Molecular Mechanism for Familial Protein C Deficiency and Thrombosis in Protein C<sub>Vermont</sub> (Glu<sup>20</sup> → Ala and Val<sup>34</sup> → Met)*

(Received for publication, August 1, 1994, and in revised form, September 16, 1994)

Deshun Lu, Edwin G. Bovill, and George L. Long

From the Departments of Biochemistry and Pathology, University of Vermont College of Medicine, Burlington, Vermont 05405-0068

The role of two protein C γ-carboxyglutamic acid domain mutations in familial thrombosis, protein C<sub>Vermont</sub> (Bovill, E. G., Tomczak, J. A., Grant, B., Bhushan, F., Pillemer, E., Rainville, I. R., and Long, G. L. (1992) Blood 79, 1456-1465), was investigated. Two single mutations (Glu<sup>20</sup> → Ala and Val<sup>34</sup> → Met) and the naturally occurring double mutation were created by site-directed mutagenesis and were expressed in human kidney 293 cells. Purified recombinant protein C with the mutation glutamate to alanine at position 20 and the double mutation were defective in the assay of activated partial thromboplastin time, factor Va activation, and fibrinolysis. Mutation from valine to methionine at position 34 has only a minor effect. Activation of Glu<sup>20</sup> mutants by thrombin-thrombomodulin was not enhanced by phospholipid vesicles and showed a different calcium dependence compared with the wild type, suggesting that Glu<sup>20</sup> is important in the interaction of the protein C Gla domain with a phospholipid-mediated site on the thrombomodulin molecule. Glu<sup>20</sup>-substituted protein C is not inhibited by calcium ion in its interaction with the calcium-dependent monoclonal antibody H-11, suggesting that this mutation has lost the calcium-induced, lipid-independent conformational transition of the protein C Gla domain. These data indicate that the loss of Glu<sup>20</sup> causes the major familial dysfunction of protein C to associate with phospholipid as well as to undergo Ca<sup>2+</sup>-dependent, lipid-independent conformational changes and are consistent with the importance of Glu<sup>20</sup> in both external and internal Ca<sup>2+</sup> binding based upon the x-ray-derived structure of the homologous Gla domain in bovine prothrombin.

Protein C (PC) is a vitamin K-dependent plasma protein consisting of a γ-carboxyglutamic acid (Gla) domain, two epidermal growth factor domains, and a classical trypsin-like serine protease domain (1). PC can be converted by thrombin complexed with thrombomodulin on the surface of endothelial cells (2) to activated PC (APC), which functions as an anticoagulant by proteolytically cleaving coagulant factors Va (3, 4) and VIIIa (5, 6). The first congenital PC deficiency was reported by Griffin et al. (7), and following reports (8, 9) led to the association of PC deficiency with thrombotic disease. Newborns with PC homozygous deficiency exhibit severe, life-threatening purpura fulminans (10), whereas heterozygotes from clinically affected families experience mainly thromboembolic disease in a much smaller percentage of biochemically affected individuals (11, 12). PC deficiency is classified into two categories: type I deficiency, in which both circulating PC antigenic and functional levels in heterozygotes are ~50% of the normal range; and type II deficiency, in which individuals have normal antigenic levels, but reduced functional activity. Bovill et al. (13) investigated type II PC deficiency in a family (protein C<sub>Vermont</sub>) with manifestations of both arterial and venous thrombosis. Direct DNA sequencing of PC exon 2 from genomic DNA of deficient individuals showed two nucleotide substitutions resulting in two point mutations in the Gla domain, Glu<sup>20</sup> → Ala and Val<sup>34</sup> → Met (13).

The Gla domain of PC contains 9 glutamic acid residues (14) that are post-translationally modified to γ-carboxyglutamic acid residues by vitamin K-dependent carboxylase (15). These γ-carboxyglutamic acid residues (positions 6, 7, 14, 16, 19, 20, 25, 26, and 29) are necessary for Ca<sup>2+</sup>-dependent lipid membrane association (16, 17) as well as a lipid-independent structural transition from a random to an ordered structure upon binding of divalent metal cations (18). The influence of individual amino acids of the PC Gla domain was extensively studied by Zhang and Castellino (19-21). They found that Gla residues at positions 7, 16, 19, 20, and 26 in the Gla domain of PC are essential for the anticoagulant activity of APC (19-21). To further understand the functional role of Gla domain residues and to understand the basis of PC dysfunction in familial (protein C<sub>Vermont</sub>) deficiency and thrombosis, we have studied the effects of the double mutation occurring in this affected kindred as well as each of the single mutations.

