Type I Protein C Deficiency in French Canadians: Evidence of a Founder Effect and Association of Specific Protein C Gene Mutations with Plasma Protein C Levels

Patrick Couture1,2, Christine Demers3, Jean Morissette1, Robert Delage3, Michèle Jomphe4, Louis Couture2, Jacques Simard1,5

From the 1Laboratory of Molecular Endocrinology, CHUL Research Center, Québec, Canada, 2Department of Medicine, CHUQ, Québec, Canada, 3Department of Hematology, Hôpital du Saint-Sacrement, Québec, Canada, 4Institut Interuniversitaire de Recherche sur les Populations, Université du Québec à Chicoutimi, Chicoutimi, Canada, 5Laboratory of Hereditary Cancers, CHUL Research Center, Québec, Canada

Summary

Protein C (PROC) deficiency is one of the most common autosomal codominant diseases. Although more than 150 germline mutations in the PROC gene have been described around the world, the spectrum of mutations among French Canadians is unknown. We have identified one frameshift (3363 ins C) and two missense mutations (R178Q and T298M) in 7 French Canadian families with type I PROC deficiency. In order to demonstrate a possible founder effect for the 3363 ins C mutation, we have constructed a high-resolution genetic map to locate several highly polymorphic markers close to PROC locus. We have then genotyped five markers in 36 heterozygotes for the 3363 ins C mutation. Our data suggest that these patients carry a common haplotype at the PROC locus. Immunologic plasma PROC levels of heterozygotes and genetically normal relatives were also correlated with the nature of the mutation in the coding sequence and with the genotype of three polymorphisms in the PROC promoter. We found that the mean immunologic plasma PROC levels were lower in heterozygotes for the frameshift mutation 3363 ins C compared to heterozygotes for one of the two missense mutations R178Q and T298M (0.46 vs 0.61; P = 0.0004). Moreover, this difference cannot be explained by the genetic variation of the three polymorphisms in the PROC promoter which accounts for only 10.4% of the variation of immunologic PROC levels in non-deficient subjects. These results suggest that the nature of the mutation in the coding sequence of PROC gene may modulate immunologic plasma PROC levels.

Introduction

Protein C (PROC) is a vitamin-K dependent glycoprotein and zymogen of a serine protease playing an important role in the regulation of the hemostatic system (1, 2). This inactive zymogen is synthesized by the liver as a single 462-amino acid chain that undergoes several post-translational modifications including β-hydroxylation, γ-carboxylation and glycosylation to give rise to the mature protein comprising one light and one heavy chain linked by a disulfide bridge. PROC is activated by the thrombin/thrombomodulin complex on the endothelial cell surface through cleavage of the Arg169-Leu170 bond (3). Activated PROC downregulates the coagulation cascade by selective proteolytic inactivation of the procoagulant factors Va and VIIa in the presence of protein S and phospholipids (4, 5). It also enhances fibrinolysis in part by neutralizing plasminogen activator inhibitor-1 (PAI-I).

The PROC gene is located on chromosome 2, at position 2q13-q21 (7-11), and comprises 9 exons encompassing 11 kb genomic DNA and encoding a 1795-bp mRNA (12, 13). PROC deficiency is caused by mutation in the PROC gene and is inherited as an autosomal codominant trait. More than 150 germline mutations in the PROC gene have been described around the world (14). However, in the French Canadian population, no mutation has been reported so far.

The clinical expression of PROC deficiency is highly variable and the diagnosis based on plasma measurements of PROC is often difficult because of the significant overlap between heterozygotes and non-carriers. Heterozygous subjects with plasma PROC levels between 30% and 70% of normal values possess an increased risk of developing venous thrombosis (15). The prevalence of asymptomatic heterozygous PROC deficiency in the general population ranges from 1 in 200 to 1 in 500 (16, 17) while the prevalence of symptomatic heterozygotes ranges from 1 in 16,000 to 1 in 32,000 (18, 19). Homozygous or compound heterozygous subjects with plasma PROC levels below 1% of normal usually develop neonatal purpura fulminans (20).

