Role of Individual γ-Carboxyglutamic Acid Residues of Activated Human Protein C in Defining its In Vitro Anticoagulant Activity

By Li Zhang, Ashish Jhingan, and Francis J. Castellino

To evaluate the contributions of individual γ-carboxyglutamic acid (gla) residues to the overall Ca²⁺-dependent anticoagulant activity of activated human protein C (APC), we used recombinant (r) DNA technology to generate protein C (PC) variants in which each of the gla precursor glutamic acid (E) residues (positions 6, 7, 14, 16, 19, 20, 25, 26, and 29) was separately altered to aspartic acid (D). In one case, a ca26V mutation ([gla26V]r-PC) was constructed because a patient with this particular substitution in coagulation factor IX had been previously identified. Two additional r-PC mutants were generated, viz, an r-PC variant containing a substitution at arginine (R) 15 ([R15]r-PC), because this R residue is conserved in all gla-containing blood proteins, and exists in plasma as a zymogen. The activated form of this latter case, Ca²⁺ inhibits the reaction. The mechanism of the anticoagulant activity of APC is well established. While complexes of PAI-1 and APC have been observed, the importance of this mechanism toward enhanced fibrinolysis in vivo has been questioned. More recently, it has been proposed that APC inhibits an antifibrinolytic component derived from activation of prothrombin, thus promoting a fibrinolytic response. PS also appears to be a cofactor for the fibrinolytic response of PC.

The coding regions of the human PC gene are contained in eight exons that translate into a 42-amino acid residue leader sequence, followed consecutively by a 155-amino acid residue light chain and a heavy chain of 262-amino acids. The catalytic triad of H211, D255, and S360 exists in this latter domain region. A dipeptide, K156-R157, is present in the translatable protein that connects the two chains, and its removal during normal processing is critical to forming an activatable form of PC. Other processing steps that generate mature plasma PC include cleavage of the leader polypeptide, γ-carboxylation of the nine gla precursor E residues, present at amino acid sequence positions 6, 7, 14, 16, 19, 20, 25, 26, and 29 of human PC, β-hydroxylation of D71, and glycosylation of N97, N248, N313, and N329. Based upon consideration of the positions of the introns in the gene, and amino acid sequence homologies with other proteins, it is clear that PC contains several domains. These include the γ-carboxyglutamic acid (gla)-rich amino terminal polypeptide, followed by two consecutive regions homologous to epidermal growth factor, an activation peptide, and the serine protease catalytic region. Fully processed recombinant (r) human PC and r-APC have been expressed in mammalian cells, accomplishments that allow structure-function investigations of
r-PC and r-APC to be designed through site-directed mutagenesis.

The posttranslational processing of appropriate precursor E residues to gla residues allows vitamin K-dependent blood coagulation proteins to bind to Ca\(^{2+}\) and PL, interactions that are essential to their proper functioning.\(^{21}\) Regarding PC, it is clear that incomplete γ-carboxylation of random gla-precursor E residues,\(^{22}\) as well as proteolytic removal of the entire gla domain of plasma PC, leads to a substantial diminution of the anticoagulant activities of their activated forms, as shown by the reduction of the APC-induced prolongation of activated partial thromboplastin times (APTT) of plasma. Because thromboembolic complications in patients arising from PC deficiencies are well-known clinical findings,\(^{25,26}\) and the increasing use of polymerase chain reaction (PCR) technology has led to the identification of patients with defined amino acid mutations in this protein,\(^{23,24}\) we have embarked on a program aimed to show the identity of specific amino acids that play major roles in the anticoagulant activity of PC. To date, using recombinant DNA technology, we have shown that double mutations that prevent γ-carboxylation at the E pairs present at r-PC sequence positions 6 and 7,\(^{19}\) and 19 and 20,\(^{20}\) residues conserved in all vitamin K-dependent coagulation proteins, greatly reduce the anticoagulant properties of the resulting variant r-APC molecules. More recently, we have constructed, expressed, and purified r-PC molecules containing individual mutations at all gla-precursor E residues. This allowed us to identify which of these are critical to the anticoagulant properties of APC. This report is a summary of our findings.

MATERIALS AND METHODS

Proteins. Human 293 kidney cell-expressed wild-type (wt) r-PC was generated and purified as described previously.\(^{19}\) Human plasma PC was donated by Enzyme Research Laboratories, Inc (South Bend, IN).

All r-PC and variant r-PC activations were performed as described earlier,\(^{19}\) using the Agkistrodon contortix contortix venom activator of PC, Protac C, purchased from American Diagnostica (New York, NY). The temporal progress of each activation was monitored spectrophotometrically by the appearance of amidolytic activity of r-APC toward the substrate, L-pyroglutamyl-L-prolyl-L-arginine-p-nitroanilide (substrate, 52366; Helena Laboratories, Beaumont, TX).

Murine monoclonal anti-PC, C3,\(^{27}\) was provided by Dr John Griffin (La Jolla, CA) and used in Western analysis for screening clones of transfected 293 cells for r-PC production. Murine monoclonal antibody (MoAb), L1, a Ca\(^{2+}\)-independent antibody that recognizes a determinant in the protease domain of PC, was generated in this laboratory\(^{28}\) and used to prepare immunoaffinity chromatography columns to assist in the purification of some of the variant r-PC molecules.

Restiction endonucleases were purchased from Fisher Scientific (Springfield, NJ) and BRL (Gaithersburg, MD). These enzymes were used according to the manufacturers’ recommendations.

Genes for wt and mutant r-PC molecules. The cDNA coding for wt-r-PC was provided by Dr Earl Davie (Seattle, WA) in pUC119. The changes that we incorporated into the cDNA for use with our 293 kidney cell expression system and the plasmid expression vector used (pCIS2M) have been described in detail.\(^{19}\)

The cDNAs coding for each of the r-PC mutants described were constructed from the wt cDNA for human PC, or an r-PC mutant cDNA, by site-directed mutagenesis using synthetic oligonucleotide primers. Screenings of positive transformants were accomplished by the gain or loss of suitable restriction endonuclease sites.

