Five Novel Mutations Located in Exons III and IX of the Protein C Gene in Patients Presenting With Defective Protein C Anticoagulant Activity

By Sophie Gandrille, Martine Alhenc-Gelas, Pascale Gaussem, Marie-Françoise Aillaud, Evelyne Dupuy, Irène Juhan-Vague, and Martine Aiach

We describe five families presenting with type II hereditary protein C deficiency characterized by normal antigen and amidolytic activity levels but low anticoagulant activity. All the exons and intron/exon junctions of the protein C gene were studied using a strategy combining amplification by the polymerase chain reaction (PCR), denaturing gradient gel electrophoresis of the amplified fragments, and direct sequencing of fragments displaying altered melting behavior. We detected five novel mutations. Three were located in the C-terminal part of the propeptide encoded by exon III: Arginine (Arg)-5 to tryptophan (Trp), Arg-1 to histidine (His), and Arg-1 to cysteine (Cys) mutations. The two others, located in exon IX, affected Arg 229 and serine (Ser) 252, which were respectively replaced by glutamine (Gln) and asparagine (Asn). DNA studies of the other exons from affected individuals showed no other abnormalities. These novel mutations provide further insight into the importance of the affected amino acids located close to the active site, near Asp 257, one of the three amino acids of the catalytic triad. The low anticoagulant activity of the abnormal protein C indicated that Arg 229 and Ser 252 play a key role during the interaction between protein C and its cofactor protein S, phospholipids, or factors Va and VIIIa. The Arg-1 to Cys mutation led to the dimerization of protein C with another plasminatic component, as evidenced by the presence in the plasma of a high molecular weight form of protein C that disappeared after reduction. No molecular mass abnormalities were observed in heavy and light chains of all other protein C mutants. In the five families explored, 9 (64%) of the 14 subjects bearing the mutations reported thrombotic events. This suggests that the protein C amino acids affected by the mutations are very important for the in vivo expression of the antithrombotic properties of protein C.

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PROTEIN C and its modulator protein S play a major role in regulating the blood coagulation pathway. Protein C, which is vitamin K dependent and is synthesized by hepatocytes as a 461-amino acid polypeptide, undergoes several cotranslational and post-translational intracellular modifications before secretion. These modifications include the γ carboxylation of nine glutamic acids at the N-terminal segment, the glycosylation of four asparagine residues, proteolytic cleavage of a 42-amino acid propeptide, and the endoproteolytic removal of 2 amino acids that convert the single-chain precursor into a heavy and a light chain held together by a disulfide bond.1 The nucleotide sequence of the protein C gene spans 11.2 kb and comprises nine exons, according to Plutzky and collaborators.2 The circulating protein C zymogen is converted into an active enzyme, activated protein C (APC), by limited proteolysis of the 12 N-terminal amino acids of the heavy chain. Maximal activation of protein C occurs at the endothelial cell surface as a result of thrombin binding to its membrane cofactor protein thrombomodulin in the presence of calcium ions. APC ensures the proteolytic inactivation of activated factors V and VIII, two factors necessary for thrombin formation, in a reaction that is stimulated by Ca2+, phospholipids, and protein S.3

Several types of plasma assays are used to diagnose hereditary deficiencies, which are often associated with an increased thrombotic tendency. Quantitative (type I) deficiencies are characterized by a simultaneous decrease in protein C antigen and its biologic activities. In qualitative (type II) deficiency, protein C has a normal antigen concentration but displays abnormal biologic activity. Two kinds of functional assays allow the identification of type II deficiencies: (1) amidolytic assays, which measure the hydrolysis by APC of a small synthetic substrate but only detect abnormalities of the catalytic site of the serine protease, and (2) coagulation assays, which detect more subtle abnormalities of APC interaction with Ca2+, phospholipids, protein S, and factors V and VIII.