The present study was designed to investigate the spectrum of mutations in the PROC gene in the French Canadian population, the likelihood of the existence of a founder effect for these mutations as well as to establish correlation between PROC genotype and the biochemical phenotype. We have first studied seven apparently unrelated French Canadian families with hereditary PROC deficiency in which at least one person had had thrombosis. The molecular diagnosis of the deficiency was based on the presence or absence of specific point mutations in the coding sequence of the PROC gene. Then, immunologic plasma PROC levels of heterozygous subjects and genetically normal relatives were correlated with the nature of the mutation in the coding sequence and also with the genotype of three polymorphisms in the PROC promoter. Finally, we have constructed a high-resolution genetic map to locate several highly polymorphic markers close to the PROC locus, thus providing the necessary tools to investigate the origin of the most common mutation retrieved in four of the seven families.
**Materials and Methods**

**Patients**

Seven unrelated adults patients of French Canadian descent were identified for suspected PROC deficiency based on the following classical criteria: plasma levels of PROC antigen below 69% of normal and documented evidence of deep venous thrombosis. All available family members were then contacted and blood sample was taken for PROC measurement and mutation analysis. This study was approved by the Ethic Committee of participating hospitals and informed consent was obtained from all participants.

**Coagulation Assays**

Plasma PROC antigen levels were measured by enzyme-linked immunosorbent assay (ELISA) using the Asserachrom PROC kit (Diagnostica Stago Co., Afinieres-Sur-Seine, France) and clotting functional assays for PROC were purchased from Ortho diagnostic systems (Markham, Ontario, Canada) and performed according to the manufacturer’s protocols. Antigen levels were calculated relative to normal pooled plasma levels (100%). Normal range (mean ± 2 SD) was previously determined on 55 normal subjects (0.66 to 1.34 for functional PROC and 0.69 to 1.31 for immunologic PROC).

**PCR Amplification, SSCP Analysis and DNA Sequence Analysis**

Genomic DNA was extracted from peripheral blood leukocytes by standard methods (21). Selective PCR amplification of each individual nine exons and the intron-exon splicing boundaries of the PROC gene were performed using primers previously described (22). For SSCP analysis, [35S]dATP was used for labeling PCR fragments. Each PCR reaction was subjected to 35 cycles with a temperature cycle consisting of 1 min at 95°C, 1 min at 65°C and 1 min at 72°C. The PCR product was added to formamide loading buffer, heated at 95°C for 5 min, and electrophoresed (8 W for 16 h at room temperature) in a Hydrolink-MDE polymer gel (AT Biochem, Malvern, PA). Gels were dried and exposed to x-ray film. DNA sequencing of the exons displaying aberrant electrophoretic patterns by SSCP analysis were performed as previously described (23).

**Mutation Detection Using Restriction Enzyme Fragment Analysis**

Symmetric PCR is performed under the same conditions described above using the primer pairs: 6u-mismatch 5’CTCTCTGAGCAACGGCGCTGCAGG-3’ and 6d 5’-CAGCAGTATCTCCTGGGAGAT-3’ for the 3363 ins C mutation or 7d 5’TGGGTGAAGGAGCCCACAGCCCTC-3’ and 7u-mismatch 5’TCAATTCAGCGCGCCAGGCCACCTCGTCA-3’ and 9a 5’-GGTGGTAAAGGAGCCACACCCCTCA-3’ for the T298M mutation. The PCR products were digested with the appropriate enzyme, as recommended by the supplier (New England Biolabs) and the resulting fragments were size-separated by electrophoresis on an 1.5% agarose gel. The restriction enzyme Acil was used to detect the R178Q mutation while the restriction enzyme AciI was used to detect the R178Q mutation. The allelic frequency distribution of these five markers was subsequently used to estimate the independent contributions of the different PROC promoter genotypes, age, gender, BMI to the variance of immunologic PROC levels. The covariates were selected because of their univariate associations with immunologic PROC levels. These analyses were performed using the JMP statistical software (release 3.2.2, SAS Institute).