The presence of the proper nucleotide insertions in the relevant cDNAs was evaluated by DNA sequence analysis before their placement in the expression vectors. Insertion of the variant cDNAs into the expression plasmid, pCIS2M, was accomplished as for the wt-PC gene.\(^{19}\)

Transfection in adenovirus-transformed human kidney 293 cells (ATCC CRL 1573). The 293 cell growth conditions, the procedures for their transfection with pCIS2M containing the cDNA of interest, the selection and propagation of clones expressing wt-r-PC or the relevant r-PC mutants, and the harvesting of conditioned cell media were as published previously.\(^{19}\)

Purification of the recombinant proteins. Purification of wt-r-PC with selection for a subpopulation of PC molecules that contained the maximal level of gla residues was conducted as described earlier.\(^{19}\) Similar techniques were used for the purification of all mutants, with minor operational modifications depending on the elution behavior from the anion exchange columns and their purities as shown by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (DodS04/PAGE).\(^{29}\) The following general procedure sufficed in all cases to provide r-PC and r-PC mutants containing gla residues at all available precursor E residues.

Benzamidine-HCl (5 mmol/L, final concentration) was added to the conditioned media of the 293 cells. Approximately 1.5 L of this solution was dialyzed against a buffer of 20 mmol/L Tris-HCl/150 mmol/L NaCl, pH 7.4 (column buffer), containing 5 mmol/L benzamidine-HCl/4 mmol/L EDTA, and passed over a 5 mL column of Pharmacia Fast Flow Q (FFQ) anion exchange resin (Pharmacia, Piscataway, NJ), equilibrated with column buffer/4 mmol/L EDTA at 4°C. After washing the resin with 3 column volumes of this buffer, followed by 3 column volumes of the same buffer without EDTA, the r-PC, or r-PC variant, was eluted in one step with column buffer/30 mmol/L CaCl\(_2\), pH 7.4. At this stage, the total sample volume was approximately 20 mL. Several such samples were combined and, after dialysis against column buffer containing 4 mmol/L EDTA, the dialysate was repassaged over a column containing 3 mL of this same resin equilibrated with column buffer at 4°C. The relevant r-PC or r-PC mutein was eluted at a flow rate of 0.25 mL/min with a CaCl\(_2\) gradient (start solution, 30 mL of column buffer; limit solution, 30 mL of column buffer/30 mmol/L CaCl\(_2\), pH 7.4), as described.\(^{23}\) Fractions (1 mL) were obtained and those containing r-PC, which were readily identified by DodS04/PAGE, were pooled and the CaCl\(_2\) removed by dialysis against column buffer. Next, the protein was readсорbed to 3 mL of a second FFQ column, equilibrated in column buffer at 4°C, after which the r-PC was eluted at a flow rate of 0.25 mL/min with an NaCl gradient (start solution 30 mL of column buffer; limit solution, 30 mL of 20 mmol/L Tris-HCl/500 mmol/L NaCl, pH 7.4). r-PC-containing fractions (1 mL) were identified by DodS04/PAGE and pooled. Some r-PC variants show slightly altered column behavior from the wt-protein, and from each other, but use of this procedure results in readily identifiable, highly homogeneous material. We have repeatedly observed for a variety of single and double gla mutant proteins that the r-PC material at this stage contained maximally γ-carboxylated protein (at least seven residues of gla per mole of PC). Subpopulations of PC molecules containing lower levels of γ-carboxylation, the amounts of which were considerable with some recombinant mutants, displayed very different chromatographic behavior on each column. Generally, incompletely γ-carboxylated subforms were much more weakly adsorbed to the first column.

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In the few cases in which a small amount of non-r-PC material was present after this last chromatography step, final purification was accomplished using affinity chromatography with a column (0.6 x 5 cm) of Sepharose-MoAb LI, equilibrated in column buffer. After washing with this same buffer, the material that eluted from the column upon treatment with 0.1 mol/L glycine, pH 2.7, was dialyzed against 20 mmol/L Tris-HCl, pH 7.4. The partially purified r-PC or r-PC variant was then reapplied to the FFQ anion exchange column, which was equilibrated in this same buffer. The column was then washed with a solution of 20 mmol/L Tris-HCl/200 mmol/L NaCl, pH 7.4, and eluted with a buffer of 20 mmol/L Tris-HCl/500 mmol/L NaCl, pH 7.4. The highly purified r-PC preparation was then dialyzed against 50 mmol/L NH₄HCO₃, followed by H₂O.

Gla determinations. These experiments were performed by amino acid analysis after alkaline hydrolysis. The hydrolysates were conducted with 2.5 mol/L KOH for 20 hours at 110°C, and KOH was removed from the samples as reported previously. Amino acid analysis by reverse-phase high performance liquid chromatography (HPLC) was performed after precolumn derivatization with o-phthaldehyde-p-mercaptoethanol, using a 4.6 mm (ID) x 70 mm Ultrasphere XL ODS column (3.0 µ) with a precolumn (Beckman Instruments, Fullerton, CA) step. The buffer used was 90% 0.1 mol/L NaOAc, pH 7.2/9.5% methanol/0.5% tetrahydrofuran. The flow rate of the column was 1.5 mL/min. This chromatographic step resulted in baseline resolution of glna (retention time, 0.8 minutes), D (retention time, 2.0 minutes), and E (retention time, 4.1 minutes). The ratios of glna/D and glna/E were used to obtain the number of glna residues per mole of protein.