Several type II protein C deficiencies have been described in terms of the plasma phenotypes involved.5,11 However, only three mutations have been characterized: one of them is responsible for defective cleavage of the activation peptide,8,12 and the two others cause abnormal carboxylation.14,15 We report here five novel mutations identified in patients whose protein C activity was low in a coagulation assay but was within the normal range in amidolytic and...
Patients, Materials, and Methods

Screening of Patients

For plasma assays, blood was taken by venipuncture using evacuated tubes containing 0.11 mol/L sodium citrate. Approval was obtained from the International Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki. The plasma samples were kept frozen (−30°C) until assayed. The hereditary protein C abnormality was diagnosed by three types of assays: immunoenzymatic (Asserachrom Protein C, Diagnostica Stago, Asnieres, France), amidolytic (Biorad Protease, Behring, Rueil-Malmaison, France), and coagulation (Staclot Protein C, Diagnostica Stago), all performed as recommended by the manufacturer. In the amidolytic and coagulation assays, plasma protein C was activated by Protac snake venom. Once activated, it was made to react with a synthetic chromatographic substrate in the amidolytic assay and in the coagulation assay, with a natural substrate; ie, plasma depleted of protein C as a source of factor V, factor VIII, and protein S in the presence of cephaline and Ca²⁺. The calibration curve was constructed by diluting a pool of 20 to 30 donor plasmas in buffer. A lyophilized control (Diagnostica Stago) allowed the calculation of interseries variations, which were 3% for the amidolytic assay and 8% for the coagulation assay.

Immunoblotting for Protein C

Samples containing 0.5 μL of patient or normal plasma underwent 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). The proteins were transferred onto a nitrocellulose membrane using a minisystem (Bio-Rad, Ivy-sur-Seine, France). The blot was treated as recommended by the manufacturer, made to react with an antiprotein C serum diluted 1/1,000 (Diagnostica Stago), thoroughly washed, and incubated with a 1/3,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Biorad). Aluminium hydroxide adsorption and subsequent elution were performed according to Bertina.¹⁶

Identification of the Mutation by DNA Analysis

General method and strategy. Thermus aquaticus (Taq) polymerase (5 U/μL) was from Perkin Elmer–Cetus Instruments (Norwalk, CT).

The four deoxynucleotides (deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxycytosine triphosphate, and deoxyguanosine triphosphate) and deoxycytidine triphosphate) were from Pharmacia Fine Chemicals (Uppsala, Sweden). The OPC columns were from Applied Biosystems (Roissy, France). The sequencing kit was from the United States Biochemicals Corporation (Cleveland, OH). T4 nucleotide kinase from Thermus aquaticus (Taq) polynucleotide kinase (5 U/μL) was from Perkin-Elmer–Cetus Instruments (Norwalk, CT).

DNA was extracted from the patients’ leukocytes and purified as described in a previous study.¹⁷

Oligonucleotides were synthesized on a gene assemner (381 A DNA synthesizer, Applied Biosystems, Roissy, France). The 20 mers were purified by ethanol precipitation and the oligonucleotides containing a GC-clamp on an OPC column before ethanol precipitation.

Enzymatic amplification of each PC gene exon and its splice junctions were performed essentially as described by Saiki et al.¹¹ using a set of oligonucleotides comprising a 20 mers and a primer to which a G + C-rich sequence (GC-clamp) with a variable number of nucleotides was added.¹⁸ The oligonucleotide sequences are shown in Table 1 and their locations in Fig 1.

The locations of the polymerase chain reaction (PCR) primers

immunologic assays. Three of the five mutations were substitutions of amino acids in the propeptide’s C-terminal segment, in which tryptophan (Trp) was substituted for arginine (Arg)-5, histidine (His) for Arg-1 and cysteine (Cys) for Arg-1. The two other substitutions were located in the serine protease domain, in which glutamine (Gln) was substituted for Arg-229 and asparagine (Asn) for serine (Ser) 252.

