First De Novo Mutations in the Protein C Gene of Two Patients With Type I Deficiency: A Missense Mutation and a Splice Site Deletion

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In a series of 40 patients with symptomatic protein C deficiency, we identified two sporadic cases with novel mutations that probably affect gene expression. The mutations, a 5-bp deletion of the donor splice site of intron f (nucleotides 3455 to 3459) and a mutation of nucleotide 8523 in exon IX leading to the substitution of Ser 270 by Pro, were not found in the protein C gene of the patients' parents. Transmission of the paternal and maternal protein C alleles was apparently normal on the basis of frequent polymorphisms in exons I, VI, and VIII. We also checked the transmission of the chromosomal material by analyzing the β-globin gene frameworks and three variable number of tandem repeats (VNTRs). By combining the results of intragenic polymorphism, VNTR and β-globin gene framework analyses, we were able to exclude nonpaternity and confirm the de novo origin of the mutation.

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MATERIALS AND METHODS

Patients. Plasma and DNA samples were obtained from 65 normal subjects and from subjects belonging to 40 families with a quantitative (type I) protein C deficiency.

Protein C deficiency was diagnosed by using immunoenzymatic (Asserachrom Protein C, Diagnostica Stago, Asnières, France), amidolytic (Berichrom Protein C, Behring, Rueil-Malmaison, France), and coagulation assays (Staclot Protein C, Diagnostica Stago), all according to the manufacturer’s recommendations. In both coagulation assays, protein C was activated by Protac. A lyophilized plasma (Diagnostica Stago) was used to calculate between-run variations (5% in the amidolytic assay and 8% in the anticoagulant assay). Normal values ranged between 65% and 130% in all three assays.

Blood sampling. Venous blood was collected in evacuated tubes containing 0.129 mol/L trisodium citrate (1:10) for protein C assays, and the plasma was kept frozen until use. Venous blood was collected in ethylene diamine tetraacetic acid for DNA studies and kept at 4°C. Leukocytes were isolated within 48 hours and stored frozen until DNA extraction according to Bell et al.

Materials. Thermus aquaticus (Taq) polymerase (5 U/μL) was from Perkin Elmer Cetus Instruments (Norwalk, CT). The four deoxyribonucleotides—deoxyguanosine triphosphate (dGTP), deoxyctosine triphosphate (dCTP), deoxyadenosine triphosphate (dATP), and deoxythymidine triphosphate (dTTP)—were from Pharmacia Fine Chemicals (Uppsala, Sweden). The Sequenase kit was from United States Biochemicals Corp (Cleveland, OH). The Centricron apparatus was from Amicon (Denver, CO). α-32P(dATP) was from Amersham (Buckinghamshire, UK). The size marker PhiX174 RF DNA-HaeIII digest and the restriction endonuclease SmaI were from Biolabs (Ozyme, Montigny-le-Bretonneux, France). Oligonucleotides were from Genset (Paris, France).

Protein C gene analysis. The protein C gene was analyzed as described in detail elsewhere. Briefly, the exon VI and exon IX fragments containing the two mutations described here were amplified by means of the PCR, using two sets of oligonucleotides: PRC6A + GC PRC6B and GC PRC 9B1 + PRC 9B2 for exon VI and the middle part of exon IX, respectively. Controls without input DNA were routinely included to rule out amplification of contaminating DNA.

The amplified fragments were submitted to denaturing gradient gel electrophoresis (DGGE) as described elsewhere.

Fragments with abnormal melting behavior were asymmetrically amplified, and the PCR products were desalted, the excess of oligonucleotides removed by ultrafiltration on the Centricon 100 apparatus, and sequenced according to the method of Sanger et al. The asymmetric amplification and sequencing conditions have been described in a previous study.

Mutations were confirmed by HphI digestion of the PCR product obtained with PRC6A and GC PRC6B, or by Smal digestion of the PCR product obtained with PRC9A1 and the mutagenic primer PRC9B*270 (5‘CGGGAGGCAGATGGGACATGTGGTCCCCG-3‘).

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To exclude copying errors due to Taq polymerase, DGGE was repeated with two new PCR templates and sequencing was carried out on two distinct asymmetric PCR products.

Complete coding sequence analysis was applied to samples from both propositi.  

Protein C gene haplotype analysis. Protein C allele segregation was analyzed by PCR and subsequent DGGE analysis of amplified exons I, VI, and VIII. Amplification and DGGE of amplified exons I and VIII were performed as described elsewhere.  