EXPERIMENTAL PROCEDURES

Materials—The PC Gla peptide (ANSFLγLYRSSL) was a generous gift from Dr. F. J. Castellino. Human plasma protein C was purified as described elsewhere (22). The copperhead snake Agkistrodon contortrix activator of PC (Protac<sup>27</sup>) and rabbit thrombomodulin (in 0.1% Lubrol detergent) were purchased from American Diagnostica Inc. (Greenwich, CT). Human factor V, human α-thrombin, and factor Xa were generous gifts from Dr. P. B. Tracy. Dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide was purchased from Haematologic Technologies Inc. (Essex Junction, VT) and was a generous gift from Dr. P. B. Tracy. Phospholipid vesicles composed of 75% L-palmityl-2-oleoylphosphatidylcholine and 25% L-palmityl-2-oleoylphosphatidylserine (PCPS) were prepared as described (23). Human melanoma two-chain tissue-type plasminogen activator (tPA) was purchased from Sigma. PC murine monoclonal antibodies HPC-2 and H-11 were provided by Dr. W. R. Church and J. Amiral (Diagnostica Stago, Paris, France), respectively. Horse anti-human PC polyclonal antibody was a generous gift from Dr. K. G. Mann.
Construction of Wild-type PC Expression Vector and Site-directed Mutagenesis—Full-length human PC cDNA, a product of two PC cDNA fragments obtained from primer-specific PCR amplification and cDNA/Ag11 library clones, respectively, was cloned into the EcoRI site of a SV40/adeno/pBR322-derived expression vector, pDJL24 (24). Site-directed mutagenesis was performed by the PCR-mediated method developed in the laboratory (25, 26) with the wild-type PC expression vector as template and is shown schematically in Fig. 1. The external PC primers used were as follows: B, 5'gggctcgagataaggga-CAATTGAGGAGGCTCACCTC-3'; and C, 5'gggtgaattcccgGGCGAACAAATaTGGATGACAC-3' for VaP4 (27), 29003

Activation of PC to APC and Amidolytic Assay—Activation of PC with the snake venom activator Protac was performed as described (35) with 10 PM to 20 PM of PC and 0.2 units of Protac per ml at 37 °C for 5 min at 4 °C for 16 h in 1 ml of 50 mM Tris (pH 7.4), 100 mM NaCl to achieve full activation. Activation of PC by thrombin was performed as described elsewhere (36) by incubating PC with a-thrombin (1:10, w/w) at 37 °C for 2 h in 20 mM HEPES (pH 7.4), 150 mM NaCl, and 5 mM EDTA. The mixtures were diluted with an equal volume of water and passed over a sulfopropyl-Sephadex column to remove the thrombin. APC was recovered from flow-through fractions by eluting the column with 10 mM HEPES (pH 7.4), 75 mM NaCl. The achievement of PC to APC was confirmed by the mobility difference between PC and APC on nonreduced SDS-PAGE visualized by Western blotting. The amidolytic activity of APC was measured by the hydrolysis of the synthetic substrate PCa as a function of time. Three-hundred microliters of synthetic substrate PCa (4 mM in H2O) was mixed with 800 μl of 1.0 mM CaCl2, 400 μl of 1.0 mM Tris (pH 7.4) and used as a stock to measure the APC chromogenic activity. The ability of APC to hydrolyze synthetic substrate was determined by adding 180 μl of various concentrations of the synthetic substrate PCa to 30 μl of APC (0.2 μg/ml) and monitoring the para-nitroaniline release by 405 nm absorbance increase using a microplate reader (Molecular Devices) at room temperature.

Activated Partial Thromboplastin Time (APTT) Assay—Quantitative determinations of APC were based on the prolongation of APTT. Staclot AT-III clotting assay of Diagnostic Stago (37). The prothrombinase assay mixture consisted of 2 μl HEPES/Tyrode's buffer (pH 7.4) with 5 μM CaCl2, 20 μM PCPs, and 100 nM human factor Va preincubated at 37 °C. Inactivation was initiated by the addition of APC to 0.5 nM, and 10 μl of the mixture was allowed to stand therefor 5 min at 37 °C. The clot formation was used to measure the cofactor activity of factor Va. The factor Va activity was measured as the cofactor required for thrombin formation in the prothrombinase assay (37). The prothrombinase assay mixture consisted of 2 ml HEPES/Tyrode's buffer, 1.39 μM prothrombin, 5 nM factor Xa, 20 μM PCPs, 3 μM dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide, and 5 mM CaCl2. The generation of fluorescence from binding of dansylarginine-N-(3-ethyl-1,5-pentanediyl)amide to the active site of thrombin was measured in a Perkin-Elmer LS-3B fluorescence spectrometer with an excitation wavelength of 335 nm and an emission wavelength of 565 nm. The initial rate of thrombin formation was proportional to the remaining cofactor activity (pH 7.4) factor Va under these conditions.

