. Thus, each of these two amino acids was altered to Gln.

Both mutant proteins, *viz.*, [F^4Q]r-PC and [L^8Q]r-PC, were effectively expressed and characterized. Under the conditions of our expression methodology in 293 human kidney cells, we routinely obtained 1−2 mg of each of these proteins/L of media, which was readily purified in the same manner as we have published with a number of other variants of r-PC (Christiansen & Castellino, 1994; Christiansen et al., 1995; Yu et al., 1994; Zhang & Castellino, 1990, 1991, 1994; Zhang et al., 1992). $NaDodSO_4$/PAGE gels, under

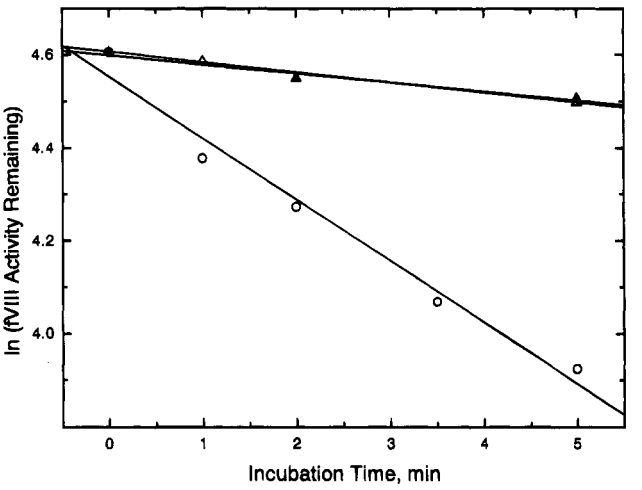

FIGURE 6: Rate of inactivation of human fVIII by r-APC. The r-APC mutants and wtr-APC were present at the same amidolytic activities. The buffer contained 20 mM Hepes−NaOH/150 mM NaCl, pH 7.4, at 37 °C. A solution of fVIII (5.6 nM, final concentration) was incubated with r-APC or the r-APC mutant protein (*ca.* 0.5 nM, final concentration) in the presence of (final concentrations) PL (40 μM) and Ca^{2+} (2.5 mM). Following this, fIXaβ (6.5 nM) and fIIa (0.1 unit/mL) were added to activate the remaining fVIII. The amount of fVIIIa present was assayed in the complete fX *in vitro* activation system. In this phase of the assay, the fVIIIa solution was added to a solution containing final concentrations of 40 μM (in phosphate) PL, 2.5 mM $CaCl_2$, and 180 μM fXa chromogenic substrate, S2222. The final volume was 790 μL. After the baseline was recorded for 1 min, substrate hydrolysis was accelerated by addition of 10 μL of a solution of 30 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 was plotted as a function of the incubation times of fVIII with the particular r-APC mutant, 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 mutant proteins. A control incubation of fVIII with r-PC in place of r-APC demonstrated that the loss of activity of fVIII was <10% over the time period illustrated on the graph. Symbols: (○) wtr-APC; (△) [F^4Q]r-APC; (▲) [L^8Q]r-APC.

reducing and nonreducing conditions (Figure 1), indicated that the proteins obtained were mainly present in their proper two-chain forms. The doublet band present in the nonreduced PC mutant proteins and in the corresponding heavy chains of their reduced forms is ascribed to glycoforms of the proteins (Yan et al., 1990). These data show that normal processing had occurred in the mutant proteins, a fact that is further emphasized by the findings that Gla and Hya were present in the expected amounts (Table 1).

