Protein C Deficiency caused by Homozygosity for a Novel PROC D180G Mutation - in vitro Expression and Structural Analysis of the Mutation

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Keywords
Protein C deficiency, mutation, venous thrombosis, skin necrosis, serine proteinase

Summary
Homzygosity for a novel D180G mutation in the protease domain of protein C, associated with plasma protein C activity and antigen levels of 8% of normal was identified in a thrombosis prone family. Transient expression of protein C in HK-293 cells and analysis of protein C antigen in culture media and cell lysates showed that the secretion of mutant protein as compared with wild-type protein was reduced by 79% while the intracellular contents were similar. Computer analysis of the X-ray structure of activated protein C and of a theoretical model of the zymogen predicts that the mutation destabilises the molecule locally. Our results are compatible with a relatively unstable mutant molecule that could be trapped inside the cell and degraded. However, if secreted the mutant molecule could have a relatively normal catalytic activity and structure consistent with the plasma levels of protein C activity and the late onset of thrombosis.

Introduction
The vitamin K-dependent plasma glycoprotein protein C is the precursor of the anticoagulant serine proteinase activated protein C that plays a role in regulating blood coagulation (1, 2). Activated protein C exerts its anticoagulant function by proteolytic inactivation of the blood coagulation cofactors V and VIII in the presence of protein S and of negatively charged membrane surface. Protein C (419 amino acid residues) is produced mostly in the liver as a single-chain inactive zymogen, which is proteolytically processed (removal of K156 and R157) to a two-chain molecule composed of a light and a heavy chain covalently bound through a disulphide bond (1). The light chain (residues 1 through 155) is organised in several structural domains. From the NH2-terminus to the C-terminus, they are the Gla-domain and two epidermal growth factor modules (EGF). The heavy chain (residues 170 to 419) consists of the serine proteinase domain. Under physiological conditions, activated protein C is generated after a specific proteolytic cleavage within its activation peptide by thrombin (between R169 and L170), preferentially when the latter is bound to thrombomodulin. Upon activation, the 12 amino acid activation peptide of protein C is released (residues 158 to 169).

Protein C deficiency is an autosomally inherited disorder associated with an increased risk of venous thrombosis (1, 2). Homzygosity and compound heterozygosity for protein C deficiency is a rare condition (prevalence about 1 in 200,000-400,000 individuals). In patients with undetectable levels of protein C activity in plasma, massive thrombotic complications may occur during the neonatal period. In patients with very low but detectable levels of protein C activity in plasma the clinical manifestations are milder and include late onset of thrombotic symptoms and repeated episodes of skin necrosis. Heterozygosity for protein C deficiency is characterised by an increased risk of venous thrombosis in early adulthood. Anticoagulant treatment of protein C deficient individuals with vitamin K antagonists (warfarin, coumarin, phenprocoumon) may be complicated by warfarin-induced skin necrosis (3).

In hereditary protein C deficiency more than 160 different mutations have been reported (4). The mutations responsible for homozgyote and compound heterozygote protein C deficiency have been identified in 30 and 23 index patients, respectively (4-16). In agreement with the distribution of mutation types in genetic diseases in general (17) more than 65% of the mutations identified in the protein C gene are missense mutations. In most cases, their influence on the biosynthesis and structure of protein C has not been studied.

The present work describes homzygosity for a novel D180G mutation (D26 in the chymotrypsinogen nomenclature) in the protein C gene associated with protein C deficiency, late onset of recurrent venous thrombosis and recurrent warfarin induced skin necrosis. The influence of the mutation on the biosynthesis of protein C was studied in vitro in a transient expression system using human kidney (HK) 293 cells. Structural analysis of the activated protein C X-ray structure and of a theoretical model for the protein C zymogen was also performed to evaluate further the potential roles of the identified amino acid substitution on folding and stability of the mutant protein C.

Materials and Methods
Clinical Material

Subjects under study were the proband, her parents and her 4 siblings (Table 1). The study was performed after informed consent.

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Blood Sampling

Blood for determination of protein C antigen and genomic DNA analysis was collected in K$_2$-EDTA (final concentration of 0.004 mol/l). Blood for other haemostatic parameters was collected in Na$_3$-citrate (final concentration of 0.11 mmol/l). Plasma was separated immediately after collection by centrifugation at 40°C and 2500×g for 20 min. Plasma and blood were stored at −70°C until analysed.