**Results**

Seven unrelated French Canadian adult patients with heterozygous type I PROC deficiency were selected for mutation analysis. They all had plasma levels of PROC antigen below 69% of normal and documented evidence of deep venous thrombosis or pulmonary embolism. When compared with results of control subjects, SSCP analysis of the nine exons of the PROC gene revealed aberrant electrophoretic patterns in exons 6, 7 and 9 in four, and one patient, respectively.

3363 ins C

Direct sequencing of the PCR fragments from exon 6 of the PROC gene in four index cases showed that the aberrant electrophoretic pattern in exon 6 was due to the presence of the frameshift mutation 3363 ins C. This mutation is caused by the insertion of a C just after nucleotide 3363 (12) and leads to a truncated protein with a proline instead of a histidine at amino acid 107, followed by 11 new amino acids and a stop codon. Since the 3363 ins C mutation does not alter any restriction site, we have developed a rapid detection method using a mismatch primer approach with primers 6d and 6u-mismatch, which contains a G instead of a C at position 3362 creating a HaeIII site that is abolished in control individuals. The resulting PCR digested fragments were 130 bp and 25 bp for the mutant allele and 154 bp for the normal allele.

R178Q Mutation

In two index cases, the partial nucleotide sequence of PCR fragments from exon 7 exhibits a G to A transition at nucleotide 6246 in one allele converting the codon 178 (CGG) encoding arginine into CAG encoding glutamine. This mutation abolishes an Acil site, thus diges-
tion of PCR products obtained with primers 7ue and 7d lead to fragments of 121 and 27 bp for the normal allele and 148 bp for the mutant allele. R178Q mutation was not detected in 50 control men of French Canadian origin.

T298M Mutation

Determination of the nucleotide sequence of the PCR amplicon from exon 9 in one index case showed a C to T transition at nucleotide 8608 in one allele. This mutation converts codon 298 (ACG) encoding threonine into a ATG encoding methionine. Digestion of PCR products amplified using primers 9u-mismatch and 9d2 with the enzyme NlaIII yielded two fragments of 98 bp and 32 bp for the mutant T298M allele and a single fragment of 130 bp for the uncut normal allele. T298M mutation was not detected in 50 control men of French Canadian origin.

Construction of a High-resolution Genetic Map of the PROC Gene Region

To determine with a greater precision the location of the PROC locus in the 2q13-21 region (7-11), two-point linkage analysis was done between the -1476 T/A polymorphism in the PROC gene and 459 microsatellite markers of chromosome 2. The closest linkage was observed with the marker D2S347 which yields a maximum pairwise lod score of 25.95 at a minimum sex-average recombination fraction of 0.008.

A framework map was also constructed by multipoint linkage analysis using a subset of five Génethon microsatellite reference markers (D2S1895, D2S2341, D2S347, D2S2215, D2S2256) to determine their order and location relative to the PROC locus. Multipoint linkage analysis showed that the PROC locus is in the interval flanked by D2S347 and D2S2215. Thereafter, a high-resolution genetic map was constructed. As illustrated in Figure 1, the order of the markers is cen - D2S2224 - D2S347 - D2S2339/D2S383 - D2S2271/PROC - D2S2296/D2S2215 - tel. The most likely order was supported by odds of 1000:1 or greater against any permutation of two adjacent markers.