The initial feature of interest in these mutants was to determine their overall abilities to effectively utilize Ca^{2+} and PL for stimulation of their biologically relevant activities. After complete activation of the r-PC mutant proteins to their correspondiong r-APC forms, *ex vivo* plasma-based APTT assays were conducted. The data obtained (Table 1) showed that these anticoagulant activities of the mutant proteins were not greater than 5% of that of wtr-APC. This loss of biological activity in the mutants must be considered with recognition of the fact that all of these enzymes have been adjusted to the same amidolytic activities in the assays (in these cases, the protein concentrations were also the same within *ca.* 10%, since the specific amidolytic activities of these enzymes were approximately equal to that of wtr-APC). Thus, the loss of activity in the mutants is not due to a

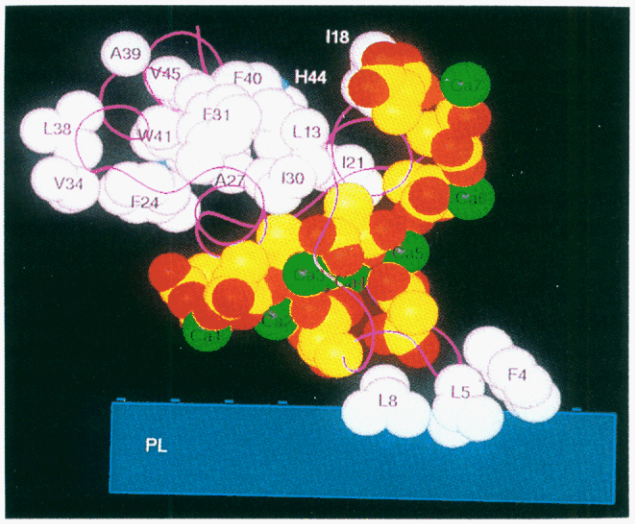

FIGURE 7: Model of the Ca^{2+}/GD_{PC} structure. Highlighted are two clusters of hydrophobic residues, the side chains of which are displayed as pink spheres. One grouping consists of F^4, L^5, and L^8, and the other cluster consists of the remaining hydrophobic amino acids in the GD_{PC}/HS_{PC} region. The amino acids are displayed beginning with their β-carbon atoms onward. The polypeptide backbone is illustrated as a thin magenta line. Peptide bonds are not shown. Gla residue side-chain carbon atoms are in yellow, while their corresponding oxygen atoms are displayed in red. The Ca^{2+} ions that interact with the GD_{PC} are shown in green. The blue design at the bottom of the figure represents the acidic PL vesicle.

defective active site but is a property of a malfunctional exo site(s). That these latter regions most probably involve Ca^{2+} and/or PL sites is seen by the decreased activity of these same mutants in the *in vitro* inactivation of fV and fVIII in systems dependent on Ca^{2+} and PL (Table 1). While these latter activity losses were not quite as dramatic as those observed in the plasma-based APTT assays, a finding similar to that seen with several other mutants of r-PC (Christiansen & Castellino, 1994; Jhingan et al., 1994), they nonetheless indicated that defects existed in the abilities of the mutant proteins to form functional complexes with Ca^{2+} and PL.

PC, along with all other vitamin K-dependent coagulation proteins, undergoes a Ca^{2+}-directed conformational change that is requisite to PL binding. This Ca^{2+}-induced alteration can be monitored by the accompanying changes that occur in the intrinsic fluorescence of the proteins (Nelsestuen, 1976). In order to determine whether [F^4Q]r-PC and [L^8Q]-r-PC were capable of adopting Ca^{2+}-dependent conformations, titrations with Ca^{2+} of the intrinsic fluorescence changes in these proteins were conducted. The data of Figure 2 and Table 1 show that the Ca^{2+}-induced conformational transition occurred in both [F^4Q]r-PC and [L^8Q]r-PC, with $C_{50,Ca}$-Fl and maximal fluorescence values very similar to those of wtr-PC. This same observation has been previously reported for [L^5Q]r-PC (Zhang & Castellino, 1994) and shows that the alterations made at these residues do not substantially influence the abilities of the mutant proteins to undergo the Ca^{2+}-induced changes characteristic of wtr-PC.

Two MAbs available for these studies were potentially enlightening with regard to Ca^{2+}-dependent transitions in the GD of the PC mutants. One of these, MAb-7D7B10, is known to preferentially interact with the Ca^{2+}-free form of PC, with the epitope localized to the amino terminus of GD_{PC} (Orthner et al., 1989). Neither [F^4Q]r-PC, [L^5Q]r-PC, nor [L^8Q]r-PC interacted with this antibody on Western immunoblots, and all were incapable of interfering with the interaction of this MAb with insolubilized wtr-PC under conditions where solution-phase wtr-PC competed effectively for binding of the antibody to wtr-PC adsorbed on microtiter plates (Figure 3). Thus, both of the wild-type amino acids of r-PC, *viz.*, F^4 and L^8, along with L^5 studied earlier, appear essential for the epitope for MAb-7D7B10. These results allow more precise identification of the amino acid residues required for full expression of the epitope on the antigen for the antibody.