### Table 1. Oligonucleotide Primers for Amplification of Exons

<table>
<thead>
<tr>
<th>Exon No.</th>
<th>Primer Name</th>
<th>Oligonucleotide Sequence</th>
<th>Nucleotide Numbering*</th>
</tr>
</thead>
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<tr>
<td>II</td>
<td>GCPRC 2A</td>
<td>5'--GGCGGATGCTGCGGACAG--</td>
<td>-72 to -53</td>
</tr>
<tr>
<td></td>
<td>PRC 2B</td>
<td>5'--GAGGCCACAGAGGTTCCC--</td>
<td>111 to 92</td>
</tr>
<tr>
<td>III</td>
<td>GCPRC 3</td>
<td>5'--GCGGCTGCTGAGGAGGAC--</td>
<td>1256 to 1275</td>
</tr>
<tr>
<td></td>
<td>PRC 3A</td>
<td>5'--CCTCGAATGACAGA--</td>
<td>1465 to 1446</td>
</tr>
<tr>
<td></td>
<td>PRC 3B</td>
<td>5'--TGAGCCCCTGGGCTGTA--</td>
<td>1565 to 1546</td>
</tr>
<tr>
<td>IV</td>
<td>PRC 45A</td>
<td>5'--ACACCGGTGCGAGGAGG--</td>
<td>2921 to 2940</td>
</tr>
<tr>
<td></td>
<td>D PRC 45B</td>
<td>5'--TGCTGTCGGCAGGCC-</td>
<td>3289 to 3270</td>
</tr>
<tr>
<td>V</td>
<td>PRC 5A</td>
<td>5'--TGGCGGTCGGACCC-</td>
<td>3043 to 3060</td>
</tr>
<tr>
<td></td>
<td>PRC 5C</td>
<td>5'--AGCCGTGCTGGCGGAC--</td>
<td>3125 to 3144</td>
</tr>
<tr>
<td>VI</td>
<td>PRC 6A</td>
<td>5'--GGCAGCCACGCCCAGC--</td>
<td>3275 to 3294</td>
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<tr>
<td></td>
<td>GCPRC 6B</td>
<td>5'--GCGGAGGACCAGGAGGAC--</td>
<td>3546 to 3527</td>
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<tr>
<td></td>
<td>PRC 6C</td>
<td>5'--TTGGGGCAGCCAGCCAGC--</td>
<td>3270 to 3289</td>
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<tr>
<td></td>
<td>D PRC 6D1</td>
<td>5'--CCACCCGACGACCAG-</td>
<td>3504 to 3485</td>
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<tr>
<td>VII</td>
<td>PRC 7A</td>
<td>5'--GACCAAGACAGGGAGGAGGCA--</td>
<td>6055 to 6074</td>
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<tr>
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<td>GCPRC 7B</td>
<td>5'--GCGGAGGACCAGGAGGAGC--</td>
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<td>7063 to 7082</td>
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<td></td>
<td>PRC 8B</td>
<td>5'--TGGGCTCACCAGCCAGC--</td>
<td>7308 to 7289</td>
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<tr>
<td>IX</td>
<td>PRC 9A</td>
<td>5'--GCTTGGTCACTCTGTCCGCCTC-</td>
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<td>5'--GCGGAGGACCAGGAGGAGC--</td>
<td>8889 to 8870</td>
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<tr>
<td></td>
<td>GCPRC 9C31</td>
<td>5'--GCTTGGTCACTCTGTCCGTCTTCTA--</td>
<td>9043 to 9024</td>
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</table>

Abbreviation: IVS, intervening sequence.

* Nucleotide numbering as in Foster et al.²

† Primer used as sequencing primer.

‡ Primers described by Reitsma et al.⁹⁶

* Nucleotide numbering as in Foster et al.²
were chosen using the computer programs MELT 87 and SQHTX, written and kindly provided by Drs L.S. Lerman and K. Silverstein.22 These softwares allow simulation of the melting behavior of DNA fragments according to their nucleotide sequence and base composition. The information acquired about this behavior was used to select positions for PCR primers that would allow the generation of fragments suitable for analysis by denaturing gradient gel electrophoresis (DGGE) and to determine the range of denaturant concentrations and electrophoresis times resulting in maximum gel resolution. One of the amplification primers always carried an additional G + C-rich nucleotide fragment at its 5' end to create a high-temperature melting domain21 and thus position the sequence of interest within the first melting domain. Because of their length, exons III and IX were studied using, respectively, two and three sets of primers. DGGE could not be used for exons IV and V because of their very high melting temperatures. These exons were therefore studied for each patient by systematic direct sequencing after asymmetric PCR.