Study of the Founder Effect

In order to investigate the origin of the frameshift mutation 3363 ins C present in four apparently unrelated French Canadian kindreds, we examined allele frequencies of the five microsatellite markers D2S347, D2S2339, D2S383, D2S2271 and D2S2215 which surround and are within 2 cM of the PROC locus in 36 patients with heterozygous type I PROC deficiency bearing this mutation as well as in 50 control French Canadian subjects with normal PROC levels. The allelic distribution of the five microsatellite markers differs significantly between control subjects and heterozygotes. Table 1 shows the presence of a significant association between the 3363 ins C mutation and alleles 286 (D2S347), 208 (D2S2339), 180 (D2S383), 160 (D2S2271), and 153 (D2S2215).

Association of PROC Gene Mutations with Variation of Plasma PROC Levels

Three mutations in the PROC gene were identified in seven French Canadian families. A total of 161 family members (mean age = 37 years, range 4 to 91 years) were available for study. These individuals were asymptomatic at the time of evaluation. Blood sample was taken in each subject for PROC and factor VII measurement as well as genetic analysis using method described above. Based on DNA analy-

Table 1 Allelic distribution frequencies of microsatellite markers in French Canadian patients (n = 36) with heterozygous protein C deficiency and control subjects (n = 50)

<table>
<thead>
<tr>
<th>Marker</th>
<th>D2S347</th>
<th>D2S339</th>
<th>D2S383</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele</td>
<td>N 2563 ins C</td>
<td>N 2563 ins C</td>
<td>N 2563 ins C</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>276</td>
<td>202</td>
<td>18</td>
<td>0.06</td>
</tr>
<tr>
<td>277</td>
<td>204</td>
<td>44</td>
<td>0.16</td>
</tr>
<tr>
<td>284</td>
<td>206</td>
<td>16</td>
<td>0.06</td>
</tr>
<tr>
<td>286</td>
<td>208</td>
<td>69*</td>
<td>0.27</td>
</tr>
<tr>
<td>289</td>
<td>210</td>
<td>00</td>
<td>0.00</td>
</tr>
<tr>
<td>272</td>
<td>212</td>
<td>00</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Significance levels for comparison between normal subjects (N) and heterozygotes calculated by the chi-square test in a pairwise manner are indicated as P-values: * P <0.001
sis, 48 individuals were classified as deficient and 113 as non-deficient. The 3363 ins C, R178Q and T298M mutations were found in 36, 10 and 2 individuals, respectively and 14 of these heterozygotes were stably anticoagulated on warfarin at the time of analysis, leaving 34 non-anticoagulated subjects. The results of functional assays for PROC activity in the plasma of non-anticoagulated subjects with heterozygous PROC deficiency were similar to those measured by immunoassay (data not shown). Interestingly, among non-anticoagulated subjects, 4 (3.7%) of the 108 non-deficient family members and 33 (97.0%) of the 34 heterozygotes had immunologic PROC levels below 0.69 (Fig. 2). Moreover, all non-anticoagulated subjects with immunologic PROC levels below 0.60 were heterozygotes for one of the three PROC gene mutations. Of those individuals with borderline PROC levels (0.60 to 0.75), 5 were heterozygotes and 11 had normal genotype. In these eleven patients, absence of mutation has been confirmed by complete sequencing of the nine exons of the PROC gene. Finally, we have also observed that the mean immunologic PROC levels were lower in non-anticoagulated patients heterozygous for the frameshift mutation 3363 ins C compared to patients heterozygous for one of the two missense mutations R178Q and T298M (0.46 ± 0.05 vs. 0.61 ± 0.06; P = 0.0004). The two genetic groups were equivalent with respect to age (P = 0.81) and gender (P = 0.20).

All individuals were also genotyped for three previously reported PROC promoter polymorphisms (C/T, A/G, and A/T). Of the 27 possible genotypes, only 5 were observed, of which 4 were most frequent. Table 2 summarizes the mean immunologic PROC levels in the different genotype groups. Analysis with one-way ANOVA in non-deficients indicated a statistically significant difference in the immunologic PROC levels among the five genotype groups (P = 0.02). Further analysis with Tukey-Kramer HSD pairwise comparison procedure revealed that the mean PROC levels were significantly lower in the CC/AG/TT genotype group compared to the CC/AA/AA genotype group. Moreover, in the CC/AG/AT genotype group, heterozygotes for one of the two missense mutations (n = 3) have higher PROC levels than heterozygotes for the 3363 ins C frameshift mutation (n = 14).

