Highly conserved residue arginine-15 is required for the Ca^{2+} -dependent properties of the γ -carboxyglutamic acid domain of human anticoagulation Protein C and activated Protein C

Abraham THARIATH and Francis J. CASTELLINO*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556, U.S.A.

The function of the rigidly conserved amino acid residue R^{15} in the Ca^{2+} /phospholipid-dependent properties of the γ -carboxyglutamic acid (Gla)-containing domain (GD) of human Protein C (PC) were investigated through site-directed mutagenesis strategies. A series of recombinant (r) mutants, namely r-[R^{15}K]PC, r-[R^{15}H]PC, r-[R^{15}L]PC, and r-[R^{15}W]PC, were constructed, expressed and purified, and their relevant properties investigated. As revealed by intrinsic fluorescence analysis, all of the variant proteins underwent Ca^{2+} -dependent structural transitions. Nonetheless, they displayed altered binding properties to acidic phospholipid vesicles, and also did not interact with a monoclonal antibody specific for the type of Ca^{2+} -dependent conformation of the GD that characterizes the wild-type protein. On conversion into their activated forms, these variant enzymes possessed less than 10% of the *ex vivo* plasma anticoagulant

activity of wild-type r-PC. Similar activities were found when the r-active PC mutants were assayed directly for inactivation of factor Va and factor VIII, in the complete prothrombinase and tenase complexes respectively. We conclude that R^{15} is a critical residue in allowing the GD of PC, and probably of other proteins of this class, to adopt a Ca^{2+} -dependent conformation that allows functional phospholipid binding, thus explaining the strict conservation of this amino acid residue in GD modules of various proteins. As a result of an analysis of structural models of the Ca^{2+} -GD complex of PC, it is postulated that hydrogen bonds between the side chain of R^{15} and the functionally important Gla^{16} residue, as well as between the side chain of R^{15} and the carbonyl oxygen in the peptide bond of H^{10} , are critical for adoption of a Ca^{2+} -dependent conformation of the GD that allows functional phospholipid binding.

INTRODUCTION

Protein C (PC) is the plasma protein precursor of the anti-coagulant enzyme, active PC (APC), a serine protease. APC functions maximally with Ca^{2+} , phospholipid and another protein, Protein S, to inactivate by limited proteolysis coagulation factor V/activated coagulation factor (f) V (fV/fVa) [1] and fVIII/fVIIIa [2], thereby inhibiting thrombin formation in the coagulation cascade. The activation of PC to APC is catalysed by thrombin [1] or thrombin/thrombomodulin [3] and results from cleavage of a single peptide bond ($\text{R}^{169}\text{-L}^{170}$) in the proenzyme. Ca^{2+} inhibits the activation of PC by thrombin [4] and thrombin/thrombomodulin [5].

PC contains a domain organization [6] that is similar to those of the coagulation proteins factor VII [7], factor IX [8] and factor X [9]. In these cases, a module consisting of a propeptide/ γ -carboxyglutamic acid (Gla)-rich region (GD) is followed consecutively by a short helical stack (approx. eight residues), two epidermal growth factor-homology units, a specific activation peptide region and two exons encoding the serine protease chain. The non-protease chains of the enzyme forms of these proteins are mainly involved with the regulation of their activities by binding of effectors, and such sites of interactions seem to be confined to individual domains. In general the Gla-containing region is responsible for binding of the proteins to Ca^{2+} -phospholipid [10–12], and in factor IX the GD also contains

specific endothelial cell [13–15] and platelet [16] receptor-binding sites; the helical stack seems to function as a spacer for separation of the active site from the membrane surface, and the epidermal growth factor-like domains are mainly involved with functional higher affinity interactions of these enzymes with a Ca^{2+} [17,18] ion and with their protein cofactors [19–23].

The residue corresponding to R^{15} in human PC is rigidly conserved in the GDs of all proteins of this type. Also, symptomatic patients have been described with R^{15}G [24], R^{15}Q [25] and R^{15}W [25] mutations, further emphasizing the biological relevance of the integrity of this residue in the Gla-rich region of PC (residues 1–37) (GD_{PC}). To understand more fully the importance of this residue in the ability of the GD_{PC} to display its proper Ca^{2+} -phospholipid-dependent properties, we constructed a series of mutations at this residue and evaluated the structure–function consequences of these changes in PC and APC. The results of this study are presented here.

EXPERIMENTAL

Proteins

All forms of recombinant (r)-PC were expressed in human kidney 293 cells and isolated by fast-flow Q chromatography as published previously [26]. The corresponding r-APCs were prepared from the appropriate zymogens by activation with the

Abbreviations used: [$\text{C}_{50,\text{Ca}}\text{-F}$], total Ca^{2+} concentration required to alter the intrinsic fluorescence in half of the protein molecules; [$\text{C}_{50,\text{Ca}}\text{-PL}$], total Ca^{2+} concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of protein; fV (fVa), (activated) coagulation factor V; fVIII (fVIIIa), (activated) coagulation factor VIII; GD, Gla-containing domain; GD_{PC} , Gla-rich region of PC (residues 1–37); Gla, γ -carboxyglutamic acid; mAb, monoclonal antibody; PC (APC), (activated) Protein C; PL, 60%/40% (w/w) sonicated dispersion of chicken egg phosphatidylcholine/bovine brain phosphatidylserine, 20 μM in phosphate; r, recombinant; S2222, benzoyl-Ile-Glu(OR)-Gly-Arg-*p*-nitroanilide; S2238, H-D-Phe-L-pipecolyl-Arg-*p*-nitroanilide; wt, wild-type.