β-Globin gene frameworks and VNTR analysis. Transmission of the paternal and maternal chromosomal material was studied by analyzing the β-globin gene frameworks, as described by Ghanem et al., and three different VNTR systems. The VNTR locus D1S80, the apolipoprotein B 3′ hypervariable region, and the intron 40 VNTR of von Willebrand’s gene were amplified using the primer sets described in the corresponding references. Ten microliters of each PCR product was electrophoresed in 6% polyacrylamide gels, except for intron 40 VNTR of von Willebrand’s gene, for which 10% polyacrylamide gel was used. The Phix174 RF DNA HaeIII digest was used as a size marker.

RESULTS

Clinical and laboratory findings. In family 1, protein C deficiency was diagnosed in a 32-year-old man who had had recurrent thrombotic episodes since the age of 19. After the first thrombotic episode, the protein C level was 34% in an amidolytic assay. Both parents had normal protein C levels and had never had venous thrombosis. The father had a myocardial infarction at the age of 55, which was probably related to several risk factors (smoking, hypertension, and diabetes). The family pedigree and protein C levels are depicted in Fig 1A.

In family 2, the propositus was a 27-year-old woman who developed thrombosis of the vena cava and of the right femoro-iliac vein at the age of 17, in the absence of risk factors. Protein C levels were 46%, 34%, and 40% in anticoagulant, amidolytic and immunoenzymatic assays, respectively.

Neither parent had a history of thrombosis, and both had normal protein C levels in several types of assay. The patient’s sister and brother also had normal protein C levels.

The family pedigrees and protein C levels are presented in Fig 2A.

Identification of protein C gene mutations. To identify the mutation responsible for the deficiency, we explored all the protein C coding sequences and their flanking domains. Briefly, DNA fragments corresponding to exons I to III and VI to IX were amplified and submitted to DGGE. Each fragment with an abnormal DGGE pattern was asymmetrically amplified and sequenced. Fragments corresponding to exons IV and V, which could not be screened by DGGE, were sequenced in both cases.

Using this strategy we detected a mutation located in the amplified fragment corresponding to exon VI and its flanking regions in the propositus of family 1, and in exon IX in the propositus of family 2. Only the propositi had abnormal DGGE patterns, as shown in Figs 1 and 2, which depict the DGGE patterns for family 1 exon VI and family 2 exon IX.

Exon VI contains a frequent polymorphism affecting nucleotide 3342 (G/T) in the triplet coding for Ser 99. Thus, in the normal population, Ser 99 can be encoded by either TCT (allele B1) or TCG (allele B2). The typical DGGE profile observed in a normal heterozygous subject is thus characterized by the presence of two homoduplexes (B1 and B2). In addition, the rearrangement of allele B2 to allele B1 lead to the formation of heteroduplexes with slower migration (the two upper bands on the gel). In family 1, subjects I.1 and II.1 had normal DGGE patterns and were heterozygous for alleles B1 and B2. Subject I.2 was homozygous for the normal allele B1. The propositus II.2 was homozygous for a normal allele and a mutated (*) allele B, as evidenced by the abnormal migration of the heteroduplexes as a single band on the top of the gel. As the genotype of the propositus (B1 or B2) was difficult to establish by the single DGGE analysis, we sequenced the DNA domain encoding Ser 99 and found that the patient was homozygous for B1. The mutated B1 allele (B1*) migrated like a B2 allele because the mutation increased the stability.

The nucleotide sequence of exon VI in the propositus of family 1 showed a deletion of five nucleotides [nucleotides 3455 to 3459] in the 3′ flanking sequence, which forms the donor splice site of intron F. The deletion abolished the splice
site consensus sequence, and thus probably impaired intron f splicing. The sequence of exon IX in the propositus of family 2 revealed a T to C transition in nucleotide 8523, converting Ser 270 (TCG) into Pro (CCG). The two patients’ sequences are depicted in Fig 3.

The 5 bp deletion identified in the propositus of family 1 was checked using the restriction endonuclease HphI, as the mutation abolished the site for this enzyme. The mutation in the propositus of family 2 was checked by amplifying the mutant fragment with PRC 9AI as upstream primer, and a downstream mutagenic primer (29 mer), in which CC was introduced instead of TG to create a new Smal site in the mutant allele (CCCKGG). Only the amplified fragment from the propositus was digested by Smal, confirming that he was the only family member to bear the mutation (data not shown).

