Protein C\textsubscript{Vermont}: Symptomatic Type II Protein C Deficiency Associated With Two GLA Domain Mutations

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This study investigates type II protein C deficiency in a family with manifestations of both arterial and venous thrombosis. Of 64 members of the kindred, 14 have been tested and 7 have PC deficiency. Among affected individuals (n = 7), mean protein C levels by different assays were as follows: enzyme-linked immunosorbet assay (ELISA), 3.8 \mu g/mL (2.1 to 4.3 \mu g/mL): amidolytic with venom activator, 115% (60% to 140%); clotting with venom activator, 42% (23% to 59%). The mean ratio of clotting to amidolytic assays for the affected individuals was 0.37 compared with a normal range of 0.8 to 1.2. Thus, the affected individuals have normal total protein C and their activated protein C has a normal active site assessed by chromogenic substrate; however, they have markedly diminished clotting activity. Immunoassay and chromatography data suggested an abnormality of carboxylation in the gamma carboxyglutamic acid (Gla) domain.

THE SERINE PROTEASE precursor protein C is a 62 Kd, disulfide-linked, two-chain vitamin K-dependent plasma glycoprotein.\textsuperscript{1} The amino acid sequence and domain organization have been derived from analysis of human liver cDNAs.\textsuperscript{2,3} The amino terminal region contains the metal ion binding \(\gamma\) carboxy glutamic acid residues (Gla) and constitutes the Gla domain. The Gla domain is essential for lipid membrane binding by protein C in the presence of \(Ca^{2+}\) ions. Lipid membrane binding is important for thrombin/thrombomodulin activation of protein C\textsuperscript{4} and for localization of the active enzyme to membrane bound cofactors V and VIII.\textsuperscript{5} Thrombin bound to the integral endothelial cell membrane protein thrombomodulin cleaves protein C at Arg\textsubscript{95}/Leu\textsubscript{96} of the heavy chain releasing an activation peptide and generating activated protein C (APC).\textsuperscript{6} The Gla domain is essential for the membrane-bound thrombin-thrombomodulin activation of protein C. APC proteolytically degrades the procoagulant cofactors Va and VIIIa, thereby downregulating the coagulation cascade. The anticoagulant activity of APC requires activation of the zymogen, and normal substrate recognition and proteolytic activity of the enzyme. Abnormalities of any of these functional components of APC may lead to decreased anticoagulant activity. Protein S, another vitamin K-dependent protein, acts as a cofactor for APC and markedly enhances protein C-mediated degradation of factors Va and VIIIa.\textsuperscript{7} APC also appears to enhance fibrinolysis, although the mechanism for this latter effect is poorly understood.\textsuperscript{8,9} Thus, protein C plays a central role in the regulation of coagulation.

Homozygous protein C-deficient individuals with less than 1% protein C activity develop life-threatening purpura fulminans at birth, which is fatal if not treated with protein C-containing blood products or concentrates.\textsuperscript{10} A number of investigators have reported families in which diminished levels of protein C, consistent with a heterozygous deficiency state, have been associated with recurrent thromboembolic disease.\textsuperscript{11,16} The majority of these deficiency states have been characterized as type I deficiencies with equal reductions of biologic and immunologic activity of protein C.

A smaller number of families have been described with type II deficiency characterized by a higher level of immunologic than biologic activity.\textsuperscript{17,18} Among the families with type II deficiency, resolution of the defect at the genomic level has been achieved in only one family,\textsuperscript{19} in which an Arg\textsubscript{360} (CGG) to Trp(TGG) disrupts the cleavage site for thrombin-thrombomodulin. Four families with a type II protein C deficiency have been described with normal plasma concentrations of protein C measured by immunologic and amidolytic assays but low concentrations of protein C measured by clotting assay.\textsuperscript{19,22,24,27} These four families may have defects involving their Glu domains because the normal amidolytic activity of their protein C tends to exclude abnormalities of either activation peptide cleavage or the serine protease catalytic site of APC, although abnormalities of substrate recognition sites could also produce similar observations. None of these four families with type II deficiency have been analyzed at the genomic level. In this study, we report a family with a hereditary thrombotic diathesis associated with type II protein C deficiency characterized by two Glu domain mutations (Glu\textsubscript{99} to Ala and Val\textsubscript{142} to Met).