* To whom correspondence should be addressed.

venom protease, Protac C (American Diagnostica, New York, NY, U.S.A.), as described earlier [26] or with thrombin in the absence of Ca^{2+} .

Human plasma fVa was purchased from Hematologic Technologies (Burlington, VT, U.S.A.), and r-fVIII was a gift from the Genetics Institute (Cambridge, MA, U.S.A.). Human thrombin, factor IX and factor X were provided by Enzyme Research Laboratories (South Bend, IN, U.S.A.). Bovine factor IXa β was obtained by activation of bovine factor IX [27].

Monoclonal antibodies (mAbs) were radiolabelled with ^{125}I by the Iodobead (Pierce Chemical Company, Rockford, IL, U.S.A.) procedure as described previously [28]. For these studies the Ca^{2+} -dependent mAb JTC1 [29] was provided by Dr. K. Wakabayashi (Tokyo, Japan). mAb-C3, specific for a Ca^{2+} -independent epitope within the first epidermal growth factor-like domain [30], which was used for screening PC antigen in transfected 293 cell colonies, was a gift from Dr. J. Griffin (La Jolla, CA, U.S.A.).

Restriction endonucleases were purchased from the Promega Corporation (Madison, WI, U.S.A.).

Lipids

Phospholipid vesicles were prepared from mixtures (60/40, w/w) of chicken egg phosphatidylcholine/bovine brain phosphatidylserine [31]. The PL vesicle concentration was determined as total organic phosphate with use of a conversion factor of 25 (w/w) to obtain the weight concentrations of the PL vesicles [32].

cDNA species

The properties of the cDNA coding for recombinant wild-type (wtr-PC (p119[PC])) have been provided previously [26].

The mutant cDNA species required for amino acid substitutions at position 15 of PC were constructed by primer-directed mutagenesis, with synthetic oligonucleotides on single-strand templates of p119[PC] [33]. For the [R¹⁵L] mutation, single-strand wtr-PC was used as the template with the following mutagenic primer (the variant bases are indicated by lower-case lettering): wtr-PC, 5'-AGC CTG GAG CGG GAG TGC ATA GAG-3'; r-[R¹⁵L]PC, 5'-AGC CTG GAGCtc GAG TGC ATA GAG-3'. The transformants were screened for the creation of a *SacI* restriction endonuclease site (underlined).

The R¹⁵K and R¹⁵W mutations were constructed in the same manner from the following two primers on the template of wtr-PC: wtr-PC, 5'-C AGC AGC CTG GAG CGG GAG TGC ATA GAG G-3'; r-[R¹⁵K]PC, 5'-C AGC AGC CTG GAG aaG GAG TGC ATA GAG G-3'; r-[R¹⁵W]PC, 5'-C AGC AGC CTG GAG tgG GAG TGC ATA GAG G-3'. Both transformants were screened for the mutations through loss of the naturally occurring *BsrBI* restriction site in the wild-type cDNA (shown in bold).

The final mutation, [R¹⁵H], was engineered into the single-strand template of r-[R¹⁵L]PC by using the following primer: r-[R¹⁵L]PC, 5'-C CTG GAGCTC GAG TGC ATA GAG-3'; r-[R¹⁵H]PC, 5'-C CTG GAG CaC GAG TGC ATA GAG-3'. These transformants were screened by loss of the *SacI* restriction site (underlined above) that had previously been placed in this mutant.

In all cases the substitutions were confirmed by nucleotide sequence analyses through the mutated regions.

Intrinsic fluorescence titrations

The ability of Ca^{2+} to induce adoption of 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 [28]. A SLM-Aminco 8100 recording spectrofluorimeter (SLM-Aminco Instruments, Urbana, IL, U.S.A.) was used for this purpose.

mAb binding

Binding of the [^{125}I]-mAbs to wtr-PC and to r-PC variants was determined by microtitre plate assays, as described previously [28].

Interaction of r-PC with acidic PL

The binding characteristics of r-PC variants to acidic PL were measured at 25 °C by 90° relative light-scattering techniques [11,34]. Our exact procedures have been published [35].

r-APC assays

Plasma-based activated partial thromboplastin time anti-coagulant assays *ex vivo* with PC-deficient human plasma, as well as fVIII inactivation assays *in vitro* in the complete tenase system, were used to determine the activities of the r-APC mutants. Our methodologies have been published [36,37].

An assay for the APC-catalysed inactivation of fV/fVa was also designed by using the complete prothrombinase complex in a two-stage assay. Here APC was employed to inactivate fVa in step 1, and this mixture was used to accelerate prothrombin activation in step 2. The amount of thrombin generated was detected by hydrolysis of the chromogenic substrate *H*-D-Phe-L-pipecolyl-Arg-*p*-nitroanilide (S2238) in a continuous assay. The amount of wtr-APC employed in stage 1 of this assay was based on its amidolytic activity. To determine an effective level of APC for this assay, the enzyme was assayed against the chromogenic substrate L-pyro-Glu-Pro-Arg-*p*-nitroanilide (S2366), and the stock concentration of the APC was then adjusted such that a rate of 0.002 absorbance unit at 405 nm/min per μl of APC was obtained. Equal volumes (5 μl) of the APC and the fVa stock (1 mg/ml) solutions were then mixed in a suspension containing (final concentrations) PL in a buffer of 20 mM Na/Hepes (pH 7.4 at 37 °C)/150 mM NaCl/2.5 mM CaCl_2 (final volume 15 μl).