Antibody MAb, JTC1, possesses a Ca^{2+}-dependent epitope within the amino terminus of GD_{PC} (Wakabayashi et al., 1986). We have previously employed this MAb extensively to study the formation of Ca^{2+}-induced transitions in GD_{PC} (Christiansen & Castellino, 1994; Christiansen et al., 1995; Yu et al., 1994; Zhang & Castellino, 1992, 1994). Similar to the situation with MAb-7D7B10, the Ca^{2+}/[F^4Q]r-PC complex did not recognize this antibody, despite the evidence from intrinsic fluorescence studies suggesting that the Ca^{2+}-dependent conformation had developed properly in this mutant. On the other hand, as was the case with [L^5Q]r-PC (Zhang & Castellino, 1994), [L^8Q]r-PC reacted weakly with this MAb in the presence of Ca^{2+}. Even this low reactivity nonetheless allowed a Ca^{2+} titration to be conducted of MAb binding to the mutant protein, thus providing the $C_{50,Ca}$ for the [L^8Q]r-PC/JTC1 interaction. The value obtained, 6.5 mM, was very similar to that for the Ca^{2+} dependencies of the wtr-PC/JTC1 (4.5 mM) and [L^5Q]r-PC/JTC1 (6.0 mM) interactions, again demonstrating that a proper Ca^{2+}-induced conformation occurred in the [L^8Q]r-PC mutant. Thus, the weak interaction of the Ca^{2+}/[L^8Q]r-PC complex with JTC1 is most probably due to elimination of an essential amino acid in the epitope and not to defects in formation of the Ca^{2+}-induced conformation of [L^8Q]r-PC. This is supported by the conclusions drawn from the intrinsic fluorescence titrations. Similar conclusions have been forwarded for these types of data that were obtained with [L^5Q]r-PC as the antigen (Zhang & Castellino, 1994) and show that the epitope for JTC1 is located in the region of the protein spanned at the minimum by F^4, L^5, and L^8, with F^4 being an essential amino acid for MAb binding and L^5 and L^8 also being most important in this regard.

The ability of Ca^{2+} to induce binding of the mutant proteins to acidic PL has been examined by titrations with Ca^{2+} of the protein/PL interaction at constant protein concentrations (Figure 4). The $C_{50,Ca}$-PL value obtained for the [F^4Q]r-PC/PL interaction was nearly the same as that for wtr-PC (Table 1), but this same value for the [L^8Q]-r-PC/PL interaction was considerably higher. This shows a more defective overall interaction with PL of [L^8Q]r-PC than of [F^4Q]r-PC, with the characteristics of the former in this regard being more similar than the latter to that of [L^5Q]-r-PC (Zhang & Castellino, 1994).

The data of Figure 4 also indicate that maximal M_2/M_1 values for [F^4Q]r-PC and [L^8Q]r-PC were smaller than that for wtr-PC, suggesting that the Ca^{2+}/mutant protein complexes interacted more weakly with PL than did wtr-PC or that they bound differently to PL as compared to wtr-PC. This was also the case for [L^5Q]r-PC/PL binding to these types of vesicles (Zhang & Castellino, 1994). In order to probe this point more rigorously, titrations of PL with the mutant proteins were conducted at a constant amount of Ca^{2+}. At a concentration of 2 mM Ca^{2+} (Figure 5), the value of $C_{50,PC}$-PL for [L^8Q]r-PC/PL binding was approximately 15-fold higher than that same parameter for wtr-PC/PL binding,

whereas that for [F⁴Q]r-PC binding was nearly the same as that for the wtr-PC/PL interaction. Even in this latter case, however, the maximal M_2/M_1 for [F⁴Q]r-PC was somewhat less than that for wtr-PC, despite the fact that the $C_{50,Ca}$-PL values for [F⁴Q]r-PC and wtr-PC were approximately equal. This demonstrates that there are differences in the nature of the binding to PL of this mutant, as compared to its wild-type counterpart, perhaps in orientation of this protein on PL. Such differences may lead to a decrease in the maximal amounts of protein bound to the lipid. These variations, while subtle, may nonetheless explain the activity loss of [F⁴Q]r-APC in Ca^{2+}/PL-based assays with biologically relevant protein substrates.