MATERIALS AND METHODS

Patient selection and clinical assessment. Participants for the study were recruited from a single kindred of 64 members over five generations. All the investigated subjects had complete medical

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histories taken by the investigators. Thrombotic events were corroborated by physician and hospital records when possible. The presence of risk factors associated with the thrombotic events (eg, pregnancy, surgery, infection, trauma, birth control pill) was noted. This study was approved by the Human Experimentation Committee of the University of Vermont College of Medicine.

Collection and storage of blood samples. For plasma collection, venipuncture was performed using a two-syringe technique with 19- or 21-gauge butterfly infusion sets. The blood was drawn into silicon coated glass Vacutainer (Becton Dickinson, Mountain View, CA) tubes containing an anticoagulant solution of 3.8% (wt/vol) trisodium citrate at a 1:9 ratio (vol/vol) of anticoagulant to blood. The blood was then centrifuged at 3,000g at 4°C for 15 minutes. The platelet-poor plasma was collected, aliquoted, and stored at −70°C in 2-mL cryovials (Corning Glass Works, Corning, NY) before assay.

Assays. Protein C antigen was measured by enzyme-linked immunosorbent assay (ELISA) as previously described. The interassay coefficient of variation (CV) of the protein C assay is 4% (n = 10) with a reference interval of 2.1 to 4.3 μg/mL or 66% to 134% of normal. Amidolytic and clotting functional assays for protein C were purchased from American Bioproducts (Parsippany, NJ) and performed according to the manufacturer’s directions. The reference ranges for these assays expressed as percent of a normal pooled plasma were 80% to 154% and 77% to 149%, respectively. The interassay CV for the functional protein C assays are 7% (n = 19) and 10% (n = 13), respectively. Both the amidolytic and clotting protein C assays use the same method of protein C activation. An extract from the venom of Agkistrodon contortrix is added to patient plasma specifically cleaving the activation peptide independently of thrombin/thrombomodulin. The activated plasma is then added to protein C-deficient plasma and the proteolytic activity of APC quantitated by either an amidolytic or clotting end point. Free and bound protein S, antithrombin III, plasminogen activator inhibitor-1, prevenous and postvenous occlusion tissue plasminogen activator, plasminogen, thrombin time, and activated partial thromboplastin time were performed as described.

Assays for desaccharbopy protein C and prothrombin were performed as modified from our previously described method using the murine monoclonal antibody (MoAb) H-11 that recognizes an epitope expressed by desaccharbopy vitamin K-dependent factors II, VII, X, and protein C. The interassay CV for the protein C H-11 assay is 4% (n = 19) and the interassay CV for the prothrombin H-11 assay is 13% (n = 17). Plasma pools from normal individuals and individuals who had been on stable warfarin therapy for at least 1 month were prepared as previously described from 25 to 35 donors. One warfarin plasma pool was used to construct a standard curve and another warfarin pool and a normal plasma pool were used as controls.

In brief, 96-well Nunc immunoplates (Roskilde, Denmark) were coated with 100 μL of 30 μg/mL murine monoclonal anti-protein C antibody HPC-2 in 25 mmol/L bicarbonate buffer, pH 9.5. The plates were then washed and blocked with 2% bovine serum albumin (BSA) in 20 mmol/L Tris, 0.15 mmol/L NaCl, pH 7.4 (TBS). Reference and test plasmas were diluted 1:2 to 1:32 in 0.1% BSA/TBS. Normal and warfarin control plasmas were run at a 1:8 dilution. The blocking solution was aspirated, the plates washed with TBS, 0.1% Tween-20, and 100 μL of appropriately diluted plasma was added to each well and the plate incubated at room temperature for 1.5 hours. The plate was then washed three times with the wash buffer and 100 μL of the horseradish peroxidase-conjugated H-11 antibody diluted in 0.1% BSA/TBS, and 10 mmol/L CaCl₂ was added to each well and incubated for 1 hour at room temperature. The plate was washed five times with the wash buffer and the o-phenylenediamine substrate was added. After 7 minutes of incubation in the dark, 50 μL of 4 mol/L H₂SO₄ was added to each well to stop the color development. The plates were read at 490 nm. The dynamic range of the assay extended from the 3% to 50% of the PIVKA protein C present in the warfarin plasma reference pool. Pooled plasma from patients on stable warfarin therapy was used to construct a reference curve. Results were reported as a percent of the reference generated by the reference pool with 100% equivalent to the abnormal desaccharbopy protein C present in 1 mL of the reference pool.