At various times of incubation of APC with fVa, 10 μl of the solution was incubated with the prothrombinase complex, containing (final concentrations) bovine prothrombin (0.1 mg/ml), PL (20 μM), CaCl_2 (2.5 mM) and substrate S2238 (180 μM) in the above buffer. The reaction was accelerated by the addition of factor Xa (final concentration 8 nM), and the thrombin-catalysed hydrolysis of S2238 was monitored continuously at 405 nm. The slope of the maximal rate (observed after a short lag time) was plotted against the time of incubation of fVa with APC in stage 1, and the inactivation rate constant was determined in the usual manner for wtr-APC and the mutant enzymes.

Analytical methods

Methods for all DNA manipulations required for these studies have been described in previous publications [38–41]. Protocols for Glu and β -hydroxyaspartic acid analyses [26], as well as for determinations of the N-terminal amino acid sequences of the mutant proteins [42] and Western immunoblotting [26], have been described in the indicated publications.

RESULTS

A series of mutations were constructed in the cDNA of human PC at the location encoding R¹⁵, and the mutant proteins were expressed and purified. SDS/PAGE chromatograms of non-reduced and reduced samples of all mutant proteins were obtained (results not shown). In all cases, appropriate disulphide-linked two-chain proteins were present primarily, with less than 10% in single-chain forms. The usual doublet bands were present in the non-reduced proteins and in the heavy chains of the reduced samples, owing to the presence of PC glycoforms [43]. This is a characteristic of all r-PC mutants prepared so far [30,36,44–46]. Additional characterizations of the variant proteins demonstrated that the N-terminal amino acid sequence was correct at least up to the mutated residue and that the mutant proteins contained 95–106% of the Glu content of wtr-PC, which was found to be 8.9 mol/mol of PC (theoretical value 9.0 mol/mol of PC). Similarly the β -hydroxyaspartic acid contents of the variants ranged between 0.7 and 1.1 mol/mol of PC (theoretical value 1.0 mol/mol of PC).

The ability of the r-PC variants to adopt their bivalent-cation-dependent conformations was examined by assessment of Ca²⁺-induced intrinsic fluorescence quenching of the r-PC variants. A representative set of data of titrations with Ca²⁺ of the intrinsic fluorescence of wtr-PC and r-[R¹⁵K]PC is illustrated in Figure 1. The total Ca²⁺ concentrations required to alter the intrinsic fluorescence in half of the protein molecules ($[C_{50, Ca} - FI]$ values) and maximal fluorescence changes were similar for these two proteins. All mutants behaved similarly to wtr-PC in these titrations, providing a range of $[C_{50, Ca} - FI]$ values spanning 0.19 to 0.24 $\pm$ 0.3 (S.D.) mM and maximal fluorescence quenching of 17–19%.

Further analysis of the Ca²⁺-induced conformation of the r-PC mutants was undertaken by determining the effects of Ca²⁺ on the binding to the variants of a Ca²⁺-dependent GD-directed mAb, JTC1. The results obtained are shown in Figure 2.

Figure 1 Effect of Ca²⁺ on the intrinsic fluorescence of PC

The results shown are for the Ca²⁺–wtr-PC (■) and Ca²⁺–r-[R¹⁵K]PC (●) interactions. The protein (approx. 1 μ M) was titrated with sequential additions of Ca²⁺, and the intrinsic fluorescence intensity was measured. The fluorescence (F_0) of the protein obtained in the absence of Ca²⁺ was adjusted to 1.0, and fluorescence values (F) were obtained after the addition of Ca²⁺. The percentage fluorescence change (quenching) was calculated as $100(F_0 - F)/F_0$ and plotted as a function of the total Ca²⁺ concentration. The $[C_{50, Ca} - FI]$ and ΔF_{max} (total fluorescence change at saturation with Ca²⁺) values were determined by non-linear least-squares iterative fitting of the data. The excitation and emission wavelengths were 283 and 340 nm respectively, with slit widths of 2 and 32 nm respectively. The buffer was 20 mM Tris/HCl (pH 7.4 at 25 °C)/100 mM NaCl.

Figure 2 Effects of the Ca²⁺ on the binding of mAb JTC1 to r-PC mutants at 25 °C

Incremental additions of Ca²⁺ were made to the proteins adsorbed on the wells of a microtitre plate in the presence of [¹²⁵I]-JTC1, and the amount of antibody bound was measured. The amount (in c.p.m.) of antibody bound to wtr-PC at 30 mM Ca²⁺ was assigned a value of 100%, and all other binding was expressed as a percentage of that value. The buffer for these experiments was 20 mM Tris/HCl (pH 7.4)/100 mM NaCl. Symbols: ■, wtr-APC; ○, r-[R¹⁵H]APC; ●, r-[R¹⁵K]APC; △, r-[R¹⁵L]APC; ▲, r-[R¹⁵W]APC.