Assay of Protein C and other Biochemical Assays

The Protein C activity and antigen concentration in plasma were analysed by Stachrom™ Protein C and Asserachrom™ Protein C, respectively (both Diagnostica Stago, Asnieres-Sur-Seine, France). Protein C antigen in cell lysate and media were analysed by ELISA using polyclonal antibodies as described (18, 19). Determination of antithrombin, activated protein C resistance and total protein S in plasma was performed using COAMATIC® Antithrombin (Chromogenix, Mölndal, Sweden), COATEST® APC™ Resistance V (Chromogenix) and Assera™-plate protein S (Diagnostica Stago), respectively. No plasma was available for determination of free protein S in plasma.

Isolation of genomic DNA from peripheral blood cells, analysis of the protein C gene and assay of the Arg506Gln Leiden mutation in the coagulation factor V gene and the G20210A transition in the prothrombin gene was performed as described (19).

Construction of Mutant and Wild-type Protein C cDNA Expression Vectors

The expression vectors were constructed as described (19) with the following modification. A MluI-KpnI fragment carrying the D180G mutation was generated by polymerase chain reaction using the wild-type protein C cDNA vector as template, a new oligonucleotide primer (PCD180Gs) and a previously reported primer (PCKpnIas) (19). The PCD180Gs (5'- GAT GAC GC T G T CG GGG AGG CAG CCC CTG GCA GGT GG-3') corresponds to nucleotide number 745-779 in protein C cDNA according to Beckmann (20) with 4 nucleotide sequence variations introduced (underlined) in order to create the MluI restriction enzyme site and the mutation at amino acid residue 180.

In vitro Expression of Protein C, Purification of Total RNA and Northern Blot Analysis

Transient expression of protein C was performed in HK 293 cells and total RNA purified and analysed by Northern blot as described (19) with the following two modifications. Firstly the transfection step was performed using Lipofectamin™ 2000 Reagent (Life Technologies, Paisley, UK). Secondly co-transfection with the pCMV-β-galactosidase plasmid was not performed.

Structural Analysis

The X-ray structures of activated protein C (21) and of prethrombin 2 (22) and a theoretical model for protein C zymogen (23) (coordinate file at http://www.klkemi.mas.lu.se/dahlback) were analysed with the Accelrys (San Diego, CA, USA) computer package running on a Silicon Graphics Fuel workstation (Mountain View, CA, USA). The modules InsightII, biopolymer, DelPhi and homology were used for the analysis of the D180G substitution.

Numbering System of Nucleotides and Amino Acids

The numbering system for protein C is according to Foster (24) except where otherwise stated. In the serine protease domain of protein C, the chymotrypsinogen numbering system mentioned in Mather et al. (21) is also used and written between brackets whenever appropriate.

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**Table 1 Genotype and plasma protein C levels**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Genotype</th>
<th>Protein C Activity</th>
<th>Protein C Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1924 (mother)</td>
<td>Heterozygote</td>
<td>0.54</td>
<td>0.42</td>
</tr>
<tr>
<td>I-1916 (father)</td>
<td>Heterozygote$^a$</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>II-1947 (sister)</td>
<td>Heterozygote</td>
<td>0.72</td>
<td>0.52</td>
</tr>
<tr>
<td>II-1949 (sister)</td>
<td>Homozygote</td>
<td>&lt;0.01</td>
<td>&lt;0.01$^b$</td>
</tr>
<tr>
<td>II-1951 (brother)</td>
<td>Normal</td>
<td>1.17</td>
<td>0.84</td>
</tr>
<tr>
<td>II-1952 (proband)</td>
<td>Homozygote</td>
<td>0.08</td>
<td>0.08$^c$</td>
</tr>
<tr>
<td>II-1956 (brother)</td>
<td>Heterozygote</td>
<td>0.71</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Normal ranges are shown in parenthesis (95% reference interval). Protein C activity and protein C antigen were analysed by Stachrom™ Protein C and Asserachrom™ Protein C, respectively (both Diagnostica Stago). $^a$ Analysed during warfarin treatment. $^b$ Analysed ten days after discontinuation of treatment with warfarin. $^c$ Genotype deduced from the pedigree. Each member is identified by generation number (Roman numeral) and year of birth. N.D. denotes no available data.
Results