In the H-11 prothrombin assay, Wells were coated with a burro polyclonal antiprosthyrombin fragment pre-1 antibody at a concentration of 10 μg/mL and blocked with 1% BSA/TBS. Reference, control, and patient samples were diluted in 0.1% BSA/TBS, 0.1% Tween-20. The reference curve was constructed from 1:32 to 1:1,024 dilutions of the reference plasma. The dynamic range of the assay extended from 0.009% to 1.5% of the abnormal desaccharbopy prothrombin present in the reference plasma pool. Patient samples were quantitated similarly to the H-11 protein C assay.

Protein purification. Protein C was purified from patient and normal plasma using the method of Yan et al., modified to include a final immunoaffinity purification step as previously described. The Q-Sepharose fast flow anion exchange resin (Pharmacia, Uppsala, Sweden) was equilibrated with a running buffer of Tris 0.02 mol/L, 0.15 mol/L NaCl, pH 7.4 (TBS), 0.005 mol/L Benzmide HCl. Citrated plasma diluted 1:1 with TBS, 0.005 mol/L Benzmanide HCl adjusted to 0.002 mol/L EDTA was batch adsorbed with a 3:100 vol/vol ratio of resin to plasma. The resin was washed until baseline 280 nm absorbance was attained with TBS, 0.005 mol/L Benzmanide HCl, 0.002 mol/L EDTA and then eluted in two steps. The first elution was with TBS containing 10 mmol/L CaCl₂ and the second elution was with TBS adjusted to 1 mol/L NaCl. Yan et al. have reported that this scheme is effective in resolving γ-carboxylated (CaCl₂ eluted) protein C from non- or under-carboxylated (NaCl eluted) protein C. The eluates were assayed for protein C by immunoassay as described above and then run over an immunoaffinity column consisting of murine monoclonal anti-protein C antibody HPC2 coupled to sepharose 4B equilibrated in the TBS, 1 mol/L NaCl as previously described. The immunoadsorbed proteins were eluted with 3 mol/L NaSCN and immediately dialyzed into TBS. The eluates were assayed for protein C concentration by immunoassay as described above and evaluated by 7.5% to 15% gradient polyacrylamide gel electrophoresis in the presence of 2% sodium dodecyl sulphate (SDS) as previously described. The immunoadsorbed proteins were eluted with 3 mol/L NaSCN and immediately dialyzed into TBS. The eluates were assayed for protein C concentration by immunoassay as described above and evaluated by SDS-PAGE and immunoblotting. The eluates were eluted with TBS containing 0.005 mol/L CaCl₂ and the second elution was with TBS adjusted to 1 mol/L NaCl. Yan et al. have reported that this scheme is effective in resolving γ-carboxylated (CaCl₂ eluted) protein C from non- or under-carboxylated (NaCl eluted) protein C. The eluates were assayed for protein C concentration by immunoassay as described above and then run over an immunoaffinity column consisting of murine monoclonal anti-protein C antibody HPC2 coupled to sepharose 4B equilibrated in the TBS, 1 mol/L NaCl as previously described. The immunoadsorbed proteins were eluted with 3 mol/L NaSCN and immediately dialyzed into TBS. The eluates were assayed for protein C concentration by immunoassay as described above and evaluated by 7.5% to 15% gradient polyacrylamide gel electrophoresis in the presence of 2% sodium dodecyl sulphate (SDS) as previously described.