Figure 3 Competitive displacement assay of the binding of mAb JTC1 to r-PC mutants at 25 °C

wtr-PC was adsorbed on the microtitre wells and incremental additions of mutant proteins were made in the presence of [¹²⁵I]-JTC1 and Ca²⁺ (20 mM). The amount of antibody bound to the adsorbed wtr-PC was then measured. The amount of antibody bound to wtr-PC at zero concentration of competing proteins was assigned a value of 100%, and antibody bound at any other competing protein concentration was expressed as a percentage of that value. The buffer for these experiments was 20 mM Tris/HCl (pH 7.4)/100 mM NaCl. Symbols: ■, wtr-APC; ○, r-[R¹⁵H]APC; ●, r-[R¹⁵K]APC; △, r-[R¹⁵L]APC; ▲, r-[R¹⁵W]APC.

This mAb recognized only wtr-PC and did not possess significant reactivity towards any of the mutant proteins. This lack of reactivity is further highlighted by the plots of Figure 3, in which only wtr-PC, but none of the mutant proteins, was able to compete for JTC1 binding to insolubilized wtr-PC at high levels of Ca²⁺. Thus, despite the fact that a Ca²⁺-dependent conformation was adopted by the variants, this conformation was apparently not recognized by the antibody.

The comparative abilities of the r-PC mutants to interact with PC in a Ca²⁺-dependent manner was examined by 90° light-scattering techniques. Plots of the increase in relative molecular masses of the PL–protein complexes as a function of addition of

Figure 4 Effect of Ca^{2+} on the binding of PC mutants to acidic PL at 20 °C

Solutions of Ca^{2+} were titrated into a protein (1 μM)/PL (6 $\mu\text{g}/\text{ml}$ in phosphate) vesicle suspension, and the resulting relative molecular masses of the protein/PL complexes ($M2/M1$) were determined by 90° relative light scattering. Excitation and emission wavelengths of 320 nm and slit widths of 4 nm were used. $[\text{C}_{50,\text{Ca}}\text{-PL}]$ was calculated by iterative non-linear least-squares minimization of the data. The buffer for these experiments was 20 mM Tris/HCl (pH 7.4)/100 mM NaCl. Symbols: ■, wtr-PC; ○, r-[R¹⁵H]PC; ●, r-[R¹⁵K]PC; △, r-[R¹⁵L]PC; ▲, r-[R¹⁵W]PC.

Figure 5 Measurement of the binding of r-PC mutants to PL in the presence of Ca^{2+}

The dependence on protein concentration of the interaction of the proteins with PL (6 $\mu\text{g}/\text{ml}$) in the presence of 20 mM CaCl_2 was evaluated. The relative molecular masses of the r-PC/PL complexes ($M2/M1$) were determined by relative light scattering after subtraction of the light scattering of the unbound r-PC. Excitation and emission wavelengths of 320 nm and slit widths of 4 nm were used. $[\text{C}_{50,\text{Ca}}\text{-PC}]$ was calculated by iterative non-linear least-squares minimization of the data. The buffer was 20 mM Tris/HCl (pH 7.4)/100 mM NaCl. Symbols: ■, wtr-PC; ○, r-[R¹⁵H]PC; ●, r-[R¹⁵K]PC; △, r-[R¹⁵L]PC; ▲, r-[R¹⁵W]PC.

Ca^{2+} to the PL/protein mixture are shown in Figure 4. The total Ca^{2+} concentrations required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of protein ($[\text{C}_{50,\text{Ca}}\text{-PL}]$ values) obtained from these plots were 1.9 ± 0.2 , 5.5 ± 0.4 , 5.0 ± 0.3 , 4.4 ± 0.4 and 5.6 ± 0.4 mM for wtr-PC, r-[R¹⁵H]PC, r-[R¹⁵K]PC, r-[R¹⁵L]PC and r-[R¹⁵W]PC respectively. Thus, in addition to the lower $M2/M1$ [molecular-mass ratio of (PL + protein)/PL] values obtained at saturation for each of the mutant proteins than for wtr-PC, an approximate 2-fold increase in the Ca^{2+} concentration required for half-maximal binding was observed. This suggests that altered binding of the mutant proteins to PL occurred as a result of the alteration at R¹⁵. Confirmation of this altered PL

Figure 6 Anticoagulant activity of APC

Assay of the overall anticoagulant activity of r-APC mutants, each present at the same amidolytic activity, by using activated partial thromboplastin times with PC-deficient human plasma at 37 °C. Stock solutions (5 $\mu\text{g}/\text{ml}$) of the relevant r-APCs were prepared and their amidolytic activities determined. Adjustments of the stock concentrations were made such that their amidolytic activities were identical (in this case the APC concentrations were also equal). A dose-response curve was obtained for each sample and plotted as the activated partial thromboplastin times (clotting time) against the concentration (nM) of the appropriate r-APC. Symbols: ■, wtr-APC; ○, r-[R¹⁵H]APC; ●, r-[R¹⁵K]APC; △, r-[R¹⁵L]APC; ▲, r-[R¹⁵W]APC.

binding by the variants was confirmed by an analysis of the effects of PC concentrations on this binding event at constant Ca^{2+} (20 mM). The results obtained are shown in Figure 5. In these cases the total PC concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of Ca^{2+} was altered from 0.62 ± 0.1 μM to 1.9 ± 0.1 μM , 3.2 ± 0.2 μM , 2.3 ± 0.2 μM and 2.6 ± 0.3 μM for wtr-PC, r-[R¹⁵H]PC, r-[R¹⁵K]PC, r-[R¹⁵L]PC and r-[R¹⁵W]PC respectively.