Analysis of Protein C Genotype and Plasma Protein C Phenotype

Table 1 summarises the results of genotype and plasma phenotype analyses of protein C in members of the protein C-deficient family under investigation. Nucleotide sequence analysis showed that the proband and her sister (II-1949) are homozygous and the mother and two siblings (II-1947 and II-1956) are heterozygous for an A6252G transition in the protein C gene, converting the normal D180 (GA) to G (GG) in the NH₂-terminal part of the serine proteinase domain. The mutation is not present in the protein C gene of the brother (II-1951). Exon 1 plus 69 nucleotides upstream from the transcription initiation site (including the putative promoter region), 2 through 6, 8 and 9 of the proband were sequenced. No abnormalities could be demonstrated. Family members with abnormal genotype had a reduction of protein C anticoagulant activity and protein C antigen levels in their plasma, consistent with co-segregation between the identified mutation and plasma protein C deficiency.

Three neutral polymorphisms corresponding to nucleotide position -1476 (A/G), 3342 (T/G) and 7228 (C/T) have been identified in the protein C gene (25). The haplotype of the proband corresponding to these polymorphism’s was A/A (position 1476), T/T (position 3342) and T/T (position 7228).

In vitro Expression of Mutant and Wild-type Protein C

To characterise the influence of the D180G mutation on protein C gene expression, mutant and wild-type protein C cDNA were transiently expressed in HK 293 cells. Table 2 shows that the level of mutant

Table 1

<table>
<thead>
<tr>
<th>Protein C antigen (arbitrary units/l; median and range)</th>
<th>Mutant (n=4)</th>
<th>Normal (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>0.033 [0.030-0.036]</td>
<td>0.155 [0.128-0.162]</td>
</tr>
<tr>
<td>Cell lysates</td>
<td>0.043 [0.036-0.052]</td>
<td>0.052 [0.035-0.056]</td>
</tr>
</tbody>
</table>

Mutant and wild-type protein C were transiently expressed in HK 293 cells. The culture media and cell lysates were harvested 72 h after transfection and subjected to analysis of protein C antigen. The protein C level in the mutant culture media as compared to the protein C level in the wild-type culture media was reduced by 79 %, while no significant difference was observed in the level of mutant and wild-type protein C antigen in cell lysates (p > 0.49). There was no significant difference (Mann Whitney-test) in number of cells (p > 0.69). The number of harvested cells per culture dish were (mean [range]) was 4.00 x 10⁶ [2.84 x 10⁶ - 5.60 x 10⁶] n = 4 (mutant) and 1.19 x 10⁶ [3.56 x 10⁶ - 5.12 x 10⁶] n = 4 (wild type).

Table 2

Transient expression of mutant and wild-type protein C in HK 293 cells

<table>
<thead>
<tr>
<th>Chymotrypsinogen numbering</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
<th>29</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant protein C</td>
<td>T</td>
<td>R</td>
<td>R</td>
<td>G</td>
<td>G</td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>Q</td>
</tr>
<tr>
<td>Human protein C</td>
<td>T</td>
<td>R</td>
<td>R</td>
<td>G</td>
<td></td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>Q</td>
</tr>
<tr>
<td>Bovine protein C</td>
<td>A</td>
<td>G</td>
<td>W</td>
<td>G</td>
<td>E</td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>Q</td>
</tr>
<tr>
<td>Rat protein C</td>
<td>T</td>
<td>K</td>
<td>Q</td>
<td>G</td>
<td>D</td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>Q</td>
</tr>
<tr>
<td>Mouse protein C</td>
<td>T</td>
<td>K</td>
<td>Q</td>
<td>G</td>
<td>D</td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>Q</td>
</tr>
<tr>
<td>Rabbit protein C</td>
<td>T</td>
<td>R</td>
<td>R</td>
<td>G</td>
<td>D</td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>Q</td>
</tr>
<tr>
<td>Pig protein C</td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>G</td>
<td>E</td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>Q</td>
</tr>
<tr>
<td>Dog protein C</td>
<td>T</td>
<td>R</td>
<td>R</td>
<td>G</td>
<td>E</td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>Q</td>
</tr>
<tr>
<td>Human prothrombin</td>
<td>A</td>
<td>E</td>
<td>I</td>
<td>G</td>
<td>M</td>
<td>S</td>
<td>P</td>
<td>W</td>
<td>Q</td>
</tr>
</tbody>
</table>