Each amplified fragment was sequenced after asymmetric PCR using the primers indicated in Table 1.

**DGGE and sequencing of the two DNA domains bearing the mutations.** The 5' end of exon III was amplified using GC-PRC 3 and PRC 3A as extension primers and the 5' end exon IX using PRC 9A1 and GC-PRC 9A2. The oligonucleotide sequences concerned and their locations are given in Fig 1.

Symmetric PCR amplifications of the 5' end of exon III and exon IX were performed as follows: each PCR mixture contained 30 pmol of upstream primer (GC-PRC 3 or PRC 9A1) and 30 pmol of downstream primer (PRC 3A or GC-PRC 9A2), 200 μmol/L of each dNTP, 1 μg of genomic DNA, 1× PCR buffer (10 mmol/L TRIS-HCl, pH 8.3, 50 mmol/L KCl, 1 mmol/L MgCl₂, and 0.01% [wt/vol] gelatin), 2.5 U of Taq polymerase, and 5% of dimethyl sulfoxide (DMSO) in a final volume of 100 μL. The reactions were run in 1 mL microcentrifuge tubes (Eppendorf, CML, Nemours, France) with Hybaid Thermal cycler (Teddington, UK).

The thermal profile included 5 minutes of denaturation at 94°C, followed by 30 cycles consisting of denaturation for 1 minute at 94°C, annealing for 1 minute at either 59°C (exon III) or 61°C (5' end of exon IX) and extension for 1 minute at 72°C. Samples were maintained at 72°C for 10 minutes. Because assay resolution is increased by heteroduplex examination, the PCR cycles were followed by 10-minute denaturation at 94°C and 30-minute annealing at 55°C to favor the annealing of allelic DNA strands. The specificity of the PCR was checked on 6% polyacrylamide gel using a minisystem (MiniProtean, Bio-Rad, Richmond, CA).

DGGE was performed as described by Attree et al.33 Amplified DNA fragments of the 5' ends of exons III and IX (272 and 360 bp, respectively) were subjected to electrophoresis for 5 hours at 160 V in 6.5% polyacrylamide gel containing a 30% to 80% denaturant gradient (100% denaturant = 7 mol/L urea and 40% formamide in TEA buffer [Tris 2 mol/L, EDTA 50 mmol/L, natrium acetate 1 mol/L, pH 7.6]) and stained with etidium bromide.

As shown in Fig 1, GC-PRC 3 was located in the intervening sequence (IVS) B and PRC 3A in exon III. The resulting 272-bp fragment contained the sequence coding for amino acids 19 to 25. PRC 9A1 was located in IVS H and GC PRC 9A2 overlapped with the sequence corresponding to amino acids 273 to 278. The 360-bp
fragment obtained with this set of primers contained the sequence encoding amino acids 224 to 278.

The asymmetric PCR developed by Gyllensten and Erlich was performed using 50 pmol of GC-PRC 3 and 1 pmol of PRC 3A or 3 pmol of PRC 9A1 and 50 pmol of GC-PRC 9A2, as described above, except that the thermal profile involved 60 cycles. This reaction led to the preferential amplification and enrichment of the noncoding strand of exon III and of the coding strand of the 5' end of exon IX. The products of this enrichment were desalted and excess deoxynucleotide was removed by spin dialysis on a Centricon 100 apparatus. The single-strand templates were then sequenced using the Sequenase kit, with PRC 3A and PRC 9A1 as sequencing primers.

**Dot-blot analysis.** Dot-blot analysis was performed after alkaline denaturation. Amplified DNAs were dot blotted onto nylon filters and hybridized with 32P-labeled allele-specific oligonucleotide (ASO) probes corresponding either to the normal sequence (−5 N CGG, −1 N CGT, 229 N CGG, 252 N AGC) or to the mutated sequences (−5 M TGG, −1 M CAT, −1 M TGT, 229 M CAG, 252 M AAC). The sequences of these ASO probes are listed in the legend to Fig 6. Probes were labeled, hybridized, and washed as previously described except that temperatures were adapted to the probes used.