We used as reference standards both a commercial o-phthalaldehyde-derivatized amino acid standard mixture as well as a peptide. ANSFL(glna)KLSVL synthesized by standard Fmoc chemistry (the α-N-Fmoc-γ'-ditBu-L-glna-OH used in the peptide synthesis for placement of glna in the peptide was chemically synthesized by Dr Sushil Sharma in this laboratory). Use of this peptide for this purpose allowed us to obtain recovery factors after alkaline hydrolysis of the peptide and to determine very accurate conversion factors of peak areas to concentrations of glna residues in the hydrolyzed samples. The glna/D ratio in this peptide of 2.0 (N is converted to D as a result of hydrolysis), along with the concentration response factor for D from a commercial standard amino acid solution, was used to obtain the concentration response factor for a glna residue. Checks of the method were made by performing glna residue analyses on human plasma PC and bovine plasma factor X.

Amidolytic activity of various PC molecules. Automated amino terminal amino acid sequence analysis of each r-PC sample was conducted as previously described. In all cases, protein sequencing was successful through 35 residues, steps that were sufficient to obtain sequence information through the entire glna-domain.

Amidolytic assays of r-APC and its variants. Optimal conditions found for activation of r-PC and the variants described herein have been described earlier. For amidolytic assay of the resulting r-APCs, solutions were prepared with 2 to 400 μL of substrate S2366 (stock solution, 4.2 mmol/L in H₂O), 40 μL of Tris-HCl, pH 7.4 (stock solution, 1 mol/L), and 80 μL of NaCl (stock solution, 1 mol/L). A quantity of H₂O was added to adjust final volumes to 0.79 mL. The amidolytic reaction was accelerated as a result of addition of 10 μL of enzyme and rates of p-nitroanilide release were monitored by the increase of absorbancy at 405 nm. Km and kcat values were calculated from Lineweaver-Burk plots of the variation in initial velocities as a function of S2366 concentrations.

APTT assays. For this assay, the concentrations of wt-APC, human plasma APC, and all recombinant variants investigated were adjusted so that each APTT assay was conducted at the same amidolytic activity of the particular APC under study. The concentration range of human plasma APC in the assay solutions was 0.025 to 0.4 μg/mL and was readjusted for the muteins, depending on the clotting times observed.

APTT assays were conducted at 37°C with PC-deficient plasma using the APTT assay kit (Sigma Diagnostics, St Louis, MO), essentially as described earlier. Controls in this assay were performed in the absence of APC and with unactivated r-PCs in place of their respective APCs. For the assay, a volume of 100 μL of APTT reagent was added to the reaction vessel and allowed to temperature-equilibrate at 37°C for 1 minute. This was followed by the addition of 10 μL of the desired concentration of the r-APC under examination and 90 μL of PC-deficient human plasma. After an additional 3 minutes of incubation, clotting was initiated by the addition of 100 μL of a 25 mmol/L stock solution of CaC₂, pre-equilibrated at 37°C. Clotting times were measured with a commercial fibrometer (Fisher Scientific, Springfield, NJ). Our standard clot times (approximately 45 seconds) were essentially the same as those reported by the manufacturer of the assay kit.

For calculation of the results, plots of the logarithm of clotting time versus the logarithm of the dilution of the stock solutions of the relevant APC were constructed for each APC sample. The displacement of the sample line from the wt-APC sample (the dose-response curves for the different r-APC mutants were parallel) was used to calculate the relative potencies of each sample. The specific activity of the wt-APC sample in each assay was obtained by comparison with the same graphs constructed for plasma APC and wt-APC. The specific activity of wt-APC was calculated using the value of 250 U/mg for human plasma APC and the percent activity of wt-APC relative to plasma APC. The specific activities of each variant r-APC were calculated from the specific activity of wt-APC and from the above described direct determinations of the relative activities of wt-APC and the variant r-APC of interest.

Analytical methods. Our procedures for oligonucleotide synthesis, cDNA purification and sequencing, cell transfections, site-specific mutagenesis, and Western analysis of the expressed PC molecules have been previously described in detail.

RESULTS

A number of strategically important recombinant glna-domain variants of PC have been constructed, expressed, and purified. These are listed in Table 1, along with the oligonucleotides used for mutagenesis and the restriction endonuclease sites used for screening positive bacterial transformants. The translated amino acid sequence of the glna-domain of human PC is illustrated in Fig 1, with indications of the sequence positions of the amino acids mutated in this investigation. After translation of each cDNA in human kidney 293 cells, it was necessary not only to purify the r-PC, but also to isolate the subpopulation of r-PC molecules that contained glna residues at all precursor E residues. We have found that some of the variants, particularly [glna20D]r-PC, [glna16D]r-PC, and [glna26V]r-PC, possessed only a small percentage (approximately 10% to 20%) of maximally γ-carboxylated r-PC antigen, whereas in other variants, viz., [glna6D]r-PC and [glna7D]r-PC, a much higher percentage (approximately 80% to 90%) of the total r-PC antigen wasγ-carboxylated at all available precursor E residues. Regardless of this circumstance, the purification procedure that we used was very satisfactory for isolation of maximally γ-carboxylated subpopulations of r-PC variants,
Table 1. Construction of Human r-PC Variants