After activation of the r-PC mutants, their overall *ex vivo* whole plasma anticoagulation activities in PC-deficient plasma were determined, and compared with that of wtr-APC. In each case the anticoagulant activities were normalized to equal amidolytic activities of the mutants for comparative purposes. From the results shown in Figure 6 it is clear that very little activity (less than 10%) compared with that of wtr-APC was present in any of the mutants. Assays *in vitro* in both the prothrombinase (Figure 7) and tenase (Figure 8) complexes, which, as designed here, isolated the activities of these mutants on fVa and fVIII respectively, showed that there were nearly complete losses of activities of the variants; the R¹⁵K mutant displayed the highest activity, at approx. 15% of that of wtr-APC.

DISCUSSION

The sequence L/V-Gla-R¹⁵-Gla (human PC numbering) is rigidly conserved in all species of vitamin K-dependent coagulation proteins, a fact suggestive of the importance of its integrity in this class of proteins. In agreement with this conclusion, the only known mutations at R¹⁵ in patients are to a Gly [47], a Gln [25] or a Trp [25] residue. These symptomatic patients possess type II pedigrees in PC. We sought a molecular understanding of this marked effect on biological activity of the alteration of a single residue that is not directly involved in the active site of APC, or seemingly directly involved in the Ca^{2+} -binding properties of the GD_{PC}. To investigate these critical consequences of alterations at

Figure 7 Inactivation of fVa by APC

Time course of inactivation of human fVa by r-APC mutants, each present at the same amidolytic activity. The buffer contained 20 mM Na/Hepes (pH 7.4 at 37 °C)/150 mM NaCl. After various times of incubation of (final concentrations) fVa (0.1 μ M) with the APC (0.5 nM) in the presence of PL (20 μ M) and Ca^{2+} (2.5 mM), an aliquot of the mixture was added to a cuvette containing (final concentrations) prothrombin (1.39 μ M), CaCl_2 (2.5 mM), PL (20 μ M) and S2238 (180 μ M), in a final volume of 790 μ l. The baseline was recorded for 1 min, after which 10 μ l of a solution of 8 μ M factor Xa was added to accelerate S2238 hydrolysis. Activation of prothrombin was accelerated by the addition of factor Xa (8 nM), and the rate of amidolysis of this latter substrate by thrombin was determined spectrophotometrically. The maximal rates of thrombin 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 logarithm of the fVa concentration 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 the particular mutant to that of wtr-APC was employed to calculate its percentage activity. A control incubation of fVa with r-PC demonstrated that the loss of activity of fVa was less than 5% over the period illustrated. Symbols: ■, wtr-APC; ○, r-[R¹⁵H]APC; ●, r-[R¹⁵K]APC; △, r-[R¹⁵L]APC; ▲, r-[R¹⁵W]APC.

R¹⁵ we evaluated a series of conservative and radical point mutations at this position and investigated the consequences of these alterations on several Ca^{2+} -PL-dependent properties of the resulting r-PC and r-APC mutants.

A large degree of post-translational processing of PC occurs normally, and it is important to determine, for any variant generated, whether the mutations have affected any of these reactions. The fact that most r-PC variants consisted of two-chain protein suggested that endoproteolytic liberation of the K¹⁵⁶R¹⁵⁷ dipeptide was accomplished in all of the r-PC variants. N-terminal amino acid sequencing confirmed that this cleavage occurred, and also demonstrated that the propeptide was efficiently cleaved from the proteins. The Glu and β -hydroxyaspartic acid contents of the mutants were similar to those of wtr-PC, showing that mutations at R¹⁵ did not affect the recognition site of the γ -carboxylase or the β -hydroxylase that catalyse these amino acid modifications. The fact that at least two glycoforms exist for all of the mutants, similarly to wtr-PC, demonstrates that glyco-processing has occurred. Thus we conclude that those types of processing event that are important to the types of studies conducted herein have generally taken place in these particular R¹⁵-based mutants.

The addition of Ca^{2+} to vitamin K-dependent coagulation proteins such as PC results in a quenching of their intrinsic fluorescence [48]. At least two contributions to this effect are believed to be present [49]: a small quenching due to higher-affinity Ca^{2+} site(s) outside the GD, and a second, larger, quenching that is thought to be dependent on the lower-affinity Ca^{2+} sites within the GD. The first stage is difficult to observe in intact PC because of the much larger quenching of the second

Figure 8 Inactivation of fVIII by r-APC

Time course of inactivation of human fVIII by r-APC mutants, each present at the same amidolytic activity. The buffer contained 20 mM Hepes/NaOH (pH 7.4 at 37 °C)/150 mM NaCl. A solution of fVIII (final concentration approx. 6 nM) was incubated with the relevant APC (final concentration approx. 0.5 nM) in the presence of (final concentrations) PL (40 μ M) and Ca^{2+} (2.5 mM). Next, factor IXa β (approx. 6.5 nM) and thrombin (0.10 unit/ml) were added 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 μ M PL (in phosphate), 2.5 mM CaCl_2 and 180 μ M of the factor Xa chromogenic substrate benzoyl-Ile-Glu(O²)-Gly-Arg-p-nitroanilide (S2222) in a final volume of 790 μ l. The baseline was recorded for 1 min, after which 10 μ l of a solution of 32 μ M factor X was added to accelerate the hydrolysis of S2222. The rate of amidolysis of the latter substrate by factor Xa was determined spectrophotometrically. The maximal rates of factor Xa 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 concentration 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 the particular mutant to that of wtr-APC was employed to calculate its percentage activity. A control incubation of fVIII with r-PC demonstrated that the loss of activity of fVIII was less than 5% over the period illustrated. Symbols: ■, wtr-APC; ○, r-[R¹⁵H]APC; ●, r-[R¹⁵K]APC; △, r-[R¹⁵L]APC; ▲, r-[R¹⁵W]APC.