Table 3 presents the results of the multiple regression analysis of the immunologic PROC level changes of two independent variables in non-deficient subjects. Age was directly correlated with PROC levels. The cumulative r-square (R²) coefficient, which represents the proportion of the variation of PROC levels attributed to the independent variables, reached 23%. The proportion of the variation of PROC levels explained by the variation of promoter genotype reached 10.4% after controlling for age. BMI and gender were not associated with a significant variation of PROC levels.

**Discussion**

In the present study, we have identified two missense mutations (R178Q and T298M) in exons 7 and 9, respectively, and one frameshift mutation (3363 ins C) in exon 6 of the PROC gene following mutation analysis in 7 unrelated French Canadian subjects with heterozygous type I PROC deficiency. Each of these mutations have already been reported in other populations of the world (22, 32-37) and their presence co-segregated with the biochemical phenotype of heterozygous PROC deficiency in the family members studied. Moreover, none of the two missense mutations has been retrieved in a control population of 50 adult men of French Canadian origin suggesting that these mutations are not linked polymorphisms. Indeed, the effects of these two amino acid substitutions on the structure-function relationship of the PROC has already been studied. According to these studies the R178Q muta-

---

**Table 2** Plasma PROC antigen levels according to promoter genotype in non-deficient and deficient subjects

<table>
<thead>
<tr>
<th>Promoter Genotype</th>
<th>Missense Mutation (5)</th>
<th>Nonsense Mutation (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC/AG/TT</td>
<td>0.95 ± 0.04 (31)</td>
<td>0.40 ± 0.03 (9)</td>
</tr>
<tr>
<td>CC/AG/AT</td>
<td>1.01 ± 0.04 (25)</td>
<td>0.40 ± 0.02 (14)</td>
</tr>
<tr>
<td>CT/AG/AT</td>
<td>0.99 ± 0.05 (22)</td>
<td>0.51 ± 0.05 (4)</td>
</tr>
<tr>
<td>CT/AA/AA</td>
<td>1.11 ± 0.05 (20)</td>
<td>0.59 ± 0.06 (4)</td>
</tr>
<tr>
<td>CC/AA/AA</td>
<td>1.19 ± 0.07* (8)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.

*Significantly different from the promoter genotype CC/AG/TT using Tukey-Kramer HSD as multiple pairwise comparison procedure, with an overall alpha level of 0.05

**Table 3** Multiple linear regression analysis showing the contribution of independent variables to the variance of plasma PROC antigen levels in non-deficient subjects

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent variable</th>
<th>Partial R² X 100</th>
<th>Total R² X 100</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROC Ag</td>
<td>Age</td>
<td>12.9</td>
<td>23.3</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Promoter genotype</td>
<td>10.4</td>
<td></td>
<td>0.05</td>
</tr>
</tbody>
</table>

PROC Ag, protein C antigen; R squared, coefficient of multiple determination indicating the proportion of the variance in the protein C changes explained by the independent variables; P, probability value.
tion probably leads to disruption of the calcium-binding loop formed by the carboxyl groups of Asp-227 and Arg-230, as well as the side chains of Glu-225, -232, and -235 secondary to misfolding of the domain (38). It is also proposed that this mutation would influence the removal of the connecting dipeptide during intracellular processing and impaired secretion of the protein (39). On the other hand, the T298M mutation would destabilize the structure of the hydrophobic pocket formed by Ser-181, Trp-183, Gin-184, Leu-318, Phe-320 and Pro-363 because the hydroxyl group of T298 is normally hydrogen-bonded to the side chain of Q184 (38).