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer</th>
<th>Screen</th>
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<tbody>
<tr>
<td>gla6D</td>
<td>5'-TCCTTCCTGGAAAGAATCGGTCACAGC -Sac I</td>
<td></td>
</tr>
<tr>
<td>gla7D</td>
<td>5'-CCTGGAGGACCTCCGTCACAGC -Sac I</td>
<td></td>
</tr>
<tr>
<td>gla14D</td>
<td>5'-GCACGCTGGAAAGCAGTGCGATAGA +Nru I</td>
<td></td>
</tr>
<tr>
<td>R15L*</td>
<td>5'-AGCCTGGAGGCACTCCGTCACAGC + Sac I</td>
<td></td>
</tr>
<tr>
<td>gla16D</td>
<td>5'-CAGCTGGAGGCGAGTGCGATAGC -Sac I</td>
<td></td>
</tr>
<tr>
<td>gla19D</td>
<td>5'-TGACATAGAACGCTGAGTGTGAGT -Bgl II</td>
<td></td>
</tr>
<tr>
<td>gla20D</td>
<td>5'-TGCATAGAACGCTGAGTGTGAGT -Bgl II</td>
<td></td>
</tr>
<tr>
<td>gla25D</td>
<td>5'-TGTGACTTCGAGGCAAGGAAATT -Svi I</td>
<td></td>
</tr>
<tr>
<td>gla26V</td>
<td>5'-CTTCGGAGGACGGCAAGGAAATT +Sac I</td>
<td></td>
</tr>
<tr>
<td>gla29D</td>
<td>5'-AGGCCAAGGAAATTCTTCCAAAAATT +EcoRV</td>
<td></td>
</tr>
<tr>
<td>F31</td>
<td>5'-GCCAAGGAAATTCTTCCAAAAATT +EcoRV</td>
<td></td>
</tr>
</tbody>
</table>

The lower case letters represent the mutagenic bases. The mutation convention used is the normal amino acid and its sequence position in PC, followed by the single letter code for the new amino acid placed in that sequence position by mutagenesis. Gla refers to γ-carboxyglutamic acid.

*The starting wt-cDNA was first mutated at the third base of the codon for L8 to eliminate the interfering Sac I restriction endonuclease site.
†The starting cDNA was [R15L]-r-PC.

and in all cases described herein isolation of appropriate proteins was accomplished.

The first step of the purification used Ca²⁺-based elution from an FFQ anion exchange column, an example of which is shown in Fig 2A, and provided PC molecules containing at least seven residues of gla in the protein material cluted with the Ca²⁺ gradient. The second step of the purification (Fig 2B) is based on elution from the same chromatography resin with NaCl and results in removal of small amounts of contaminating protein. The elution positions of the different variants described herein may differ somewhat from each other and from the example provided in Fig 2, but the major protein peaks observed are the desired r-PC molecules. Finally, when needed (eg, [gla16D]-r-PC, [gla20D]-r-PC, and [gla26V]-r-PC), a further step on an immunoaffinity column resulted in highly purified protein. From our selected clones, the overall yields of the maximally γ-carboxylated r-PC variants described varied considerably, with the lowest amount obtained for [gla16D]-r-PC of approximately 50 μg/L of conditioned cell culture medium, to the highest amount obtained for [gla6D]-r-PC of approximately 500 μg/L of conditioned medium.

Fig 2. Purification of maximally γ-carboxylated r-PC on FFQ anion exchange chromatography at 4°C. The example provided is for [gla6D]-r-PC. (A) After batch elution with 30 mmol/L CaCl₂ of the [gla6D]-r-PC contained in 1.5 L of conditioned human kidney 293 cell culture medium, three such preparations were combined. After dialysis against 20 mmol/L Tris-HCl/150 mmol/L NaCl/4 mmol/L EDTA, pH 7.4, the resulting sample was reapplied to the same column (3 mL), equilibrated with a buffer of 20 mmol/L Tris-HCl/150 mmol/L NaCl/4 mmol/L EDTA, pH 7.4 at 4°C. The column was washed with this buffer, followed by this buffer without EDTA, after which the indicated CaCl₂ gradient was applied (60 mL, total volume). Fractions (1 mL) were collected at a flow rate of 0.25 mL/min. The major peak contains the desired material. (B) The partially purified [gla6D]-r-PC isolated in (A), above, was dialyzed against a buffer of 20 mmol/L Tris-HCl/150 mmol/L NaCl, pH 7.4 at 4°C, and reapplied to a 3 mL column of FFQ resin equilibrated in 20 mmol/L Tris-HCl/150 mmol/L NaCl, pH 7.4 at 4°C. The indicated gradient of NaCl was applied (60 mL, total volume) and 1 mL fractions were collected. The major peak contains highly purified [gla6D]-r-PC.

DodSO₄/PAGE gels for the r-PC variants used in this
investigation are provided in Fig 3. In all cases, highly homogeneous maximally γ-carboxylated material has been obtained. The two components observed for each PC represent the usual case,19,22,26 and are related to variable glycosylation of the four relevant N residues in the protein.35 We determined the gla content of each protein, and the results listed in Table 2 show that all of the mutants contained the expected levels of gla. Additionally, amino-terminal amino acid sequence analysis for each isolated recombinant protein has been performed through 35 residues. In all cases, the desired mutation was present at the anticipated location, and no more than a 5% repetitive yield of E was observed at any of the sequence positions that should have undergone γ-carboxylation. These absences of gla-precursor E residues showed that the r-PC variants that we have isolated and investigated contained the maximal levels of gla residues resulting from processing of the appropriate precursor E residues.

We have converted each r-PC variant to its respective APC by activation with the snake venom protease, Protac C. This reaction does not require Ca\(^{2+}\), and it was expected that the gla-deficient r-PC mutants, despite having probable variations in Ca\(^{2+}\) binding properties, would be nonetheless fully converted to their respective r-APC forms. While we have not as yet investigated possible differences in activation kinetics of this reaction for the r-PC variants, we did find that each r-PC was completely activatable to its corresponding r-APC. This included the [R15L]-r-PC and [F31L, Q32G]-r-PC mutants. Additionally, we determined Km and kcat values, at pH 7.4 and 37°C, for wrt-APC and representative variants that were active ([gla19D]-r-APC) and inactive ([gla7D]-r-APC) in APTT assays (vide infra). For these proteins, the Km value for substrate S2366 ranged from 1.1 to 1.4 mmol/L and the kcat for this same substrate ranged from 170 to 182 s\(^{-1}\). This shows that the amidolytic activities are essentially the same, a fact that would be expected from literature precedents.17,19,26