Southern hybridization of genomic DNA. Genomic DNA was isolated from peripheral blood cells, digested, and transferred to NitroPlus 2000 (Micron Separations Inc, Westborough, MA) membranes as previously described. Membranes were baked at 80°C for 2 hours. Southern hybridization was performed using the Genius nonradioactive DNA detection kit (Boehringer Mannheim, Indianapolis, IN), according to the manufacturer’s instructions, with the following modifications: membranes were prehybridized, hybridized at 65°C, and then rinsed at 65°C in 1X SSC (150 mmol/mL NaCl, 15 mmol/L sodium citrate, pH 7.0), 0.1% SDS, followed by incubation for 4 hours with anti-digoxigenin-alkaline phosphatase conjugate diluted 1:2,000 in 100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5, 0.5% (wt/vol) blocking reagent. The protein C probe consisted of a 1.75-kb human protein C cDNA fragment containing the entire coding region of protein C. The fragment was labeled by random-primed incorporation of digoxigenin-11-β-UTP per the manufacturer’s instructions (Boehringer Mannheim).

Polymerase chain reaction (PCR) amplification. Primers were synthesized on an ABI Model 381A DNA synthesizer (Applied Biosystems, Foster City, CA) and purified using oligonucleotide
purification cartridges (ABI). PCR reactions were performed in 100 μL volumes containing 10 mmol/L Tris HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% (wt/vol) gelatin, 0.2 mmol/L each dNTP (dATP, dCTP, dGTP, dTTP; Pharmacia), 0.2 μmol/L each primer, and 500 ng genomic DNA. Mixtures were overlaid with 100 μL mineral oil and heated to 94°C for 10 minutes. Amplification was then initiated by the addition of 0.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 35 cycles of denaturation (92°C, 1 minute), annealing (62°C, 1 minute), and extension (72°C, 2 minutes) were performed in a Perkin-Elmer Cetus Thermal Cycler, followed by a final 10 minutes of incubation at 72°C. Reaction products were chloroform extracted, ethanol precipitated, and analyzed on 1% agarose gels. For second-round PCR, reactions were performed as above, using 0.5% of the first-round reaction product as template.

Strand separation and DNA sequencing of PCR products. Dried PCR products (85% of total) were dissolved in loading solution (0.5X TBE, 50% dimethyl sulfoxide [DMSO], 0.05% Bromphenol-blue and Xylene Cyanole), heated 3 minutes in a 100°C water bath, quick cooled on ice, and loaded on a 1.2-mm thick nondenaturing 5% polyacrylamide sequencing gel (18 x 38 cm) according to Maxam and Gilbert. Samples were run into the gel at 300 V until Xylene cyanole had migrated 5 mm, and then electrophoresed at 1.5 W for ~20 hours at room temperature. Under these conditions not only are the complementary single strands resolved, but conformational polymorphisms consisting of only one base differences can be separated. After electrophoresis, the gel was stained with ethidium bromide and the desired single-stranded bands cut out and crushed eluted, followed by ethanol precipitation. Thirty percent of each extracted DNA band was sequenced by the M13 Taq cycle sequence method (Applied Biosystems) using dye-labeled M13 sequencing primers. Sequencing reactions were performed in a Perkin-Elmer Cetus thermocycler with 15 cycles of denaturation at 92°C for 30 seconds, annealing at 60°C for 1 second, and extension at 70°C for 1 minute, followed by 15 additional cycles of denaturation at 95°C for 30 seconds and extension at 70°C for 1 minute. Extension products were resolved and analyzed on an ABI Model 370A DNA sequencer. RESULTS

Clinical and biochemical characterization. We have investigated 14 of 64 members of a kindred with a high incidence of thrombotic disease (Fig 1). The 14 investigated individuals were asymptomatic at the time of this investigation. Seven of the 14 individuals had evidence for type I protein C deficiency with normal levels of protein C determined by immunologic and amidolytic assays but low levels by clotting assays (Table 1). The amidolytic and clotting assays