Fig. 1 Alignment of amino acid sequences of mutant and normal human protein C, protein C from other species and human prothrombin corresponding to residues 22 to 30 within the chymotrypsinogen numbering system (21). The affected amino acid in the protein C molecule is shown in bold. The sequences used are from the SwissProt accession numbers P04070, P00745, P31394, P33587, Q28661, P00734 and the GeneBank accession numbers AF191307 and CAA05126.
protein C antigen in culture media was decreased by 79% compared with the level of wild-type protein (p < 0.03), while there was no significant difference between the levels of mutant and wild-type protein C antigen in cell lysats (p > 0.49). Northern blot analysis of total RNA in cell lysates showed no significant difference in amounts between mutant and wild-type protein C mRNA (p > 0.18). The amounts of protein C mRNA (median; range; number of experiments) were 0.79 arbitrary units; 0.56-1.29 arbitrary units; n = 6 (mutant) and 0.50 arbitrary units; 0.45-1.06 arbitrary units; n = 5 (wild-type). Control experiments showed that non-transfected cells and cells transfected with the expression vector without the protein C insert did not express protein C or protein C mRNA.

Structural Analysis

Potential impacts of the mutation on the X-ray structure of Gladinomainless activated protein C and of a theoretical model for protein C zymogen were investigated via structural analysis. At the amino acid sequence level, a negatively charged residue at position 180 is always present in the protein C sequences but is not strictly conserved in the serine protease family (Fig. 1). This point illustrates the importance of a negatively charged group in this region of protein C and suggests that this residue could be important during folding or for the stability of the molecule, via, for instance, ionic interactions and hydrogen bonds. D180 is located in the serine protease domain,
The D26G mutation is likely to destabilise locally, both the zymogen and activated forms of the molecule. A neutral substitution would affect protonation state of residues located in the catalytic groove. Electrostatic computations carried out on the wild type activated protein C X-ray and mutant (assuming similar overall fold) did not show differences of the electrostatic potential values in the active site area.

Because protein C is first produced as a zymogen, it is also important to evaluate some possible effects of the mutation on this molecular structure. The location in space of protein C residues 147 to 169 can be approximated using the related experimental structure of prethrombin 2 (Fig. 3). Because of the low resolution of this segment, exact definition of the amino acid interactions can not be listed. However, assuming that residues K156 and R157 are still present, structural analysis of the mutated zymogen suggests that several positively charged amino acids could have electrostatic interactions with D180 (Fig. 3). Such network of salt bridges and hydrogen bonds should contribute to the folding and stability of this region of the molecule.

**Clinical Phenotype**

The proband suffered at the age of 21 years a spontaneous attack of phlebography verified thrombosis affecting the left deep femoral vein. Few days after initiation of heparin and warfarin treatment she developed skin necrosis of her left femoral area. While on warfarin treatment she suffered episodes of skin necrosis of her right breast and mucosal necrosis of the rectum and the urinary bladder with histologically verified multiple thrombosis of small vessels in necrotic tissue. The mucosal necrosis occurred in relation to warfarin overdose, INR >5. After these episodes warfarin treatment was discontinued and low molecular heparin treatment initiated. While on low molecular heparin (dalteparin doses between 70 and 230 anti-Xa IU/kg/24 h) the proband had several image-verified attacks of thrombosis affecting the left deep femoral vein as well as recurrence of thrombosis of her left profound leg veins, the common iliac vein, the right femoral vein, the right internal jugular vein as well as recurrence of thrombosis of her left profound femoral vein and clinical signs of pulmonary embolism. Since these attacks she has used dalteparin 300 anti-Xa IU/kg/24 h and have had no additional thromboembolic manifestations. The proband had no concurrent disease.

The father of the proband had a retinal thrombosis at the age of 50 years and a deep vein thrombosis of an inferior limb at the age of 63 years. The parents were first cousins. The sister (II-1949) had her 50 years and a deep vein thrombosis of an inferior limb at the age of 21. She had no concurrent disease.
first deep vein trombosis of a lower limb verified by phlebography, at the age of 24 years. She was held on warfarin except during pregnancies, where she was treated with unfractionated heparin. One pregnancy was complicated with a leg vein thrombosis. Neither the mother nor the father or the sister (II-1949) had any concurrent disease. The three siblings (II-1947, II-1951 and II-1956) had no history of thromboembolism or any other disease.