With the availability of the complete set of single gla-mutated r-APC molecules, as well as some other strategically mutated r-APC variants, the major goal of this investigation was to assess the role of each gla residue as a determinant of the in vitro anticoagulant activity of APC. For this purpose, we chose to use the clinically relevant APTT assay with PC-deficient human plasma. We believed it most important to differentiate this specific function of APC from its general amidolytic activity, and thus chose to adjust the APC solutions in the assay such that they were present at equal amidolytic activities, which essentially corresponds to the same protein concentrations in the cases of these particular mutations. As an example of the data obtained, an illustration of the clotting time data obtained for wrt-APC and one of the gla variants, [gla25D]-r-APC, is provided in Fig 4. This type of analysis has been made for every gla mutant protein investigated. The resulting anticoagulant activities of each of the recombinant variants are summarized in Table 3 and compared with wrt-APC, which has been expressed in the same cell line. The results clearly show that anticoagulant activity has been greatly reduced for recombinant variants that lack gla residues at any of the following sequence positions, viz, 7, 16, 20, and 26, whereas 80% to 92% of the anticoagulant activity of wrt-APC has been observed in recombinant variants that lack gla residues at any of the following positions, viz, 6, 14, and 19. The remaining two mutants containing substitutions at gla

### Table 2. γ-Carboxyglutamic Acid and Contents of Various r-PC Mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expected</th>
<th>Obtained</th>
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<tbody>
<tr>
<td>Bovine factor X</td>
<td>12.0</td>
<td>11.7 ± 0.4</td>
</tr>
<tr>
<td>Human PC</td>
<td>9.0</td>
<td>8.8 ± 0.3</td>
</tr>
<tr>
<td>wrt-PC*</td>
<td>9.0</td>
<td>8.9 ± 0.3</td>
</tr>
<tr>
<td>[gla6D]-r-PC</td>
<td>8.0</td>
<td>8.3 ± 0.2</td>
</tr>
<tr>
<td>[gla7D]-r-PC</td>
<td>8.0</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>[gla14D]-r-PC</td>
<td>8.0</td>
<td>7.8 ± 0.3</td>
</tr>
<tr>
<td>[R15L]-r-PC</td>
<td>9.0</td>
<td>9.0 ± 0.3</td>
</tr>
<tr>
<td>[gla16D]-r-PC</td>
<td>8.0</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>[gla19D]-r-PC</td>
<td>8.0</td>
<td>8.0 ± 0.3</td>
</tr>
<tr>
<td>[gla20D]-r-PC</td>
<td>8.0</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>[gla25D]-r-PC</td>
<td>8.0</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>[gla26V]-r-PC</td>
<td>8.0</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>[gla29D]-r-PC</td>
<td>8.0</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>[F31L, Q32G]-r-PC</td>
<td>10.0</td>
<td>10.0 ± 0.3</td>
</tr>
</tbody>
</table>

The mutation convention used is the [normal amino acid, its sequence position in PC, single letter code for the new amino acid placed in that sequence position by mutagenesis], followed by r-PC. Gla refers to γ-carboxyglutamic acid.

*Expressed in human kidney 293 cells.

![Fig 3. Nonreduced sodium dodecyl sulfate polyacrylamide gel electrophoretograms of all purified r-PC variants used in this study. The samples displayed are as follows. (A) Lane 1, human plasma PC; 2, 293 cell-expressed wrt-PC; 3, [gla6D]-r-PC; 4, [gla7D]-r-PC; 5, [gla14D]-r-PC; 6, [R15L]-r-PC; 7, [gla16D]-r-PC. (B) Lane 1, human plasma PC; 2, 293 cell-expressed wrt-PC; 3, [gla19D]-r-PC; 4, [gla20D]-r-PC; 5, [gla25D]-r-PC; 6, [gla26V]-r-PC; 7, [gla29D]-r-PC; 8, [F31L, Q32G]-r-PC.](image)
PROTEIN C MUTAGENESIS

Residues 25 and 29 showed significantly reduced levels of anticoagulant activity, yielding values of 24% and 9%, respectively, of wtr-APC. It has also been shown that R15 is an important residue in allowing Ca²⁺-dependent anticoagulant activity to be expressed, because only a small amount of anticoagulant activity has been found, ie, 19% relative to wtr-APC, consequent to the R15L mutation. Finally, inclusion of a gla residue at sequence location 32 did not significantly influence the anticoagulant activity of the resulting gla32-derived recombinant variant.

In addition, because some of the mutants have been purified by adsorption to an MoAb affinity column and eluted at low pH, we examined the effect of this treatment on the APTT activities of wtr-APC and two active mutants, [gla6D]r-APC and [gla14D]r-APC. These samples were adsorbed and eluted from the column as described, and activated to their respective r-APCs. The specific activities were 320 U/mg, 284 U/mg, and 290 U/mg, respectively. This shows that this purification step does not affect the functional property of interest.

**DISCUSSION**

We have prepared and successfully expressed each of the nine possible r-PC variants containing mutations at E residues that would become γ-carboxylated in the mature protein. The fact that some of the recombinant mutants contained a considerable amount of under-γ-carboxylated r-PC molecules may suggest that some recognition sites for the γ-carboxylase that catalyzes these reactions exist in the gla-domain, an observation made earlier, but which has been challenged. However, our investigation was not designed to fully explore this question. In any case, a highly homogeneous subpopulation of r-PC has been isolated for each mutein. Each of these proteins contained the appropriate content of gla residues. Further, amino acid sequence analysis clearly shows that the mutation placed in the corresponding cDNA was properly translated and that each mutein was γ-carboxylated at all available gla-precursor E residues. Thus, the purified proteins were appropriate for the planned investigations intended to show the importance of individual gla residues of r-APC in its Ca²⁺-dependent anticoagulant activity.