The fact that R178Q and T298M mutations occurred at CpG dinucleotides that are well established “hot spots” for point mutation (40) supports the notion that these two missense mutations are recurrent. In fact, the independent occurrence of the R178Q mutation has recently been demonstrated using analysis of eight RFLP(s) in the PROC gene in three PROC deficient Spanish families (41).

The 3363 ins C mutation which was present in 4 of the 7 French Canadian families studied has previously been reported in a French patient with heterozygous type I PROC deficiency (35) and in a large Vermont kindred with a high incidence of venous thrombosis (36, 37). Interestingly, our genotype analysis at the PROC locus suggests that this mutation is carried by a single allele in the French Canadian population. Preliminary genealogic studies also suggest that this mutation was probably introduced in North America by a couple of French settlers who established themselves in the Québec city region during the seventeenth century. The early introduction of the 3363 ins C mutation in the Québec settlement process would imply that this mutation has a high prevalence in the French Canadian population. Epidemiological studies are needed to address this question since the prevalence of PROC deficiency is unknown in the Province of Québec. Furthermore, the French-Canadian emigration in New England namely between 1850 and 1930 (42) would explain the presence of the 3363 ins C mutation in Vermont (36). In order to confirm our hypothesis, it would be necessary to analyse the haplotype of heterozygotes from France and Vermont.

The present study also shows that genotypic variations of the PROC promoter are associated with significant variations of immunologic PROC levels. In non-deficient, the genetic variation in the PROC promoter accounts for 10.4% of the variation of PROC levels after controlling for age. Our data confirm the results previously reported by Spek et al. (24) which showed a similar association between the variations in PROC levels and these three PROC promoter polymorphisms. Moreover, these results also suggest that the 26% (0.61 vs. 0.45) difference in PROC levels between heterozygotes for one of the two missense mutations and heterozygotes for the 3363 ins C frameshift mutation could not be explained by the genotypic variation of the PROC promoter alone. In fact, immunologic plasma PROC levels could also be affected by the nature of the mutation in the coding sequence of the PROC gene. The mechanisms responsible for this difference are as yet obscure but could be related to the severity of the functional repercussion of the 3363 ins C frameshift mutation on the PROC synthesis. However, further studies with a larger number of subjects are required to confirm this observation and to determine if such difference in immunologic plasma PROC levels between these two genetic groups would influence significantly the severity of the clinical phenotype.

In this study, we have also demonstrated the presence of a significant overlap between low normal levels and mild deficiency in PROC. Such overlap may make the diagnosis of heterozygous PROC deficiency confusing using available commercial immunologic PROC assays. The anticoagulation with coumarin derivatives also confounds the diagnosis of PROC deficiency (43). Thus the rapid PCR restriction enzyme fragment analysis presented here offers several advantages over classical protein-based methods for the diagnosis of PROC deficiency especially in the French Canadian population where a limited number of mutations in a gene usually accounts for the majority of cases (42). These methods should be useful to facilitate the clinical diagnosis of PROC deficiency in the French Canadian population and in those harvesting the same mutations. Finally, the present study demonstrates that the PROC gene is closely linked with markers D2S347, D2S2339, D2S383, D2S2271 and D2S2215. This localization is in good agreement with previous mapping of PROC gene to the 2q13-21 region (7-11). The fine genetic mapping of the PROC gene close to several highly informative microsatellite markers should provide a more efficient approach to segregation studies designed to trace the origin of PROC gene mutations present in different populations and to identify new PROC gene mutations and the major susceptibility loci linked to interindividual variations in plasma PROC levels.

Acknowledgements

This research was supported by grants from the Medical Research Council of Canada and “Hydro Québec-FRSQ”. We are indebted to the patients and their parents involved in this study. P.C. is recipient of a fellowship award from the Medical Research Council of Canada.

References


Received October 29, 1997 Accepted after resubmission June 22, 1998