Fibrinolysis—tPA-induced fibrinolysis was performed as described by Bajzar et al. (38) with thrombin-activated, plasma-derived PC or rPC. The reaction was performed in a 96-well plate. Human plasma was dialyzed against 20 mM HEPES (pH 7.4), 150 mM NaCl and then diluted one-third with the same buffer. Aliquots of plasma were added to each well of the plate containing CaCl2, human melanoma tPA, and thrombin at final concentrations of 10 mM, 0.6 mM, and 6 mM, respectively, in a final volume of 250 μl in the absence and presence of 20 mM APC. The profile of clotting initiated by thrombin and subsequent fibrinolysis induced by tPA was measured by monitoring the turbidity at 405 nm at 3-min intervals by using a Thrombo reader. The lysine was judged as the time required for turbidity to decrease to 50% of plateau.

Calcium Dependence of PC Activation—Activation of PC by 20 mM thrombin or by 2 mM thrombin, 20 mM thrombomodulin (preincubated for 5 min at 37 °C) was performed by incubation of PC (0.5 μM) with thrombin or the thrombin-thrombomodulin complex (plus or minus 100 μM PCPs) in 20 mM Tris (pH 7.4), 150 mM NaCl, and 0.1% gelatin in the presence of varying concentrations of CaCl2 at 37 °C. At different time intervals after the start of activation, 30-μl aliquots were stopped by the addition of 10 μl of 0.5 μM antithrombin III and 150 units/ml heparin. APC was quantified by adding 100 μl of chromogenic substrate PCa (0.8 mM) and recording the increase in absorbance at 405 nm as a function of time. The activity of APC was defined as the change in absorbance/minute, and the initial rate of APC production was obtained by determining the slope of change in absorbance/minute versus time of activity for the determination of APC.

Epitope Mapping of PC—Binding of PC to monoclonal antibody H-11 (18) was performed in both solid and solution phase. All solutions were determined as described by Przybieski et al. (34). NH2-terminal sequence of PC were determined with an Applied Biosystems Model 470A protein sequencer.
The elements of the expression vector pDX are indicated SV40 enhancer; mutations in the PC Gla domain sequence; "Experimental Procedures." partition lender; injection of calcium ion. with 100 μl of o-phenylenediamine/hydrogen peroxide (8 mg, 8 μl in 25 ml of citrate/phosphate buffer (pH 5.0)) for 15 min, which was then stopped by the addition of 50 μl of 4 N sulfuric acid. The absorbance at 490 nm of the product was measured with a kinetic microplate reader. For the solution-phase binding assay, 1.1 ng PC and 4 ng H-11 were incubated at 37 °C for 3 h in the absence or presence of varying concentrations of CaCl₂ in 0.1% BSA/TBS and aliquoted to a 96-well plate that had been coated with horse anti-human PC polyclonal antibody (5 μg/ml) and blocked with 1% BSA. Free and H-11-complexed PCs were captured by PC antibody, and free H-11 was washed away with three washes of TBS, 0.1% Tween 20. After overnight incubation at 4 °C, H-11 complexed with PC on the plate was detected as described above. The controls in both solid- and solution-phase studies were without PC or H-11 in the system. The final absorbance of each well was generated by the subtraction of the mean of two controls. Ca²⁺-inhibited binding of H-11 to PC was expressed as the percentage of absorbance compared with the absorbance without CaCl₂.

RESULTS

PCR-mediated site-directed mutagenesis was used to generate two single mutants (Glu²⁹ → Ala and Val³⁴ → Met) and the double mutant (Glu²⁹ → Ala/Val³⁴ → Met) of protein C (Fig. 1). Wild-type PC cDNA and corresponding segments with the above mutations were ligated into the SV40/adenovirus mammalian expression vector pDX and used to derive stable cell cultures to express rPC. After the selection and screening, the expression levels of stable transfectants used for the production of recombinant protein were 2.8, 2.1, 4.0, and 1.6 μg/ml, for wild-type rPC and Glu²⁹ → Ala, Val³⁴ → Met, and Glu²⁹ → Ala/Val³⁴ → Met mutant rPCs, respectively.