**RESULTS**

**Clinical and Laboratory Investigations**

We explored 28 individuals from five families, 14 of whom had a ratio of anticoagulant to amidolytic protein C activity below 0.6 and were suspected to be heterozygous for this defect. The results of the plasma assays are given in Table 2 and the family pedigrees in Fig 2.

**Family A.** The propositus developed deep venous thrombosis (DVT) after surgery at the age of 37. His father reported a history of recurrent pulmonary embolism starting at the age of 43 and one episode of myocardial infarction at the age of 60 followed by DVT. One of his siblings also exhibited the corresponding abnormal phenotype and had suffered from recurrent paraphlebitis since the age of 27.

**Family B.** The propositus, a 54-year-old woman, presented with a left sural and iliac DVT after prolonged car travel. She had previously suffered from three thrombotic episodes. The first episode, postpartum left iliac DVT with pulmonary embolism, occurred when she was 25 years old. Her daughter (I12) displayed the same plasma protein C deficiency but was still asymptomatic. Her son exhibited phlebitis after a traumatic leg fracture that occurred when he was 19 and has been treated by plaster cast immobilization, but his protein C levels were normal. The propositus...
starting oral contraception. Two of the other nine family members explored (II2 and II5) had low protein C anticoagulant activity. The propositus’ mother (II2) reported one episode of postsurgical DVT.

**Family E.** The propositus had had leg phlebitis when she was 21 years old. This patient had borderline antigen levels in two separate explorations and an abnormally low ratio of anticoagulant to amidolytic activity. She was therefore included in the series of patients with qualitative protein C deficiency. Her mother (I2) and two brothers (II1 and II2) had normal protein C plasma levels and were free of clinical symptoms of thrombosis. Unfortunately, we could not examine her father.

**SDS-PAGE and Immunoblot Analysis**

The blots for protein C antigen obtained by 10% SDS-PAGE showed normal migration of plasma from the affected subjects belonging to families B, C, D, and E. Protein C migrated as a triplet owing to different degrees of glycosylation. An example of the patterns observed in each family is shown in Fig 3 (upper panel). The proportions of the three compounds α, β, and γ were apparently identical in the normal and patient plasmas, and α and β were the major compounds. Beside these normal compounds, the affected patients in family A exhibited two additional bands with a higher molecular mass. Therefore, additional experiments were performed on semipurified samples obtained from plasma processed by aluminium hydroxide adsorption, a procedure that allowed better resolution of the different compounds. The migration of the aluminium hydroxide eluates in 7% polyacrylamide gels confirmed the normal migration pattern displayed by the affected subjects from families B, C, D, and E. The two abnormal bands observed in family A migrated with apparent molecular masses of 91 and 116 Kd, which might correspond to α and β dimers (Fig 3, middle panel). The absence of molecular mass abnormalities in the heavy and light chains was further confirmed by the migration of reduced aluminium hydroxide eluates in 12% polyacrylamide gels. Under reducing conditions, the molecular masses of both chains were apparently normal in all the families. Furthermore, the disappearance of the high molecular mass compounds from the plasma of the patients belonging to family A suggested that the abnormal compounds found in this family were formed by the binding of protein C to another protein through a disulfide bond (Fig 3, lower panel).

**Identification of Protein C Gene Mutations**

The fact that the abnormal protein C responsible for the qualitative deficiencies observed in the five families described here had a normal molecular weight suggested a missense mutation in the coding sequence of the protein C gene. Therefore, to identify the mutations resulting in the expression of protein C with reduced anticoagulant activity in these families, we explored all the protein C coding sequences, ie, exons and their flanking domains, in 25 subjects who included members of all the families (A, B, C, D, and E).

DNA fragments corresponding to exons I, II, III, VI, VII,
VIII, and IX were enzymatically amplified and submitted to DGGE. Each fragment exhibiting an abnormal DGGE pattern was asymmetrically amplified and then sequenced. Fragments corresponding to exons IV and V, which could not be screened by DGGE, were sequenced for all the patients.