<table>
<thead>
<tr>
<th>Pedigree No.</th>
<th>Age/Sex</th>
<th>Age at 1st Event (yr)</th>
<th>Thrombotic Event</th>
<th>PC Antigen (2.1 to 4.3 μg/mL)*</th>
<th>PC Amidolytic (80% to 154%)</th>
<th>PC Clotting (77% to 149%)</th>
<th>PCcl/PCamid*† (0.8 to 1.2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-4</td>
<td>49/M</td>
<td>36</td>
<td>MI(R)</td>
<td>4.0</td>
<td>114</td>
<td>59</td>
<td>0.6</td>
</tr>
<tr>
<td>III-15*</td>
<td>40/F</td>
<td>17</td>
<td>DVT(R), pregnant</td>
<td>4.0</td>
<td>30</td>
<td>17</td>
<td>0.6</td>
</tr>
<tr>
<td>III-16</td>
<td>40/M</td>
<td>30</td>
<td>TIA</td>
<td>4.0</td>
<td>123</td>
<td>56</td>
<td>0.5</td>
</tr>
<tr>
<td>IV-22</td>
<td>24/F</td>
<td>17</td>
<td>DVT(R), pregnant</td>
<td>4.6</td>
<td>97</td>
<td>55</td>
<td>0.6</td>
</tr>
<tr>
<td>IV-23</td>
<td>20/F</td>
<td>—</td>
<td>Nil</td>
<td>3.2</td>
<td>101</td>
<td>23</td>
<td>0.2</td>
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<tr>
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<td>18/F</td>
<td>—</td>
<td>Nil</td>
<td>3.2</td>
<td>136</td>
<td>32</td>
<td>0.2</td>
</tr>
<tr>
<td>V-2</td>
<td>4/F</td>
<td>—</td>
<td>Nil</td>
<td>3.7</td>
<td>120</td>
<td>27</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Abbreviations: DVT, deep venous thrombosis; SVT, superficial venous thrombosis; R, recurrent; MI, myocardial infarct; TIA, transient ischemic attack; SAT, subclavian artery thrombosis; CVA, cerebrovascular accident.

*Normal ranges are shown in parentheses.
†Ratio of protein C clotting assay activity to protein C amidolytic assay activity.
‡Patient on warfarin.
were measured against the same pooled reference plasma, thus facilitating the comparison of the two assays as a ratio. The ratios of the clotting assays to the amidolytic assays ranged from 0.2 to 0.6 in the affected individuals compared with 0.9 to 1.2 in the unaffected individuals. The propositus (pedigree no. III-15) had a ratio of clotting to amidolytic assay of 0.6; however, she was on warfarin therapy and had a measured protein C plasma concentration by the amidolytic assay below the lower end of the reference interval. The remaining six affected individuals had normal protein C levels measured by the amidolytic assay.

A variety of other analytes associated with hereditary thrombotic diatheses were measured in this kindred. The other two major inhibitors of the procoagulant pathway, protein S (free and bound) and antithrombin III, were normal. The thrombin time, plasminogen, and plasminogen activator inhibitor-1 levels were also normal. In one of the affected patients, a slightly decreased release of tissue plasminogen activator after venous occlusion was noted but no other abnormalities were observed.

Two of the women (III-15 and IV-22), mother and daughter, have experienced recurrent deep vein thromboses (DVTs) associated with pregnancy. Patient III-15 developed a superficial thrombophlebitis at age 42 and was started on warfarin. One day after the initiation of warfarin therapy, she developed a left subcaval artery thrombosis and an associated small magnetic resonance imager-proven cerebrovascular accident. Ultrasound evaluation of her heart was unremarkable. The thrombus was removed surgically and she was subsequently treated with heparin and warfarin. This latter episode precipitated the present investigation. Patient IV-22 was successfully managed with subcutaneous heparin through her third pregnancy without recurrent DVT. The two affected younger sisters (IV-23 and IV-24) of patient IV-22 have each been successfully managed through a pregnancy with prophylactic subcutaneous heparin. Two of the brothers of the propositus (III-4 and III-16) have experienced myocardial infarction and transient ischemic attacks, respectively, at young ages (Table 1). Neither of the brothers has evidence of lipid abnormalities, although one of them is a smoker.