All forms of PC were purified to homogeneity based upon SDS-PAGE (Fig. 2). Chemical analysis of Gla and 8-hydroxyaspartate indicated that each construct was properly modified (Table I). NH₂-terminal analysis of all forms of PC (first 10 amino acid residues) revealed the appropriate sequences of light and heavy chains for each cycle, indicating that the recombinant proteins were appropriately processed into two-chain forms and that the propeptide had been properly removed (data not presented).

Both Protac- and thrombin-activated PCs were assessed by chromogenic substrate PCa hydrolysis as a function of time. The fully activated PC achieved similar specific activities with the chromogenic substrate for all of the plasma-derived and recombinant PCs. The turnover numbers of the synthetic substrate PCAs were 153 ± 5 and 137 ± 6 Δ OD/min/μg of APC for plasma-derived PC and four rPCs under our conditions, respectively. Based upon equal chromogenic substrate activity of each construct, APCs were adjusted to the same level of serine protease activity for use in the functional assays described below.

Protac-activated PCs with equal chromogenic activities were used for APTT and factor Va inactivation assays. The prolongation of clotting time for each APC species is shown in Fig. 3.
The wild-type rAPC prolonged clotting time was identical to that for plasma-derived APC, and the clotting time obtained with Val34→Met mutant rAPC was slightly shorter than that obtained with wild-type rAPC. However, clotting times for Glu20→Ala mutant APC and double mutant APC were the same as that for the buffer control alone. These results indicate the Glu20 mutation as the cause of PC dysfunction. Similar results were obtained for the APC inactivation of factor Va, as shown in Fig. 4. In the presence of 20 μM PCPS, factor Va retained 20% of the initial cofactor activity after 15 min of incubation with wild-type rAPC or plasma-derived APC, and Val34→Met mutant rAPC inactivated factor Va identically to wild-type rAPC. In contrast, factor Va still retained 80% of the initial cofactor activity after 15 min of incubation with Glu20→Ala mutant rAPC or double mutant rAPC (Fig. 4).

In addition to its anticoagulant activity, the tPA-induced fibrinolytic activity of APC was also tested in a cell-free system (38). The lysis time was shortened in the presence of plasma-derived APC or wild-type rAPC and Val34→Met mutant rAPC, but only slightly by the Glu20→Ala mutant rAPC, but only slightly by the Glu20 single and double mutants (Fig. 5). The profibrinolytic effect of APC has been attributed specifically to inhibition of the activation of prothrombin in cell-free plasma (39). The decrease in fibrinolytic activity and the factor Va inactivation exhibited by Glu20→Ala mutant APC in our studies are both consistent with this hypothesis.

All forms of PC were studied for activation by thrombin alone and by the thrombin-TM complex in both the absence and presence of phospholipid vesicles. Our results (Fig. 6) indicate that thrombin alone activates all constructs into APC in a similarly slow pattern and that the thrombin-TM complex accelerates the activation process (data shown for only wild-type rPC and Glu20→Ala mutant rPC). The presence of phospholipid vesicles had an accelerating effect on the activation process by the thrombin-TM complex for wild-type rPC (Fig. 6A) and Val34→Met mutant rPC, similar to that for plasma-derived PC (data not shown). In contrast, phospholipid vesicles had no effect on activation of Glu20→Ala mutant rPC (Fig. 6B) and double mutant rPC (data not shown). The Ca2+-dependent activation of wild-type rPC (Fig. 6A) and Glu20→Ala mutant rPC (Fig. 6B) by the thrombin-TM complex is shown in Fig. 7. As indicated, activation by the thrombin-TM complex of wild-type rPC was hyperbolic as a function of CaCl2 concentration in the absence of phospholipid, whereas the addition of PCPS changed the CaCl2 dependence into a sigmoidal curve. Similar curves were obtained for Val34→Met mutant rPC (data not shown).