The mutations observed in these 25 patients were all located in exons III and IX. DGGE, performed as indicated in the Materials Methods section and Fig 1, showed abnormal migration of the fragment corresponding to the 5' end of exon III in three families and abnormal migration of the fragment corresponding to the 5' end of exon IX in two.

The DGGE patterns observed for the 5' end of exons III and IX in all the 25 individuals studied from families A, B, C, D, and E are given in Fig 4. In all 14 patients with abnormally low protein C anticoagulant activity, these patterns were typical of heterozygous subjects and showed (1) the presence of two homoduplex bands resulting from a shift in the mobility of the alleles bearing a nucleotide substitution and (2) the presence of two other heteroduplex bands generated during the PCR reaction by the matching of normal alleles with alleles bearing a mutation.

The nucleotide sequence of exon III showed a C to T transition in family D converting Arg-1 into His, and a C to T transition in family E converting Arg-5 into Trp. The nucleotide sequence of exon IX allowed the identification of a G to A transition converting Arg-229 into Gln in family B and a G to A transition converting Ser-252 to Asn in family C (Fig 5).

To exclude the possibility of a neutral polymorphism, we analyzed the amplified 5' end of exon III and IX from 61 healthy individuals with normal protein C levels. They all had normal DGGE patterns, which indicated the absence of sequence variation. The mutations were confirmed by hybridization of the amplified 5' end of exon III and IX from all the coding domains corresponding to exons II, III, VI, VII, VIII, and IX were sequenced.

Figure 7 indicates the location in the amino acid sequence of the five novel mutations described here.
DISCUSSION

We described here five novel mutations of the protein C gene that were responsible for a defective APC anticoagulant activity. The protein C concentrations measured by immunoenzymatic and amidolytic assays were above 90% in all the affected individuals explored except in the family E propositus, whose protein C concentrations were at the lower limit of normal. This strongly suggests that the amino acid substitutions observed do not affect either the mature protein C secretion by hepatocytes or serine protease site expression following protein C activation. However, the mutant protein did not participate in the development of the proteolytic inactivation of coagulation factors V and VIII in plasma because all the heterozygous subjects bearing one of the mutations described here had a ratio of anticoagulant to amidolytic activity between 0.38 and 0.59.

The five mutations detected were located in two functional domains of protein C: three were observed in exon III and affected the C-terminal part of the propeptide, and the two others affected the N-terminal part of the serine protease domain encoded by exon IX (Fig 7).

The possible presence of additional mutations was ruled out both by the results of DGGE and by sequencing of all the gene domains of functional significance.

The substitution of His or Cys for Arg-1 might have been expected to impair the processing of the protein, which normally is cleaved by liver endoproteases after the dibasic His-Arg sequence. Furthermore, mutations of the homologous Arg-1 in factor IX led to the secretion of a protein circulating with the propeptide. However, the normal migration of the protein C light chains observed in families A and D after SDS-PAGE and immunoblot argues against such impairment. In addition to consensus Lys-Arg or Arg-Arg sequences, mammalian endoproteases recognize other motifs such as Lys/Arg-X-Lys/Arg. It is possible that the presence of the sequence Arg-Ile-Arg-Lys upstream of the mutated Arg-1 allowed the detection of the Arg-Lys
Fig 6. Dot blot analysis of the PCR-amplified genomic DNA using the normal (N) and mutated (M) probes for exon III in families A, C, and D and for exon IX in families B and E. The allele-specific oligonucleotide probes were as follows: PC-5 N CGC 5'-CAGGTGCTGCGGATCCGCA-3; PC-5 M TGG 5'-TGCGGATCCACAGCACCCTG-3; PC-1 N CGT 5'-GAGTTGGCACTTTGCGGA-3; PC-1 M CAT 5'-TCCGCAACATGCAACTC-3; PC-1 M TGT 5'-AGTTGGCACATTGCAGGG-3; PC-229 N CGG 5'-TCCCAGCGCCGCAGGTCAT-3; PC-229 M CAG 5'-ATGCATGCTGCTGCTGCTGGA-3; PC 252 N AGC 5'-TCGGTGCTCTTGTGCTGT-3; PC 252 M AAC 5'-ACAGCAAGAACACCACCGA-3.