Analysis of Other Risk Factors for Venous Thrombosis

The plasma of the proband was analysed for other haemostatic parameters than protein C that may predict an increased risk of venous thromboembolism. These parameters were antithrombin, protein S, activated protein C resistance, fibrinogen, thrombin time and APTT. These parameters were all within normal range. Because only total protein S was measured coinheritance of free protein S deficiency could not be excluded. The proband and the members of the family were examined for the presence of the Arg506Gln Leiden mutation in the coagulation factor V gene and the G20210A transition in the prothrombin gene. The results showed that the proband and all family members were homozygous for the normal genotypes.

Discussion

This study describes homozygosity for a novel D180G (D26 in the chymotrypsinogen nomenclature) mutation in the protein C gene associated with protein C deficiency, late onset of thrombotic episodes and recurrent warfarin induced tissue necrosis. As is the case with many other missense mutations, it was not directly evident whether this mutation was the cause or just linked to the plasma protein C deficiency. In order to clarify this question mutant protein C cDNA was transiently expressed in HK 293 cells. The data showed that the secretion of mutant protein C was decreased by 79% indicating that the D180G mutation was indeed the cause of the protein C deficiency. There was no significant reduction in the steady state level of mutant protein C mRNA or mutant protein in cell lysates, indicating that mutant protein C mRNA was translated.

We decided to analyze further the above experimental data via structural investigation of both, the experimental structure of activated protein C and a proposed model for the zymogen form of the molecule. Such analysis helps to rationalize potential impacts of the mutation on folding and/or stability. First, the importance of protein C residue D180 is underlined by its conservation (or conservative substitution) in the protein C sequences (Fig. 1). Second, this residue is essentially buried in the activated protein C X-ray structure and is likely to be fully buried in the zymogen form (in prothrombin the corresponding residue is a Met and is fully buried in the X-ray structure of prothrombin 2). It is known that the probabilities that a buried residue will not be substituted by any other residue type during evolution is higher than a surface position and this observation strongly applies to Aspartate residue (26). More specifically, several slightly destabilizing mechanisms and/or folding problems may superimpose in the case of the D180G substitution. In activated protein C and even more so in protein C, D180 seems to be part of a stabilizing hydrogen bond-salt-bridge network (Figs. 2 and 3). In addition, salt-bridges and hydrogen bonds can contribute to the specificity of the fold and as such, substitution of polar and/or charged residues can create local to global folding problems. Further, residue 180 in protein C follows a Glycine and is located in a turn structure. Two subsequent glycines as in the D180G substitution could increase the flexibility of this region and be both, destabilizing and unfavorable to folding (e.g., the folding reaction toward the native state could be delayed). In the present situation, like often observed in the field of biostructural pathology research (27), the D180G mutation is not expected to fully impede folding and the destabilization could be in the order of about 2-5 Kcal/mol. Yet, such mutated proteins can easily be trapped inside the cells by molecular machinery that detects structural fluctuations. Indeed, there is increasing evidence that moderate (to strong) impairment of protein folding and/or destabilization are the major molecular disease mechanism of a great proportion of missense mutations and certain other mutation types found in genetic disorders (28, 29). In the present situation, we expect the mutant protein C to be less stable than the wild type, but if secreted, to have relatively normal catalytic activity and 3D structure. This would in fact be consistent with the in vitro study and antigen levels of the cell lysate, the detectable plasma levels of protein C antigen and activity, and late onset of thrombosis in the proband. If the protein was totally misfolded it would not be recognized by the antibodies and it would have no catalytic activity and thus, thrombotic events should occur earlier in the life of the proband.

The development of skin and mucosal necroses during the initiation of warfarin treatment and during fluctuations of treatment intensity as observed in the proband is a known phenomenon in protein C deficient patients (3). It is probably caused by an imbalance between the levels of the vitamin K dependent proteins, factor VII (procoagulant) and protein C (anticoagulant), both of which have a biological half-life of a few hours. In order to reduce the risk of warfarin induced skin necrosis in risk patients such as protein C deficient patients and patients with previous episodes of warfarin induced skin necrosis it has been suggested to initiate the treatment with low doses of warfarin under the cover of therapeutic doses of heparin (3).

Overall, our analysis underlines the importance of this region of protein C and should help investigate further how sequence relates to structure and function of this important anticoagulant enzyme.

Acknowledgements

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References