In contrast, hyperbolic curves were obtained for Glu20→Ala mutant rPC and double mutant rPC (data not shown) in the pres-
A single mutant rPC. Mutation is also seen by epitope mapping of PC by the calcium-Ala mutant rPC) in familial thrombosis were the first reported naturally occurring proteins. As shown in Fig. 8, increasing concentrations of CaCl₂, dependence of lipid-independent monoclonal antibody H-11 (18, 19) or with 2 nM thrombin, 20 nM TM in the presence (A, B; [Glu --- Ala] rPC) of 100 µM PCPS. Aliquots were drawn at the time intervals indicated, and APC was assayed as described under "Experimental Procedures." Error bars in the curves represent the standard deviation from three independent experiments. Essentially identical results (not shown in the figure) were obtained for the Val⁴⁰ → Met mutant versus wild-type rPC and for the double mutant versus Glu²⁰ → Ala single mutant rPC.

DISCUSSION

Two missense mutations (Glu²⁰ → Ala and Val⁴¹ → Met) found in a kindred (protein C Vermont) with severe type II protein C deficiency and exhibiting a high penetrance of PC-associated familial thrombosis were the first reported naturally occurring mutations in the Gla domain of PC (13). To understand the structural and functional consequences of the mutations, both appropriate single mutant, double mutant, and wild-type PC recombinant proteins were expressed in human kidney 293 cells and purified to homogeneity. Chemical characterization indicated that the recombinant proteins appeared to be properly processed in regard to γ-carboxylation of glutamic acid residues, β-hydroxylation of aspartic acid, and proteolytic processing. SDS-PAGE also indicated that a similar amount of glycosylation had also occurred in all forms.

Abolition of clotting time prolongation in the APTT assay in the case of the Glu²⁰ mutants and only a minor change for the Val⁴¹ mutant indicate that the mutation Glu²⁰ → Ala is primarily responsible for the dysfunction of PC in the affected family members. In vitro studies involving the inactivation of purified human factor Va more specifically address the issue of Glu²⁰ mutation. Cleavage by APC at the lipid-dependent Arg²⁴⁸ site of the human factor Va heavy chain, required for full inactivation of factor Va cofactor activity, is markedly decreased for the Glu²⁰ mutant (41). Finally, in a cell-free system (38), the Glu²⁰ mutants are ineffective in enhancing tPA-induced fibrinolysis. Consequently, the results from both the anticoagulant and fibrinolytic studies strongly suggest that the physiological defect in the reported naturally occurring PC variant is the lack of Glu²⁰ with subsequent loss of the Ca²⁺-dependent conformational change and lipid association. This interpretation is consistent with reports from Zhang et al. (19) and Zhang and Castellino (20, 21) involving the systematic replacement of amino-terminal Glu residues by Asp in PC.

Fig. 6. Stimulation of the thrombin-TM complex-catalyzed activation of rPC by PCPS. rPC at 0.5 µM (A, wild-type rPC; B, Glu²⁰ → Ala mutant rPC) in 20 mM HEPES, 150 mM NaCl, 0.1% gelatin, 5 mM CaCl₂ (pH 7.4) was incubated at 37 °C either with 20 nM α-thrombin (- - - -) or with 2 nM thrombin, 20 nM TM in the presence (A, B; [Glu --- Ala] rPC) of 100 µM PCPS. Aliquots were drawn at the time intervals indicated, and APC was assayed as described under "Experimental Procedures." Error bars in the curves represent the standard deviation from three independent experiments. Essentially identical results (not shown in the figure) were obtained for the Val⁴⁰ → Met mutant versus wild-type rPC and for the double mutant versus Glu²⁰ → Ala single mutant rPC.

Fig. 7. Ca²⁺ dependence of protein C activation by the thrombin-TM complex. The initial rate of PC activation at varying concentrations of CaCl₂ was related to that at saturating CaCl₂. A, wild-type rPC was activated by the thrombin-TM complex in the presence (Δ) or absence (□) of 100 µM PCPS. B, Glu²⁰ → Ala mutant rPC was activated by the thrombin-TM complex in the presence (■) or absence (○) of PCPS.
with various concentrations in calcium-dependent activation and conformational change of thrombin-TM complex (Fig. 7).