Each r-PC was converted to its corresponding r-APC as a consequence of activation by Protac C, a reaction that does not require Ca²⁺. The amidolytic activity of the variant r-APC preparations did not substantially differ from each other or from wtr-APC, a result consistent with earlier studies that showed that extensively under-γ-carboxylated r-APC expressed in the presence of warfarin possessed a specific amidolytic activity similar to its wt counterpart. Each generated r-APC was then assayed for its Ca²⁺-dependent anticoagulant activity. From the results obtained, shown in Table 3, it is concluded that gla residues at amino acid sequence positions 7, 16, 20, and 26 are essential for the anticoagulant activity of r-APC, whereas those present at amino acid sequence positions 6, 25, and 29 are also of some importance to this same activity. The muteins expressing the highest anticoagulant activity, ie, 80% to 92% of wtr-APC, were [gla6D]r-APC, [gla14D]r-APC, and [gla19D]r-APC, suggesting that gla at sequence positions 6, 14, and 19 in wtr-APC are the least important to its specific function. These findings correlate well with our previous studies showing that pairwise mutations of gla residues present at amino acid sequence positions 6 and 7, as well as 19 and 20, led to a virtual complete elimination of the anticoagulant activity of each mutant r-APC. In the former recombinant mutant, it is now clear that this previously reported loss of activity was a result of mutation at gla7, whereas in the latter case, mutation of gla20 alone would have been sufficient for the observed loss of anticoagulant activity. Of significance, it also appears that R15, a

![Graph showing assay of overall anticoagulant activity of APC using APTT times with PC-deficient human plasma at 37°C.](image)

**Table 3. Anticoagulant Properties of r-APC Variants**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Specific Activity (U/mg)</th>
<th>wtr-APC Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtr-APC*</td>
<td>325 ± 14</td>
<td>100</td>
</tr>
<tr>
<td>[gla6D]r-APC</td>
<td>279 ± 12</td>
<td>86 ± 4</td>
</tr>
<tr>
<td>[gla7D]r-APC</td>
<td>19 ± 5</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>[gla14D]r-APC</td>
<td>299 ± 10</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>[R15L]r-APC</td>
<td>62 ± 9</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>[gla16D]r-APC</td>
<td>&lt;6</td>
<td>&gt;2</td>
</tr>
<tr>
<td>[gla19D]r-APC</td>
<td>260 ± 11</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>[gla20D]r-APC</td>
<td>&lt;6</td>
<td>&lt;2</td>
</tr>
<tr>
<td>[gla26D]r-APC</td>
<td>78 ± 9</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>[gla26V]r-APC</td>
<td>&lt;6</td>
<td>&lt;2</td>
</tr>
<tr>
<td>[gla29D]r-APC</td>
<td>29 ± 6</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>[F31L, Q32gla]r-APC</td>
<td>319 ± 13</td>
<td>98 ± 4</td>
</tr>
</tbody>
</table>

The mutation convention used is the [normal amino acid, sequence position in PC, single letter code for the new amino acid placed in that sequence position by mutagenesis], followed by recombinant r-APC. Glα refers to γ-carboxyglutamic acid.

*This protein and all muteins were expressed in human kidney 293 cells.
residue conserved in all vitamin K-dependent human coagulation proteins, is extremely important to the Ca\(^{2+}\)-dependent anticoagulant activity of APC (Table 3), because only 19% of the activity of wt-PC remained after alteration of this amino acid to a noncharged variant containing an L15 residue. Additionally, alteration of the Q32 in r-APC to contain E32, which did in fact undergo \(\gamma\)-carboxylation during processing, led to a variant r-APC that contained full anticoagulant activity. While positioning of gla residues in other vitamin K-dependent blood coagulation proteins is highly conserved up to amino acid sequence position gla29 (or its homologous residue), there are differences at sequence locations beyond this point. For example, human factor IX contains additional gla residues at sequence positions 33, 36 and 40,\(^{39}\) human prothrombin possesses an additional gla32,\(^{40}\) and human factor VII contains gla35.\(^{41}\) Because inclusion of a gla at residue 32 of r-APC did not affect its anticoagulant activity, it appears that APC represents a functioning vitamin K-dependent protein with the minimum necessary levels of gla residues. Proteins containing additional gla residues may require such amino acids for their specific functions and/or their specific cellular properties.

The results reported herein will be of great value in relating mutations in the gla domain of APC, which are being increasingly discovered with PCR technology in symptomatic patients, with the thrombotic complications observed in these same patients. As examples, in the recent past, several gla mutations have been found in vitamin K-dependent proteins in patients with clotting abnormalities. In factor IX-Nagoya 4, the nucleotide sequence of the subject DNA was predictive of a translated protein with an E21 (equivalent to E20 in PC) K mutation. This patient, clinically classified as a severe B-type hemophilia, possessed a Ca\(^{2+}\)-dependent factor IX coagulant (fIX:c) activity less than 1% of normal.\(^{42}\) This low level of residual activity is analogous to the situation with [gla20D]r-APC, wherein the gla-dependent activity of this latter protein in a clotting assay was essentially eliminated despite a much more conservative mutation at this same gla residue. Similarly, in other severe hemophilia B patients with known gla-domain variations, viz, factor IX-Seattle \(^{34}\) and factor IX-Chongqing,\(^{44}\) DNA mutations that would be predicted to translate into proteins with E27 (equivalent to E26 in PC) K (fIX:c < 1%) and E27 (equivalent to E26 in PC) V (fIX:c < 1%) alterations, respectively, have been found. We generated an r-PC with this latter mutation, i.e., [gla26V]r-PC, and found that the corresponding [gla26V]r-APC also nearly completely lost its Ca\(^{2+}\)-dependent activity in a clotting assay (Table 3). Thus, gla residues 20 and 26 in human APC possess similar essential features to the corresponding residues 21 and 27 in human factor IXa, despite the fact that factor IXa exhibits procoagulant activity, whereas APC possesses anticoagulant activity. This suggests that the integrity of gla at these sequence positions is of great importance to the Ca\(^{2+}\)-dependent functions of both proteins, probably related to common essential features of Ca\(^{2+}\) binding, and this may be a uniform functional property of other vitamin K-dependent coagulation proteins. One gla mutation in PC has been discovered, viz, [gla20A] (along with [V34M]), in a patient with symptomatic type 2 PC deficiency.\(^{45}\) Assuming that the thrombotic complication was due to the gla20A mutation, and not that of V34M, this finding would be entirely predictable from our data (Table 3), because the much more conservative mutation, gla20D, led to nearly complete loss of the Ca\(^{2+}\)-dependent anticoagulant activity of the mutant r-APC. Another symptomatic patient with a dysfunctional PC, PC Yonago, has been identified, with an R15G mutation in the gla-domain.\(^{46}\) Our results with [R15L]r-APC, which possessed only 19% of the activity of wt-APC, also are predictive of a thrombotic state in a patient homozygous for this mutation.