Normal total protein C antigen levels in the family excluded a type I deficiency. The ability of the patients' APC to cleave small peptide substrates demonstrated by the amidolytic assay tended to exclude abnormalities of activation peptide release and/or the serine protease active site. Consequently, an abnormality in the Gla domain was hypothesized. As an initial test of this hypothesis, we measured the plasma levels of abnormal descarboxy protein C and descarboxy prothrombin with two ELISAs based on a murine MoAb (H-11) that recognizes a shared epitope expressed by descarboxy protein C and prothrombin in the presence of calcium ions but not expressed by the fully carboxylated proteins in the presence of ionic calcium. The specificity of these two ELISAs was conferred by the solid-phase capture antibodies specific for either protein C or prothrombin.28 The H-11 assay directly measures the plasma concentration of either abnormal descarboxy protein C or abnormal descarboxy prothrombin from a calibration curve constructed from a pool of plasmas from patients on stable warfarin therapy. In the H-11 assay, affected family members had levels of abnormal protein C (Table 2) ranging from 1.5 to 3.3 times greater than the amount measured in pooled plasma from patients on stable warfarin therapy. None of the affected family members had measurable levels of abnormal descarboxy prothrombin. Unaffected family members had neither abnormal descarboxy protein C nor abnormal prothrombin.

The variant form of the protein was purified as described above. The purified protein comigrated on 5% to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with normal protein C (data not shown). Protein C purified from normal and affected family member’s plasma eluted differently from Q-sepharose. Ninety-seven percent of protein C from normal individuals eluted from Q-sepharose with 10 mmol/L CaCl₂, with only 3% eluted at 1 mol/L NaCl; whereas 50% of protein C from variant family members eluted from the resin with 10 mmol/L CaCl₂ and the remaining 50% eluted with 1 mol/L NaCl. These data strongly suggest that the variant protein is not fully γ-carboxylated.29

Genomic analysis. The PCR amplification of genomic DNA containing exon 2 of human protein C using flanking intronic primers (A and B) resulted in an initial 806-bp product (shown schematically in Fig 2A). Subsequent second-round amplification using an internal hybrid primer (C/M13) yielded a product migrating at the proper position for a predicted size of 685 bp (Fig 2B). The internal primer contains M13 sequencing primer sequence, thereby providing product template suitable for sequencing primer annealing and direct DNA sequencing without subcloning. Figure 2B also shows the resolution of single-stranded DNA into four distinct bands (two different alleles) for protein C-deficient family members, in contrast to only two bands (two identical alleles) for normal individuals.

Material from strand separation gels was extracted and directly sequenced. Figure 3A presents the sequence profile for both the normal and mutant alleles. The complementary strands were also sequenced using the same strategy presented in Fig 2A, and the results were consistent with

<table>
<thead>
<tr>
<th>Pedigree No.</th>
<th>Protein C (%)</th>
<th>Prothrombin (%)</th>
<th>Pedigree No.</th>
<th>Protein C (%)</th>
<th>Prothrombin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III-4</td>
<td>200</td>
<td>0</td>
<td>III-9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>III-15</td>
<td>150</td>
<td>130*</td>
<td>III-12</td>
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<tr>
<td>III-16</td>
<td>200</td>
<td>0</td>
<td>III-18</td>
<td>0</td>
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</tr>
<tr>
<td>IV-22</td>
<td>160</td>
<td>Insufficient sample</td>
<td>IV-16</td>
<td>0</td>
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<tr>
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<tr>
<td>V-2</td>
<td>210</td>
<td>0</td>
<td>V-1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Patient on warfarin.
those derived from the data in Fig 3A (data not shown). Each of the other seven protein C-coding exons were amplified by the scheme shown in Fig 2a, using appropriate flanking intronic primers, strand separated, and sequenced. None of these exonic products showed gel electrophoretic conformational polymorphism or changes in DNA sequencing profiles from the normal and expected patterns (data not shown).

Figure 3B shows the nucleotide and translated amino acid sequence for exon 2 of protein C. As indicated, two single-base substitutions were observed in DNA amplified from protein C-deficient family members. The codon for Glu\textsubscript{35} contains an A → C mutation resulting in an Ala codon. Glu\textsubscript{35} in the normal protein is γ-carboxylated and resides approximately in the middle of the Gla domain. The second mutation (G → A) results in a Val\textsubscript{44} → Met substitution.