stage; we believe that this latter quenching phenomenon is primarily being observed in Figure 1. If that is indeed so, it then appears as though all of the R¹⁵-based mutants are capable of undergoing a Ca^{2+} -mediated transition at similar concentrations of this cation. Thus at least those Ca^{2+} sites in the GD_{PC} that are reporters of the fluorescence quenching seem unaltered in these mutant proteins. Of course, no conclusions can be drawn about the integrity of those Ca^{2+} -sites not responsible for the fluorescence quenching of the protein and not involved in the concomitant conformational changes of the proteins.

The fluorescence studies strongly suggest that a Ca^{2+} -dependent conformation is adopted by the mutants, but this conformation is apparently not recognized by the mAb, JTC1, directed to the Ca^{2+} -dependent conformation of the GD_{PC} (Figures 2 and 3). A similar finding has been reported from a plasma sample of a patient with a R¹⁵G mutation in PC [47]. However, this latter protein was not rigorously characterized, and it is not possible to conclude whether processing problems that could have affected this result existed in the patient. Nonetheless, although the results shown in Figures 2 and 3 indicate that the Ca^{2+} -induced conformation in the mutants is not precisely the same as that for wtr-PC, it is alternatively possible that R¹⁵ is a necessary residue for the chemical epitope of JTC1, and the absence of this particular residue resulted in a loss of binding. Although we find this explanation plausible, we tend to reject it, because we found that an increase in the mAb concentrations in the assays for each of the mutants still did not lead to significant interactions in the presence of Ca^{2+} , even in the more conservative R¹⁵K mutant. It is difficult to imagine that a single

Figure 9 Model of the Ca^{2+} -GD structure of PC

The model [50] is based on the X-ray crystal structure of the Ca^{2+} -bovine prothrombin fragment 1 complex [51]. The backbone of residues A¹ to T³⁷ are displayed. The peptide bond carbonyl of H¹⁰ is shown, along with the side chains of R¹⁵ and Gla¹⁶ (γ stands for Gla). The disulfide bond (yellow) between C¹⁷ and C²² is also illustrated. Carbon atoms are in black; oxygen atoms are in red; nitrogen atoms are in blue. The broken lines are the likely contact points between the side chains of R¹⁵ with H¹⁰ and Gla¹⁶.

amino acid substitution would result in a complete loss of the chemical epitope for the mAb, unless that same substitution also led to an altered Ca^{2+} -dependent conformation, thus more generally affecting the integrity of the mAb-binding site.

Confirmation that altered Ca^{2+} binding and possible altered Ca^{2+} -induced conformations might be consequences of the mutations at R¹⁵ is obtained from the PL-binding characteristics of the mutant proteins with acidic PL (Figures 4 and 5). Regardless of the substitution made at R¹⁵, the $[\text{C}_{50,\text{Ca}}\text{-PL}]$ values (Figure 4) for the mutants were approx. 2–3-fold higher than the same value for wt-PC. Thus additional Ca^{2+} is required for the protein to bind to PL when R¹⁵ is removed. The similarity of the values for the mutant proteins, possessing a wide variety of replacements, shows that the effects are due to the amino acid that has been deleted rather than to the particular substitutions made.

Not only is the Ca^{2+} dependence of PL binding influenced by the absence of R¹⁵, but the saturation values of Figure 4 also seem to be depressed for each of the mutants. This indicates that the Ca^{2+} -loaded variant proteins do not interact as well with the PL as wt-PC, a hypothesis directly examined by determination of the concentrations of mutant proteins required to induce binding to phospholipid vesicles of 50 % of the protein molecules at a constant, high, $[\text{Ca}^{2+}]$. Calculation of these protein saturation midpoint values from the data of Figure 5 in fact demonstrates that binding of the Ca^{2+} -protein complex to PL was diminished in the mutants by approx. 3–5-fold. Thus R¹⁵ is an important amino acid for binding of PC to acidic PL, by indirectly influencing Ca^{2+} binding to PC and/or by acting as a determinant

for generation of a proper Ca^{2+} -induced conformation of this protein.

These alterations at R¹⁵, although showing subtle effects on the ability of r-PC to interact with Ca^{2+} and PL, are far more marked in their influence on the Ca^{2+} -PL-related activities of r-APC. Although the amidolytic activity of r-APC was not significantly affected by mutations at R¹⁵, the plasma anti-coagulant activities *ex vivo* of all of the variants were 8–15 % of that of their wild-type counterpart (Figure 6), when present in the assay at equal amidolytic activities. To confirm whether these losses of activity in the mutants were due to effects on the normal substrates of wt-APC, namely fV/fVa and fVIII/fVIIIa, and to evaluate whether the activities of the mutant enzymes were similar towards these two related substrates, the individual cofactors were reassayed in the complete prothrombinase and tenase complexes respectively (Figures 7 and 8). The results clearly show that very little activity was observed towards inactivation of the fVa or fVIII. Thus substitution at this, the single residue in the non-catalytic chain of r-APC, is sufficient to virtually eliminate the biological activity of APC.