A final factor IX mutation of interest to this investigation has been found in a symptomatic patient with factor IX HB9, wherein a gla33D replacement (fIXc = 4%) has been discovered.\(^{47}\) Because this gla residue was found to be of such importance for the clotting activity of factor IX, and because PC does not contain a gla precursor E residue at this location, we decided to substitute an E-residue for the equivalent Q32 in a mutant r-PC to determine whether r-APC activity would be influenced by this change. As seen in Table 3, the anticoagulant activity of this mutein was found to be approximately the same as that of wt-APC. Thus, a gla residue at this location does not appear to be of similar importance to APC anticoagulant function as it is for factor IX coagulant activity. In this r-APC variant, an additional F31L mutation was incorporated due to the nature of the oligonucleotide primer used that was found to be highly suitable for screening bacterial transformants. Because such a high level of anticoagulant activity was obtained for this variant, we decided not to realter L31 to its natural F residue. Additionally, L is present at this position in human prothrombin and is obviously not harmful to expression of Ca\(^{2+}\)-dependent properties of this protein at that location.

It should be emphasized that we have intentionally engineered very conservative gla mutations into r-APC because our purpose was to discover which of the gla residues in this protein were essential to its Ca\(^{2+}\)-dependent anticoagulant properties. We believe that when such activity is lost with D-replacements for gla residues, it is likely that any other substitution will also result in at least this level of activity loss. As additional patients are discovered with other amino acid replacements for apparently nonessential gla residues, these will be inserted and investigated in this manner, as was accomplished here with the gla26V mutation.

When examining human subject data of these types, it is extremely important to be able to decide whether the mutation in itself is lethal to the particular activity involved or whether the particular mutation caused activity loss because of other direct or secondary effects, such as the ability of a particular residue to influence the extent of \(\gamma\)-carboxylation of the protein. In the case of PC, it has been shown that at least seven residues of gla are needed for APC anticoagulant activity,\(^{18}\) and thus a nonspecific loss of ability to \(\gamma\)-carboxylate appropriate precursor E residues of
this protein would have a deleterious effect on the Ca\textsuperscript{2+}-dependent anticoagulant activity of the resulting r-APC. Thus, there is a need to conduct an investigation of this type with recombinant mutant proteins that are \( \gamma \)-carboxylated at all nonmutated precursor E residues, a fact that we have considered paramount in deciding to rigorously purify subpopulations of r-PC that were maximally \( \gamma \)-carboxylated. Were this investigation only to have considered total mutant r-PC antigen as the source of APC, the results would certainly not have been properly interpreted, because in virtually all cases a heterogeneous population of incompletely \( \gamma \)-carboxylated r-PC molecules was produced by the transfected cells. This is likely also the case in plasma of symptomatic patients with these mutations, thus complicating exact correlation of the thrombotic complication with the observed amino acid mutation(s) in the protein. An ancillary benefit of this work will be its assistance in providing accurate and systematic correlations of this type with patient plasmas. Further, these results will be invaluable for clinical assessment of patients who may be heterozygous for these mutations, because in these cases a wide variety of functional and malfunctional molecules may be present in plasma.

While elucidation of the exact mechanism(s) of the variable anticoagulant effects of the r-APC mutants in whole plasma was not the purpose of this study, such effects are undoubtedly related to the relative propensities of the muteins to inactivate coagulation cofactors F\( \text{V} \), F\( \text{V} \)a, F\( \text{VII} \), and F\( \text{VIII} \)a, as well as their relative abilities to be stimulated by protein S in plasma. It is also possible that the interaction of APC with its plasma inhibitor could be influenced by mutations at gla residues. All of these functional properties of APC are Ca\textsuperscript{2+}-dependent, and it is of great interest to this laboratory to determine in purified assays whether all mutants exert their effects through the same functional properties. This long-term effort is currently ongoing and will be the subject of future communications.

Finally, with the assistance of the crystallographic coordinates of the Ca\textsuperscript{2+}/bovine prothrombin fragment 1 complex, a region of prothrombin that contains its gla domain, it is possible to comment on the role of particular gla residues in the binding of Ca\textsuperscript{2+} and to consider the relationship between this binding and the Ca\textsuperscript{2+}-dependent functional properties of APC. Because bovine prothrombin and human PC possess gla residues at equivalent locations of the molecule, and a high degree of identity exists in the remainder of the gla-domain, it is likely that Ca\textsuperscript{2+}-coordination in the gla domain is similar for both proteins. Indeed, using a human prothrombin MoAb that recognizes the Ca\textsuperscript{2+}-induced conformational alteration common to several vitamin K-dependent clotting proteins, including PC, it has been found that the antigenic determinant for this antibody is expressed on human prothrombin and human PC. The epitope for this antibody is similarly masked in both of the above proteins after complexation with Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, and Mg\textsuperscript{2+}, an observation that argues for a similar conformational alteration in these proteins after coordination with Ca\textsuperscript{2+}.