Independent PCR amplifications of the same affected individual as well as several other protein C-deficient family members (shown in Fig 2b) all resulted in identical DNA sequences (determined for individuals III-15, IV-22, and IV-23) containing the two substitutions. This observation, along with the fact that sequenced material represents the entire PCR product and is not the result of product subcloning, strongly argues for the mutations not being the result of artificial PCR-induced base substitutions.

As shown in Fig 3B, the A → C mutation in the Glu\textsubscript{35} codon also results in the abolition of a Bgl II restriction endonuclease recognition site (AGATCT) found in the normal sequence. To unequivocally confirm the Glu\textsubscript{35} → Ala mutation, nonamplified genomic DNA from protein C-deficient and normal family members was analyzed by the method of Southern hybridization after Bgl II digestion and agarose gel resolution of resulting DNA fragments. Figure 4B clearly shows the presence of a new 8.9-kb band in variant protein C DNA, consistent with the results predicted schematically in Fig 4A, and thereby confirms the Bgl II polymorphism at amino acid residue Glu\textsubscript{35} in deficient family members. The appearance of the new band (8.9 kb) and a reduction to ~50% of the 5.6- and 3.3-kb bands, relative to that of the reference adjacent 6.8-kb band, is shown qualitatively in Fig 4C.

**DISCUSSION**

The missense mutations (Glu\textsubscript{35} to Ala and Val\textsubscript{44} to Met) expressed in this family with type II protein C deficiency are the first reported naturally occurring mutations in the Gla domain of protein C. The protein C genes from about 50 protein C-deficient families have been analyzed, but the number of identified mutations have been surprisingly few.\textsuperscript{36}
Fig 3. DNA sequence for variant and normal protein C exon 2 alleles. (A) Direct sequencing of conformationally polymorphic bands from protein C-deficient individual IV-22 (Fig 1). Shown is the output from the ABI Model 370A automated sequencer. The nucleotide sequence (left to right, top to bottom) of exon 2 (within the arrowed vertical bars: \[\rightarrow\] ) is the inverse complement of the coding sequence. Short vertical lines under letters denote manual assignments. Vertical closed arrows (\[\Rightarrow\] ) show the position of nucleotide substitution between the variant and the normal alleles. (B) DNA and amino acid sequence of protein C exon 2. The nucleotide (5' to 3' coding direction) and amino acid sequence are shown for the normal allele. Shown above and below are the base and amino acid substitutions, respectively, in the variant allele. Also shown is the \(Bgl\ II\) site abolished by the \(A \rightarrow C\) mutation. The vertical arrow indicates the proteolytic cleavage site resulting in the removal of the propeptide from the amino terminus of the mature protein. Amino acid numbering is based on that of the mature protein. \(\gamma\)-Carboxylated residues in the mature protein are noted (\(\gamma\)).
Fig 4. Southern hybridization of protein C genomic DNA. (A) Schematic Bgl II restriction enzyme digestion map for the protein C gene. Vertical bars and intervening thin lines represent exonic and intronic segments of the gene, respectively. Numbers refer to the amino acids found in each exon. Shown below the diagram of the gene are sites of Bgl II cleavage and the size of resulting fragments in kilobases. Organization of the gene is taken from Plutzky et al. (B) Bgl II digestion Southern hybridization pattern. Lane names refer to DNA source individuals: N, normal unrelated to family; IV-22 and III-15, protein C-deficient family members (see Fig 1); III-12 and IV-16, unaffected family members. Shown on the right by horizontal lines are the positions of 1 kb ladder DNA size markers (BRL) from top to bottom: 12, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 kb. See Materials and Methods for details. No differences from the normal gene were detected after digestion with the restriction enzymes BamHI, EcoRI, Pvu II, Pst I, Kpn I, Tag I, and Xba I and subsequent Southern hybridization (data not shown). (C) Densitometry tracings of Southern hybridization patterns. Lanes N (- - - -) and IV-22 (-----) shown in (B) were scanned on a Shimadzu dual-wavelength TLC scanner Model CS-930 (Shimadzu, Kyoto, Japan). The insert shows the size and percentage of total area of each of the hybridizing bands. Gel migration is from top (origin) to bottom of the nitrocellulose membrane shown in (B).
Of the vitamin K-dependent proteins, factor IX has been the most extensively analyzed at the genomic level because of its causal role in hemophilia B. Over 100 mutations have been described in the factor IX gene, including deletions and point mutations involving both coding and noncoding regions. 46 Four substitutions for Glu in the Gla domain have been described: Glu, to Ala,46 Glu, to Val,41 Glu, to Val,42 and Glu, to Lys. The Glu, to Ala substitution is associated with mild disease, whereas the more carboxy-terminal substitutions at Glu, and Glu, are associated with moderately severe to severe clinical manifestations. The importance of specific Gla residues for normal function of the vitamin K-dependent proteins has been suggested by the observation of Borowski et al 44 of an individual with a hereditary abnormality of carboxylation, in which Gla, appeared to play a key role in the high-affinity metal ion binding site necessary for phospholipid binding. Others 46,46 have observed that in dicoumarol-treated steers selected Gla residues appear to be preferentially preserved. The Glu, to Ala substitution in this family is associated with an abnormal conformation of the protein C molecule in affected family members demonstrated by reactivity with the H-11 MoAb in the presence of Ca2+ ions. The affected family members appear to have considerably more abnormal undercarboxylated protein C present in their plasma than is present in individuals on stable warfarin therapy. This latter observation is of interest because in all but one family in which a mutant protein C molecule has been described, the abnormal protein does not appear in plasma. The abnormal protein C molecule purified from this family comigrates with normal protein C on 5% to 15% SDS-PAGE, thus excluding a major structural defect in the variant molecule such as improper cleavage of the leader peptide region. The elution conditions used in the Q-sepharose purification method described by Yan et al 35 discriminate between fully carboxylated and undercarboxylated protein C. Protein C from a normal plasma was 97% eluted from Q-sepharose by 10 mmol/L CaCl2, whereas only 50% of the variant protein C was eluted with 10 mmol/L CaCl2 with the rest eluted by 1 mol/L NaCl. This latter observation was consistent with the relative amount of variant protein C predicted by the ratio of amidolytic to clotting assays, and with the molecule being undercarboxylated. Studies from several laboratories using partially γ-carboxylated recombinant protein C indicate that a small reduction in the amount of γ-carboxylation results in loss of functional activity. Zhang and Castellino have recently reported the loss of anticoagulant activity (inhibition of clot formation and VIIIa inactivation), but not chromogenic substrate hydrolysis, for recombinant protein C in which Glu, and Glu, were changed to Asp residues. 47