With the aid of a model of the GD_{PC} [50], based on the X-ray crystal structure of the Ca^{2+} /prothrombin fragment 1 complex [51], we propose a role for R¹⁵ in the maintenance of the conformational integrity of the Ca^{2+} -GD complex. As shown in Figure 9, the side chain of R¹⁵ lies sufficiently close (3.1 and 3.2 Å; 1 Å = 0.1 nm) to that of Gla¹⁶ to form two ionic bonds between these residues, as we proposed earlier [36]. Gla¹⁶ is a residue that is uniformly critical for every property so far determined for r-APC, and mutation of this residue significantly altered the Ca^{2+} -dependent conformation of r-PC and all related activities of the resulting r-APC towards biological substrates [28,35,36,52]. Thus the disruption of the salt bonds between R¹⁵ and Gla¹⁶ by the replacement of R¹⁵ could lead to the destabilization of the native conformation of the Ca^{2+} -GD complex and/or an alteration in the Ca^{2+} -binding properties of the GD via indirect effects on the conformation of Gla¹⁶. We believe that this is an important operational mechanism in this case. Additionally, as seen in Figure 9, the side chain of R¹⁵ lies sufficiently close (2.4 Å) to the peptide carbonyl of H¹⁰ to form a hydrogen bond, further implicating R¹⁵ in stabilizing the GD_{PC} domain.

In conclusion we reveal here a remarkable effect of a single amino acid residue, R¹⁵, on the structure-activity relations of r-PC and r-APC. Coupled with our previous observations on the important roles of individual Gla residues [28,35–37,53] and of other highly conserved non-Gla residues, namely F⁴ [46], L⁵ [44,54], L⁸ [46] and C¹⁷ [55], in directing the Ca^{2+} -PL functional binding properties of the GD_{PC} , a much better understanding of the structure-function relationships of the GD in this group of proteins is emerging. Such information is crucial to the rational design of agents that can function as coagulants and anti-coagulants *in vivo*.

We thank the reviewer of the manuscript for pointing out the possible hydrogen bond between the R¹⁵ side chain and the peptide carbonyl of H¹⁰. This work was supported by grant HL-19982 from the National Institutes of Health and the Kleiderer/Pezold Family endowed professorship (to F. J. C.).

REFERENCES

- 1 Kisiel, W., Canfield, W. M., Ericsson, L. H. and Davie, E. W. (1977) *Biochemistry* **16**, 5824–5831
- 2 Vehar, G. A. and Davie, E. W. (1980) *Biochemistry* **19**, 401–410
- 3 Esmen, N. L., Owen, W. G. and Esmen, C. T. (1982) *J. Biol. Chem.* **257**, 859–864
- 4 Amphlett, G. W., Kisiel, W. and Castellino, F. J. (1981) *Biochemistry* **20**, 2156–2161