Upon increase of the Ca^{2+} concentration to 20 mM Ca^{2+}, the $C_{50,PC}$-PL that is characteristic of the wtr-PC/PL and [F⁴Q]r-PC/PL interactions did not substantially change. This indicates that the binding properties of these two proteins were not influenced at high concentrations of Ca^{2+}. However, in the case of [L⁸Q]r-PC, this same binding parameter was reduced to only 2.5-fold the value of wtr-PC at this higher concentration of Ca^{2+}. As indicated by the lower $C_{50,PC}$-PL value for the [L⁸Q]r-PC/PL interaction, it appears that this binding became tighter at higher Ca^{2+} concentrations, as was also found with [L⁵Q]r-PC (Zhang & Castellino, 1994). This shows that high Ca^{2+} concentrations can induce stronger binding for this mutant protein toward PL. This finding reflects a significant difference between [L⁸Q]r-PC and wtr-PC and is in keeping with these same results for [L⁵Q]r-PC (Zhang & Castellino, 1994). These data suggest that [L⁸Q]r-PC lacks a component that strengthens its binding to PL and that at high Ca^{2+} concentrations a different type of Ca^{2+}-induced binding occurs, perhaps utilizing Ca^{2+} sites not revealed by intrinsic fluorescence changes, which leads to a loss of Ca^{2+}/PL-based activity toward biological substrates.

We thus conclude that the cluster of surface-exposed hydrophobic residues at the amino terminus of GD_{PC} plays a major role of defining PL binding and PL-based activities of the relevant r-APCs. L⁵ and L⁸ are critical amino acids in this regard. Substitution of hydrophilic residues at these locations results in altered PL binding and nearly complete activity losses of [L⁵Q]r-APC and [L⁸Q]r-APC toward natural substrates. Both of these leucine residues are needed for binding stability to PL and also likely for proper orientation of the enzymes on the PL surface. The role of F⁴ in this regard is less important and even more subtle, and indications of its influence could be due to the fact that a more dramatic mutation was needed to define its role. Finally, it appears that proper functional Ca^{2+}-dependent binding of PC to PL is not only a property of the component Gla residues within GD_{PC} but also a property of hydrophobic side chains predicted from analysis of the model of the GD_{PC}/Ca^{2+} complex to be important with regard to PC/APC binding properties. We believe that significant hydrophobic components are present in the binding of PC/APC to PL and are likely centered in L⁵ and L⁸, with some lesser contribution of F⁴.

REFERENCES

Amphlett, G. W., Byrne, R., & Castellino, F. J. (1979) *J. Biol. Chem. 254*, 6333−6336.

Amphlett, G. W., Kisiel, W., & Castellino, F. J. (1981) *Biochemistry 20*, 2156−2161.

Atkins, J. S., & Ganz, P. R. (1992) *Mol. Cell. Biochem. 112*, 61−71.

Beals, J. M., & Castellino, F. J. (1986) *Biochem. J. 236*, 861−869.

Beckmann, R. J., Schmidt, R. J., Santerre, R. F., Plutzky, J., Crabtree, G. R., & Long, G. L. (1985) *Nucleic Acids Res. 13*, 5233−5247.

Chibber, B. A. K., Urano, S., & Castellino, F. J. (1990) *Int. J. Pept. Protein Res. 35*, 73−80.

Christiansen, W. T., & Castellino, F. J. (1994) *Biochemistry 33*, 5901−5911.

Christiansen, W. T., Tulinsky, A., & Castellino, F. J. (1994) *Biochemistry 33*, 14993−15000.