A view of the crystallographic relationships between gla side chains and bound Ca\textsuperscript{2+} in bovine prothrombin fragment 1 is provided in Fig 5.\textsuperscript{46} Other relevant amino acid side chains are displayed, along with the peptide backbone of the hexapeptide disulfide loop (residues 18 through 28 in bovine prothrombin). The amino acid side chain, gla33, did not show sufficient electron density to be observed in the diffraction pattern. Interestingly, a total of seven atoms of Ca\textsuperscript{2+} form a channel through the gla-domain, with only three of these (Ca-1, Ca-6, and Ca-7) in good contact with solvent. Ca-2 is partially exposed to solvent, and Ca-3, Ca-4, and Ca-5 are not exposed to solvent. These latter three Ca\textsuperscript{2+} ions are most probably involved with structural maintenance of the complex. Gla8 in prothrombin (gla7 in human PC), along with gla residues 17 (16), 21 (20), 27 (26), and 30 (29), are involved in complex formation with Ca-2, Ca-3, Ca-4, and Ca-5. All of these amino acid residues are crucial to the Ca\textsuperscript{2+}-dependent anticoagulant activity of APC, thus showing the importance of the integrity of the coordination of these Ca\textsuperscript{2+} ions to the structure and concomitant function of APC. The fact that partial Ca\textsuperscript{2+}-dependent anticoagulant activity is observed with mutations of some of these gla residues to D may revolve around the possibility that the side chain carboxyl group of D can substitute as a coordination donor for Ca\textsuperscript{2+}. If this is the case, a reorientation of the geometry of the affected complexes to accommodate the carboxyl group of D would occur, and the partial loss of activity would not be surprising. It has been proposed that the Ca\textsuperscript{2+}-induced conformational change and binding to PL is dependent on the presence of gla16 in human prothrombin (corresponds to gla17 in bovine prothrombin and gla16 in human PC).\textsuperscript{50} The importance of gla16 to the activity of APC is directly shown in the current study, and likely correlates to the important structural role proposed above for this residue. Similarly, the essential role (Table 2) of gla20 (gla21 in bovine prothrombin) in the Ca\textsuperscript{2+}-dependent APC anticoagulant activity of APC is also obvious from Fig 5 in that this residue is highly important for coordination of Ca-5 and, perhaps, Ca-6.

The nonessential character of gla14 in APC (gla15 in Fig 5) may be due to the fact that it only provides one coordination site for Ca-7, and another nonessential gla residue, gla19 (gla20 in Fig 5), provides the other two coordination sites for this same Ca\textsuperscript{2+}. Thus, it may be concluded that these gla residues, and perhaps Ca-7, do not play an essential anticoagulant function role in APC. Because gla19 in APC also provides one donor site for Ca-6, it is not surprising that some activity may be lost with a substitution for this gla residue, and concomitant possible weakening of the Ca-6/APC interaction. However, from the fact that only a small loss in activity is observed with [gla19D]r-APC, it is concluded that either the Ca-6/gla19 coordination site is relatively unimportant to the stability of Ca-6 binding to APC, that the mutant D19 can satisfy part of this function, and/or that Ca-6 is of lesser importance to the anticoagulant activity of r-APC. The activity (24% of wt-APC) found despite the loss of gla25 (gla26 in Fig 5) in APC can be explained if Ca-1 is important to this function and the loss of the two possible donor sites provided to
Ca-1/ APC stabilization by gla25 weakens that interaction. Because Ca-1 is also stabilized by two donor sites from the essential gla29 (gla30 in Fig 5), it may be retained in the complex (albeit more weakly), thus providing the lower level of activity observed (Table 3). Similar considerations may also govern the very low level of activity observed as a result of mutation of gla29. Lastly, while R15 does not provide sites for the coordination of Ca2+, the HH21 proton of R15 (R16 in Fig 5) is sufficiently close (0.23 nm) to the oxygen of the OE4 group of gla16 (gla17 in Fig 5) to form a hydrogen bond, which may stabilize a conformational state optimal for activity.

The fact that substitution for gla6 (gla7 in Fig 5) in r-APC did not result in a significant activity loss (Table 3) requires special consideration, because its apparent role in contributing to the stability of Ca-4 and Ca-5 would seemingly be important. Only one of the two carboxyl groups of this gla residue is involved as a donor site for these two Ca2+ ions, and it is possible that the carboxyl group of the substituted D residue can provide this same kind of coordination. Another explanation would be that loss of one of the seven possible coordination sites for Ca-4 and two of the possible five such sites from Ca-5, as a result of this mutation, did not significantly affect the abilities of these two Ca2+ ions to interact in a stable manner with the gla-domain.

In conclusion, we have assessed the individual roles of all gla residues in APC that are important to the in vitro anticoagulant activity of APC. In addition, we have attempted to correlate this information with known mutations in gla residues in symptomatic patients and with structural features of the gla domain/Ca2+ complex. Whereas this latter analysis has been derived from crystallographic data on the bovine prothrombin fragment 1/Ca2+ complex, the high degree of sequence homologies between these domains of prothrombin and PC justifies the approach. Further, the fact that this crystal structure shows such a strong correlation with the currently available functional data allows firmly based hypotheses to be forwarded and tested regarding the role of various amino acids in structure-activity relationships of APC.

ACKNOWLEDGMENT

The authors express their appreciation to Dr Alexander Tulinsky for providing the coordinates of the bovine prothrombin fragment 1/Ca2+ complex to this laboratory and for many helpful discussions.
REFERENCES


47. Koeberl DD, Bottema CDK, Buerstedde JM, Sommer SS: Functionally important regions of the factor IX gene have a low rate of polymorphism and a high rate of mutation in the dinucleotide Cpg. Am J Hum Genet 45:488, 1989


Role of individual gamma-carboxyglutamic acid residues of activated human protein C in defining its in vitro anticoagulant activity

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