Lipid resulted in sigmoidal curves for wild-type rPC and Val34 naturally occurring mutant PC are due to the mutation at the activation of PC. In our studies, all forms of PC exhibited is in contrast to retained hyperbolic curves and Hill coefficients significantly greater than 1.0, indicating complex conformational interactions (42). This is in contrast to retained hyperbolic curves and Hill coefficients = 1 for both Glu

The results (Figs. 6 and 7) also suggest that the lipid-dependent component of PC activation by the thrombin-TM complex has been lost upon Glu

TABLE II

| Plasma-derived PC | Wild-type rPC | Glu\textsuperscript{30} → Ala mutant rPC | Val\textsuperscript{54} → Met mutant rPC | Glu\textsuperscript{30} → Ala/Val\textsuperscript{54} → Met mutant rPC |
|-------------------|---------------|----------------------------------------|----------------------------------------|-------------------------------------------------|
| \([\text{Ca}^{2+}]\) \(\text{mM}\) | \(\text{max}\) | \(0.21\) | \(0.20\) | \(0.13\) | \(0.29\) | \(ND\) |
| \(\text{Hill coefficient}\) | \(0.92\) | \(0.94\) | \(1.04\) | \(0.98\) | \(1.32\) | \(ND\) |
| \(\text{PCPS} - \text{PCPs}\) | \(1.07\) | \(2.01\) | \(0.20\) | \(0.20\) | \(0.97\) |

\* Concentration of \(\text{Ca}^{2+}\) with half-maximum rate of activation determined by computer fit.

\* ND, not determined.

Fig. 8. Inhibition by \(\text{Ca}^{2+}\) of murine monoclonal antibody H-11 binding to plasma-derived PC and rPC. Binding of H-11 to PC (○, plasma-derived; ▲, wild-type; ■, Glu\textsuperscript{30} → Ala; ▼, Val\textsuperscript{54} → Met; ●, Glu\textsuperscript{30} → Ala/Val\textsuperscript{54} → Met) at various concentrations of \(\text{CaCl}_2\) was determined as described under “Experimental Procedures.” A, result of solid-phase binding of H-11 to PC; B, result of solution-phase binding. The error bars represent the standard deviation of the mean from four independent experiments.

Our results suggest that Glu\textsuperscript{30} also plays an important role in the activation of PC. In our studies, all forms of PC exhibited \(\text{Ca}^{2+}\) concentration-dependent hyperbolic activation by the thrombin-TM complex (Fig. 7 and Table II) in the absence of phospholipid. However, activation in the presence of phospholipid resulted in sigmoidal curves for wild-type rPC and Val\textsuperscript{54} → Met mutant rPC and Hill coefficients significantly greater than 1.0, indicating complex conformational interactions (42). This is in contrast to retained hyperbolic curves and Hill coefficients = 1 for both Glu\textsuperscript{30} → Ala and Glu\textsuperscript{30} → Ala/Val\textsuperscript{54} → Met mutant rPCs. The implication of these observations is that differences in calcium-dependent activation and conformational change of naturally occurring mutant PC are due to the mutation at Glu\textsuperscript{30}. The results (Figs. 6 and 7) also suggest that the lipid-dependent component of PC activation by the thrombin-TM complex has been lost upon Glu\textsuperscript{30} mutation, but not the lipid-independent component. Similar curves to those presented in Fig. 7 were obtained by Galvin et al. (43) using bovine thrombin, reconstituted rabbit thrombomodulin, and bovine wild-type or Gla-domainless PC. In their study, they also observed a lipid-dependent decrease in apparent \(K_a\) for PC, but no change for Gla-domainless PC (43). Freyssinet et al. (44), using purified human components, observed lipid enhancement of the thrombin-TM complex activation of PC, but not of Gla-domainless PC, similar to our results shown in Fig. 6. However, in contrast to the results of Galvin et al. (43), the effect of phospholipid in the human system was to increase \(V_{\text{max}}\) = 3-fold, with no change in apparent \(K_a\) for PC versus Gla-domainless PC (44). Extending these results to our study may offer a plausible explanation for the apparent severity and high penetrance of thrombotic disease in family members with the Glu\textsuperscript{30} mutation. The lipid-independent binding of Gla-domainless PC (44) or Glu\textsuperscript{30} → Ala mutant PC (this study) to the thrombin-TM complex appears to be the same as for the wild-type molecule. If this were the case in vivo under conditions in which the thrombin-TM complex is limiting relative to PC concentration, binding of mutant PC would have the effect of a competitive substrate inhibitor of wild-type PC binding and activation. Consequently, Glu\textsuperscript{30} → Ala mutant PC, in addition to being ineffective in converting membrane-bound factors Va and VIIIa to their inactive forms, would also compromise the thrombin-TM activation of normal circulating PC by acting as a competitive inhibitor.