- 5 Clarke, J. H., Light, D. R., Blasko, E., Parkinson, J. F., Nagashima, M., McLean, K., Vilander, L., Andrews, J. H., Morser, J. and Glaser, C. B. (1993) *J. Biol. Chem.* **268**, 6309–6315
- 6 Foster, D. C., Yoshitake, S. and Davie, E. W. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4673–4677
- 7 O'Hara, P. J., Grant, F. J., Haldeman, B. A., Gray, C. L., Insley, M. Y., Hagen, F. S. and Murray, M. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5158–5162
- 8 Yoshitake, S., Schach, B. G., Foster, D. C., Davie, E. W. and Kurachi, K. (1985) *Biochemistry* **24**, 3736–3750
- 9 Leytus, S. P., Foster, D. C., Kurachi, K. and Davie, E. W. (1986) *Biochemistry* **25**, 5098–5102
- 10 Nelsestuen, G. L. (1976) *J. Biol. Chem.* **251**, 5648–5656
- 11 Nelsestuen, G. L. and Lim, T. K. (1977) *Biochemistry* **16**, 4164–4171
- 12 Nelsestuen, G. L., Kisiel, W. and Di Scipio, R. G. (1978) *Biochemistry* **17**, 2134–2138
- 13 Cheung, W. F., Hamaguchi, N., Smith, K. J. and Stafford, D. W. (1992) *J. Biol. Chem.* **267**, 20529–20531
- 14 Toomey, J. R., Smith, K. J., Roberts, H. R. and Stafford, D. W. (1992) *Biochemistry* **31**, 1806–1808
- 15 Geng, J.-P., Christiansen, W. T., Plow, E. F. and Castellino, F. J. (1995) *Biochemistry* **34**, 8449–8457
- 16 Ahmad, S. S., Rawala-Sheikh, R., Cheung, W.-F., Jameson, B. A., Stafford, D. W. and Walsh, P. N. (1994) *Biochemistry* **33**, 12048–12055
- 17 Handford, P. A., Baron, M., Mayhew, M., Willis, A., Beesley, T., Brownlee, G. G. and Campbell, I. D. (1990) *EMBO J.* **9**, 475–480
- 18 Rezaie, A. R., Neuenschwander, P. F., Morrissey, J. H. and Esmon, C. T. (1993) *J. Biol. Chem.* **268**, 8176–8180
- 19 Astermark, J. and Stenflo, J. (1991) *J. Biol. Chem.* **266**, 2438–2443
- 20 Sridhara, S., Clarke, B. J. and Blajchman, M. A. (1993) *Blood Coag. Fibrinol.* **4**, 505–506
- 21 Zhong, D. G., Smith, K. J., Birktoft, J. J. and Bajaj, S. P. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3574–3578
- 22 Ahmad, S. S., Rawala, R., Cheung, W. F., Stafford, D. W. and Walsh, P. N. (1995) *Biochem. J.* **310**, 427–431
- 23 Chang, J. Y., Stafford, D. W. and Straight, D. L. (1995) *Biochemistry* **34**, 12227–12232
- 24 Mimuro, J., Muramatsu, S.-I., Kaneko, M., Yoshitake, S., Iijima, K., Sakata, Y. and Matsuda, M. (1993) *Int. J. Haematol.* **57**, 9–14
- 25 Reitsma, P. H., Bernardi, F., Doig, R. G., Gandrille, S., Greengard, J. S., Ireland, H., Krawczak, M., Lind, B., Long, G. L., Poort, S. R. et al. (1995) *Thromb. Haemostas.* **73**, 876–889
- 26 Zhang, L. and Castellino, F. J. (1990) *Biochemistry* **29**, 10828–10834
- 27 Amphlett, G. W., Byrne, R. and Castellino, F. J. (1979) *J. Biol. Chem.* **254**, 6333–6336
- 28 Zhang, L. and Castellino, F. J. (1992) *J. Biol. Chem.* **267**, 26078–26084
- 29 Wakabayashi, K., Sakata, Y. and Aoki, N. (1986) *J. Biol. Chem.* **261**, 11097–11105
- 30 Yu, S., Zhang, L., Jhingan, A., Christiansen, W. T. and Castellino, F. J. (1994) *Biochemistry* **33**, 823–831
- 31 Beals, J. M. and Castellino, F. J. (1986) *Biochem. J.* **236**, 861–869
- 32 Lowry, D. H. and Lopez, J. A. (1946) *J. Biol. Chem.* **162**, 421–428
- 33 Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
- 34 Nelsestuen, G. L. and Broderius, M. (1977) *Biochemistry* **16**, 4172–4177
- 35 Zhang, L. and Castellino, F. J. (1993) *J. Biol. Chem.* **268**, 12040–12045
- 36 Zhang, L., Jhingan, A. and Castellino, F. J. (1992) *Blood* **80**, 942–952
- 37 Jhingan, A., Zhang, L., Christiansen, W. T. and Castellino, F. J. (1994) *Biochemistry* **33**, 1869–1875
- 38 Menhart, N., Sehl, L. C., Kelley, R. F. and Castellino, F. J. (1991) *Biochemistry* **30**, 1948–1957
- 39 De Serrano, V. S., Menhart, N. and Castellino, F. J. (1992) *Arch. Biochem. Biophys.* **294**, 282–290
- 40 De Serrano, V. S. and Castellino, F. J. (1992) *Biochemistry* **31**, 3326–3335
- 41 De Serrano, V. S. and Castellino, F. J. (1992) *Biochemistry* **31**, 11698–11706
- 42 Chibber, B. A. K., Urano, S. and Castellino, F. J. (1990) *Int. J. Peptide Protein Res.* **35**, 73–80
- 43 Grinnell, B. W., Walls, J. D. and Gerlitz, B. (1991) *J. Biol. Chem.* **266**, 9778–9785
- 44 Zhang, L. and Castellino, F. J. (1994) *J. Biol. Chem.* **269**, 3590–3595
- 45 Christiansen, W. T. and Castellino, F. J. (1994) *Biochemistry* **33**, 5901–5911
- 46 Christiansen, W. T., Jalbert, L. R., Robertson, R. M., Jhingan, A., Prorok, M. and Castellino, F. J. (1995) *Biochemistry* **34**, 10376–10382
- 47 Mimuro, J., Muramatsu, S., Kaneko, M., Yoshitake, S., Iijima, K., Nakamura, K., Sakata, Y. and Matsuda, M. (1993) *Int. J. Haematol.* **57**, 9–14
- 48 Amphlett, G. W., Kisiel, W. and Castellino, F. J. (1981) *Biochemistry* **20**, 2156–2161
- 49 Johnson, A. E., Esmon, N. L., Laue, T. M. and Esmon, C. T. (1983) *J. Biol. Chem.* **258**, 5554–5560
- 50 Christiansen, W. T., Tulinsky, A. and Castellino, F. J. (1994) *Biochemistry* **33**, 14993–15000
- 51 Soriano-Garcia, M., Padmanabhan, K., deVos, A. M. and Tulinsky, A. (1992) *Biochemistry* **31**, 2554–2566
- 52 Zhang, L. (1991) Ph.D. dissertation, University of Notre Dame
- 53 Colpitts, T. L., Prorok, M. and Castellino, F. J. (1995) *Biochemistry* **34**, 2424–2430
- 54 Jalbert, L. R., Chan, J. C. Y., Christiansen, W. T. and Castellino, F. J. (1996) *Biochemistry* **35**, 7093–7099
- 55 Zhang, L. and Castellino, F. J. (1991) *Biochemistry* **30**, 6696–6704