Protein C gene haplotype analysis. The protein C gene haplotypes were established by studying the three frequent intragenic polymorphisms located in exon I (at position -1476, alleles T or A named alleles Cl or C2), exon VI (at position 3342, T or G, named alleles B1 or B2), and exon VIII (at position 7228, nucleotides T or C, named alleles A1 or A2).

The results of this analysis (Figs 1 and 2) are consistent with the normal transmission of the parental alleles. The propositus of family 1 inherited the A2B1C1 allele from his father and the A2B1C2 allele from his mother. As the propositus is homozygous for the B1 polymorphism, it is not possible to deduce in which parental gamete the de novo mutation occurred. The propositus of family 2 inherited two A2B1C1 alleles, one from each parent. Although de novo mutations occur more frequently in male gametes, we were unable to ascertain in which parental gamete the de novo mutation occurred.

β-Globin gene frameworks and VNTR analysis. Transmission of the chromosomal material, assessed by analyzing three extragenic VNTRs and the β-globin gene frameworks, appeared to be normal in both families (Figs 1 and 2). Thus, by combining the results of the protein C gene haplotype analysis, the β-globin gene frameworks and the three VNTR systems studied, we were able to exclude nonpaternity and confirm the de novo origin of the mutation.

DISCUSSION

We detected two de novo mutations by screening 40 propositi belonging to families with quantitative protein C deficiencies. Both propositi were symptomatic and had low protein C levels, whereas their parents and relatives had normal protein C levels and no history of thrombotic manifestations.

By studying the entire coding region of the protein C gene, we detected a 5-bp deletion (nucleotides 3455 to 3459) in propositus 1, and a missense mutation (Ser 270 to Pro) in propositus 2. The site of the mutation was identified by screening amplified fragments in the DGGE method. The mutations were identified by direct sequencing after asymmetric PCR (Fig 3). Both propositi were heterozygous for the mutation, as evidenced by the presence of both normal and mutated sequences on the gel.

The 5-bp deletion destroyed the consensus sequence of the donor splice site of intron f (Fig 4). Such a mechanism

![Fig 2. Family 2. (A) Family tree and DGGE of amplified exon IX. HMDX, homoduplexes. (B) Same legend as Fig 1.](image-url)
Fig 4. Effect of the 5-bp microdeletion in intron f, and alignment of vitamin K-dependent protein sequences corresponding to the protein C stretch encompassing Ser 270.

![Diagram of the donor splice site consensus sequence and exons/introns of the protein C gene.]

The T to C transition converting Ser 270 to Pro occurred on conserved residue both in protein C sequences of various mammalian species and in factor X, another vitamin K-dependent serine protease (Fig 4). This residue probably plays a key role in the tertiary structure of protein C. A mutation affecting this Ser 270 (Ser 270 to Leu) has already been described in a compound heterozygous patient with type I protein C deficiency.

These mutations were the only abnormalities detected in all the exons and intron/exon junctions of the protein C gene. Furthermore, they were not detected in 130 normal chromosomes and were thus very likely the deleterious mutations.

As these two mutations were not detected in the parents of the two propositi, we checked the transmission of the paternal and maternal protein C alleles with that of the chromosomal material. We did so by studying the transmission of the β-globin gene frameworks and those of three VNTR systems. The results of these analyses were normal, and show that the probability of a nonpaternity was less than 10−4 in both cases, according to the allele frequencies published, and thus indicating that the two protein C deficiencies described here are sporadic and result from de novo mutations in one of the parents’ gamete in each case.

This is the first report of de novo mutations of the protein C gene. The latter is closely related to three other blood coagulation serine-proteases: factor IX, factor VII, and factor X. These proteases share functional domains and have similar gene structures (reviewed by Furie and Furie), indicating that they evolved from a common ancestral gene.

The mutations observed in hemophilia B and protein C deficiency have a similar spectrum, with a high proportion of missense mutations and wide heterogeneity. As originally reported by Haldane, the rate of spontaneous mutations in hemophilia B is about 30%. This explains that, as the gene frequency of the mutant gene in the population remains constant, the rate of loss of this gene must be equivalent to the spontaneous development of new mutant gene forms. In our series, de novo mutations of the protein C gene appear to be very rare (5%). However, protein C deficiency, being of lower penetrance than hemophilia, many asymptomatic heterozygous may not be diagnosed. In the two cases presented here, the thrombotic complications may be due to the association with other unrecognized genetic factors.

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