Finally, epitope mapping by calcium-dependent antibody binding of H-11 provides additional insight into the calcium ion-induced conformational change of the Gla domain of PC. \(\text{Ca}^{2+}\) has previously been shown to abrogate binding of H-11 to PC, but has no effect on H-11 reactivity toward descarboxyl-PC (28). In our studies, \(\text{Ca}^{2+}\) reduces binding of wild-type rPC and Val\textsuperscript{54} → Met mutant rPC to H-11, whereas binding of both Glu\textsuperscript{30} mutants to H-11 is significantly less responsive to the presence of \(\text{Ca}^{2+}\). This suggests that the Glu\textsuperscript{30} mutants lack the \(\text{Ca}^{2+}\)-induced, lipid-independent conformational change of the PC molecule involved in H-11 recognition; and taken with the above activation results, may represent the same conformational change required for the lipid-independent component of PC activation by the thrombin-TM complex.

Energy minimization (45, 46) has been used to generate a three-dimensional model for the PC Gla domain in the presence of \(\text{Ca}^{2+}\) ion based upon the x-ray structure for the bovine prothrombin fragment 1-\(\text{Ca}^{2+}\) complex (47). The resulting structure is not dramatically different from that for bovine prothrombin fragment 1.2 Based upon the derived structure for

\* D. Gaffney and D. Lu, unpublished results.
PC, it is possible to structurally interpret changes in the functional properties of the mutant molecules reported in this study. The x-ray structure for the xin prothrombin fragment 1-Ca\textsuperscript{2+} complex indicates that Gla\textsuperscript{20} (residue 20 in PC) critically interacts with both surface-exposed Ca-6 and buried Ca-5 (19).

Exposed Ca-6 and Ca-7 have been suggested to be involved in the Ca\textsuperscript{2+}-mediated, negatively charged lipid membrane surface-dependent processes (47). Buried Ca-1 through Ca-5, on the other hand, have been proposed to be involved in an essential Gla-dependent conformational change, which does not involve lipid membrane. The model predicts that in the case of PC, Gla\textsuperscript{20} is similarly involved in the binding of Ca-5 and Ca-6 and that mutation at this site would alter both lipid-dependent and independent properties involving Ca\textsuperscript{2+} ions. Specifically, we propose that the observed changes in APTT, factor Va inactivation, fibrinolytic activity, and a portion of the activation process, all requiring negatively charged lipid membrane, are due to the loss of exposed Ca-6 binding upon mutation of Gla\textsuperscript{20}. Furthermore, we propose that changes in H-11 monoclonal antibody binding and lipid-independent PC activation are due to the loss of Gla\textsuperscript{20} interaction with buried Ca-5 and the associated lipid-independent conformational change of the Gla domain. In regard to the naturally occurring mutation, also including Val\textsuperscript{34} → Met, the x-ray structure predicts that Met\textsuperscript{34} in PC resides in a loop joining two α-helices of the Gla domain, with the R group distal from any of the Ca\textsuperscript{2+} ions (47). Consequently, this mutation would be expected to have little effect on the structure and function of the protein, consistent with our experimental observations.

In summary, this study has investigated the functional significance of each of two naturally occurring mutations in the Gla domain of protein C in a kindred exhibiting severe type II familial PC deficiency and thrombosis. The results indicate that the functional abnormalities can be attributed to the Glu\textsuperscript{20} → Ala mutation and consequent decrease in thrombin-TM activation of PC on the vascular endothelial surface as well as diminished intrinsic APC anticoagulant and profibrinolytic activity.

Acknowledgments—We thank Dr. P. B. Tracy for directions relating to the factor Va inactivation assay. We also thank Dr. F. J. Castellino for the PC Gla peptide and Dr. W. J. VanDusen for the analysis of γ-carboxyglutamyl acid and β-hydroxyaspartic acid.