Christiansen, W. T., Geng, J.-P., & Castellino, F. J. (1995) *Biochemistry 34*, 8087−8090.

De Serrano, V. S., & Castellino, F. J. (1992) *Biochemistry 31*, 3326−3335.

De Serrano, V. S., Menhart, N., & Castellino, F. J. (1992) *Arch. Biochem. Biophys. 294*, 282−290.

Drakenberg, T., Fernlund, P., Roepstorff, P., & Stenflo, J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1802−1806.

Esmon, N. L., Owen, W. G., & Esmon, C. T. (1982) *J. Biol. Chem. 257*, 859−864.

Foster, D. C., Yoshitake, S., & Davie, E. W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4673−4677.

Foster, D. C., Sprecher, C. A., Holly, R. D., Gambee, J. E., Walker, K. M., & Kumar, A. A. (1990) *Biochemistry 29*, 347−354.

Grinnell, B. W., Berg, D. T., Walls, J., & Yan, S. B. (1987) *Bio/Technology 5*, 1189−1192.

Heeb, M. J., Schwartz, P., White, T., Lammle, B., Berrettini, M., & Griffin, J. (1988) *Thromb. Res. 52*, 33−43.

Jhingan, A., Zhang, L., Christiansen, W. T., & Castellino, F. J. (1994) *Biochemistry 33*, 1869−1875.

Kisiel, W. (1979) *J. Clin. Invest. 64*, 761−769.

Kisiel, W., Canfield, W. M., Ericsson, L. H., & Davie, E. W. (1977) *Biochemistry 16*, 5824−5831.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.

Lowry, D. H., & Lopez, J. A. (1946) *J. Biol. Chem. 162*, 421−428.

Menhart, N., Sehl, L. C., Kelley, R. F., & Castellino, F. J. (1991) *Biochemistry 30*, 1948−1957.

Meyback, B., Helm, J., Rink, H., Zimmerman, W., & Maerki, W. (1987) *Thromb. Res. 7* (Suppl.), 33.

Nelsestuen, G. L. (1976) *J. Biol. Chem. 251*, 5648−5656.

Nelsestuen, G. L., & Broderius, M. (1977) *Biochemistry 16*, 4172−4177.

Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164−4171.

Orthner, C. L., Madurawe, R. D., Velander, W. H., Drohan, W. N., Battey, F. D., & Strickland, D. K. (1989) *J. Biol. Chem. 264*, 18781−18788.

Reitsma, P. H., Poort, S. R., Bernardi, F., Gandrille, S., Long, G. L., Sala, N., & Cooper, D. N. (1993) *Thromb. Haemostasis 69*, 77−84.

Soriano-Garcia, M., Padmanabhan, K., deVos, A. M., & Tulinsky, A. (1992) *Biochemistry 31*, 2554−2566.

Vehar, G. A., & Davie, E. W. (1980) *Biochemistry 19*, 401−410.

Wakabayashi, K., Sakata, Y., & Aoki, N. (1986) *J. Biol. Chem. 261*, 11097−11105.

Walker, F. J. (1980) *J. Biol. Chem. 255*, 5521−5524.

Yan, S. C. B., Pazzano, P., Chao, Y. B., Walls, J. D., Berg, D. T., McClure, D. B., & Grinnell, B. W. (1990) *Bio/Technology 8*, 655−661.

Yu, S., Zhang, L., Jhingan, A., Christiansen, W. T., & Castellino, F. J. (1994) *Biochemistry 33*, 823−831.

Zhang, L., & Castellino, F. J. (1990) *Biochemistry 29*, 10828−10834.

Zhang, L., & Castellino, F. J. (1991) *Biochemistry 30*, 6696−6704.

Zhang, L., & Castellino, F. J. (1992) *J. Biol. Chem. 267*, 26078−26084.

Zhang, L., & Castellino, F. J. (1983) *J. Biol. Chem. 268*, 12040−12045.

Zhang, L., & Castellino, F. J. (1994) *J. Biol. Chem. 269*, 3590−3595.

Zhang, L., Jhingan, A., & Castellino, F. J. (1992) *Blood 80*, 942−952.

BI950816Q