Fig 7. Location of the mutations observed in the amino acid sequences and their corresponding location on the protein C gene. (Φ) glycosylation site; (ΦΦ) amino acid belonging to the catalytic triad.
bond. Cleavage at this second site would have yielded products with an additional amino acid not detectably different from the components of the native light chain revealed by SDS-PAGE.

The substitution observed here of Cys for Arg-1 produced protein C with an abnormal molecular weight, as shown by the presence of two high molecular weight compounds respectively located in the native plasma and in the protein C concentrates obtained after aluminium hydroxide adsorption. The normal migration observed after reduction suggests that the additional cystein residue is engaged in a disulphide bond. Although the molecular weights of the two abnormal compounds are compatible with those of the dimers respectively formed with the α and β protein C isoforms, the abnormal protein C might associate with another protein. However, the formation of a heterodimer with albumin, already described in an antithrombin III variant, was not possible here as the compounds with an abnormally high molecular weight did not react with antialbumin serum (data not shown).

The abnormal protein C anticoagulant activity observed in plasma from the subjects with Arg-1 His, Arg-1 Cys, and Arg-5 Trp mutations might be due to a subsequent modification of the GLA domain because GLA residues confer calcium ion binding properties to the vitamin K–dependent proteins and are required for their interaction with phospholipid surfaces. The exact consequences of such mutations for propeptide cleavage and carboxylation remain to be established after purification of the mutated proteins from the patient’s plasma.

The two other mutations, Ser 252 to Asn and Arg 229 to Gln, were located in the serine protease domain, respectively 5 and 28 amino acids upstream of Asp 257, one of the three amino acids of the catalytic site (Fig 7). However, they did not affect the cleavage of oligopeptidic substrates. Therefore, these mutations probably disturb the interaction of protein C with macromolecular substrates such as factors V and VIII or with protein S.

Ser 252 to Asn mutation creates a sequence (Asn-X-Ser/Thr) known to be a consensus sequence for glycosylation. The various subforms of the heavy chains observed in immunoblots of plasma or recombinant protein C26 correspond to differences in the number of glycosylation sites. Of the four potential glycosylation sites of protein C, one is located near Ser 252, at the amino acid position 248. The carbohydrate chain at this site was recently shown to increase the efficiency of the removal of the internal dipeptide that yields the two-chain form of the molecule. Here, in the plasma from the patients bearing the Ser 252 to Asn mutation, the presence of the remaining single chain in only trace amounts and of the heavy chain subforms in apparently normal amounts suggests that the glycosylation of the mutated protein did not alter.

Inhibition by synthetic oligopeptides recently showed that the amino acid sequence at position 390 to 405 played an important part in the recognition of factors V and VIII, which are natural substrates of activated protein C.35 The present results show that the domain encompassing Arg 229 and Ser 252 is also critical for the proteolysis of factors V and VIII by APC. Whether this results from a conformational change that renders access to the catalytic site more difficult for macromolecular substrates, or whether it implies a direct interaction of this domain with the target proteins requires further investigation.

Finally, the five different mutations described here that were responsible for a defect in the anticoagulant potency of protein C may be crucial for the in vivo antithrombotic activity of protein C as 9 of the 14 patients expressing an abnormal molecule had presented with thrombotic complications.

This study confirms that it is of great interest to identify the novel natural mutations of patients with well-characterized phenotypes. The consequences of the mutations observed were unexpected and open up new prospects for the understanding of several mechanisms, although the present findings require further study, to define more clearly the consequences of the mutations at the molecular level. For instance, a consensus sequence for the cleavage of the propeptide was apparently recognized, although the critical Arg-1 residue was mutated. Furthermore, the mutation of Arg 229 and Ser 252 is the first evidence of the crucial role of these two amino acids in the anticoagulant function of APC.

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