REFERENCES
1. Long, C. L. (1987) J. Clin. Invest. 80, 1379-1373
2. Broockman, A. W., Veithkamp, J. J., and Bertina, R. M. (1983) N. Engl. J. Med. 309, 340-344
3. Hovoriou, M. H., Conard, J., Bertina, R. M., and Samama, M. (1984) Br. Med. J. 288, 1285-1287
4. Seligsohn, U., Berger, A., Aven, D., Rubin, L., Attias, D., Zivelin, A., and Rapaport, S. I. (1984) N. Engl. J. Med. 310, 550-553
5. Mann, K. G., and Bovill, E. G. (1980) in Protein C and Related Anticoagulants (Bryant, D. F., and Drehman, W. N., eds) pp. 119-123, Porlou, P. G., and Long, G. L. (1992) Blood 79, 1456-1465
6. Beckmann, R. J., Schmidt, R. J., Sattarne, R. F., Finkel, J., Crabtree, G. R., and Long, G. L. (1988) Virology A, 5235-5247
7. Suttie, J. W., Hockstein, J. A., Engelke, J., Hopfgartner, A., Ehrich, H., Bang, N. U., Belagaje, S. M., Schoner, B., and Long, O. L. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 654-657
8. Nelson, M. G., Kisiel, W., and DiScipio, R. G. (1978) Biochemistry 17, 2124-2139
9. Malmberg, M. E., Church, W. R., Haley, P. E., and Krishnaswamy, S. (1990) Blood 76, 1-16
10. Church, W. R., Boulanger, L. M., Messier, T. L., and Mann, K. G. (1989) J. Biol. Chem. 264, 17882-17887
11. Zhang, L., Jiang, A., and Castellino, F. J. (1993) Blood 80, 942-952
12. Zhang, L., and Castellino, F. J. (1992) J. Biol. Chem. 267, 26078-26084
13. Zhang, L., and Castellino, F. J. (1993) J. Biol. Chem. 268, 12040-12045
14. Baji, E. R., Rapaport, S. L., Maki, S. I., and Brown, S. F. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 151-124
15. Barenholz, Y., Gibbes, D., Litmann, B. J., Goll, J., Thompsom, T. E., and Bajaj, E. G. (1990) Blood 79, 119-123
16. Bruegy, D. E, and Drohan, W. N., eds) pp. 119-123, Porlou, P. G., and Long, G. L. (1992) Blood 79, 1456-1465
17. Freshney, R. I. (1987) Culture of Animal Cells: A Manual of Basic Technique, pp. 127-154, Alan R. Liss, Inc., New York
18. Church, W. R., Bhusan, F. H., Mann, K. G., and Bovill, E. G. (1989) Blood, 74, 2418-2425
19. Van, S. C. B., Pazzano, P., Chao, Y. B., Walls, J. D., Berg, D. T., McClure, D. B., and Grinell, B. W. (1990) Bio/Technology 8, 655-660
20. Morrissey, J. H. (1984) Anal. Biochem. 137, 307-310
21. Lamsdill, U. K. (1970) Nature 279, 869-865
22. Tsewstern, H., Stachelin, L., and Gorden, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4350-4354
23. Kudowa, M., and Katayama, K. (1981) Anal. Biochem. 117, 259-265
24. Pryase, C. T., Staggers, J. E., Ramjit, H. G., Masson, D. G., Senn, A. M., Bennett, C. D., and Friedman, P. A. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 7586-7590
25. Zhang, L., and Castellino, F. J. (1990) Biochemistry 29, 1308-13084
26. Solymos, S., Tucker, M. M., and Tracy, P. B. (1988) J. Biol. Chem. 263, 14844-14909
27. Nesheim, M. E., Katzmann, J. A., Tracy, P. B., and Mann, K. G. (1981) Methods Enzymol. 90, 249-275
28. Bajaj, L. S., and Davie, E. W., and Nesheim, M. J. (1990) J. Biol. Chem. 265, 10949-10954
29. Bajaj, L., and Nesheim, M. (1993) J. Biol. Chem. 268, 8608-8616
30. Perutz, M. F. (1970) Nature 226, 726-739
31. Lu, D., Kalafatis, M., Mann, K. G., and Long, G. L. (1994) Blood 84, 687-690
32. Esmon, N. L., DeBeull, L. E., and Esmon, C. T. (1981) J. Biol. Chem. 258, 5548-5553
33. Galivan, J. B., Kurokawa, S., Moore, K., Esmon, C. T., and Esmon, N. L. (1987) J. Biol. Chem. 262, 2195-2205
34. Freyssinet, J., Gauchy, J., and Cazeneuve, J. (1980) Biochem. J. 218, 199-2017
35. Weiner, S. J., Kolman, P. A., Case, D. A., Singh, U. C., Alagana, G., Profeta, S., Jr., and Weiner, P. (1984) J. Am. Chem. Soc. 106, 765-784
36. Weiner, S. J., Kolman, P. A., Nguyen, D. T., and Case, D. A. (1986) J. Comp. Mol. Biol. 209, 230-252
37. Soriano-Garcia, M., Padmanabhan, K., de Vos, A. M., and Tulinsky, A. (1992) Biochemistry 31, 2554-2566