The importance of the mutation(s) in this family is evident from the associated clinical thrombotic disease. The clinical pattern of venous thrombotic disease in the two affected women, who are mother and daughter, is similar to the pattern described in other protein C-deficient kindreds.11,17 They experienced DVT in association with a known predisposing condition (pregnancy). The presence of arterial thrombotic disease in patients III-4, III-16, and III-15 is somewhat unusual in protein C deficiency, but has been described. 46 The possible role of the Gla domain mutations in the genesis of the arterial thrombosis remains to be determined.

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NOTE ADDED IN PROOF

Subsequent to acceptance of this report, direct confirmation of the Val, to Met mutation was obtained at the genomic DNA level by the method of mispairing PCR.31 Zhang and Castellino have recently reported that mutation of Glu, to Asp in human protein C results in severe depression of Gla content and anticoagulant activity.32

REFERENCES

4. Johnson AE, Esmon N, Laue T, Esmon CT: Structural changes required for activation of protein C are induced by calcium binding to a high affinity site that does not contain a gamma carboxy glutamic acid. J Biol Chem 258:5554, 1983
33. Yan BSC, Razzano F, Chao YB, Walls JD, Berg DT, McClure DBG, Grinnell BW: Characterization and novel purification of recombinant human protein C from three mammalian cell lines. Biotechnology 8:655, 1990
41. Koeberl DD, Boettma CDK, Buersdedde JM, Sommer SS: Functionally important regions of the factor IX gene have a low rate of polymorphism and a high rate of mutations in the dinucleotide CpG. Am J Hum Genet 45:448, 1989


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EG Bovill, JA Tomczak, B Grant, F Bhushan, E Pillemer, IR Rainville and GL Long