Prothrombin Himi: A Compound Heterozygote for Two Dysfunctional Prothrombin Molecules (Met-337 → Thr and Arg-388 → His)

By Eriko Morishita, Masanori Saito, Ichiro Kumabashiri, Hidesaku Asakura, Tamotsu Matsuda, and Kazuo Yamaguchi

A congenitally dysfunctional form of prothrombin, Prothrombin Himi, shows reduced fibrinogen clotting activity, although it retains full hydrolytic activity toward synthetic substrates. To elucidate the structural abnormality of the variant prothrombin, we first performed genetic analysis of dysprothrombin. Polymerase chain reaction amplification of the exons 8 through 14 of the proband and her family members’ prothrombin genes, which code the thrombin moiety, followed by single-strand conformation polymorphism analysis, identified two variant conformers in exon 10 specific to this family. One variant allele detected in the father was inherited by the proband and one of her sisters, and the other detected in the mother was also inherited by them. This result indicates that the proband has two different base pair changes in the gene. Sequencing showed two novel point mutations in the proband’s gene. One is a T to C transition at position 8751, resulting in the substitution of threonine for methionine at codon 337 (Thrombin Himi I). The other is a G to A transition at 8904, resulting in the substitution of histidine for arginine at codon 388 (Thrombin Himi II). By sequencing analysis of her parents, it was determined that Thrombin Himi I was inherited from the father and Thrombin Himi II from the mother. These results confirm that Prothrombin Himi is compound heterozygous for two dysfunctional prothrombin molecules.

HUMAN prothrombin is a single-chain glycoprotein composed of 579 amino acid residues. On initiation of the coagulation process, prothrombin is converted into the active α-thrombin by factor Xa in the presence of factor Va, phospholipid, and calcium. Human α-thrombin is a serine protease, which consists of two polypeptide chains of 36 (A-chain) and 259 amino acid residues (B-chain) covalently linked by a disulfide bridge.

Congenital disorders associated with prothrombin are rare. Recently, we identified a Japanese family having a dysprothrombin, designated Prothrombin Himi. The proband of this family was a 28-year-old woman, who was referred to us because of an isolated prothrombin deficiency despite the absence of a tendency to bleed. The plasma prothrombin assays in her parents suggested that both the father and mother are heterozygous for abnormal prothrombin (Fig 1). Because her parents are not consanguineous, there are two possible genotypic situations of the proband to be considered: one is a homozygote, the other a compound heterozygote for two distinct abnormal prothrombins. The Prothrombin Himi has been purified from the proband plasma in our laboratory and was found to exhibit the following properties: factor Xa catalyzes proteolysis of the purified prothrombin variant in the presence of phospholipid and calcium to yield a two-chain thrombin having normal-sized fragment 1 and fragment 2. Activation of the Prothrombin Himi by Echis carinatus venom generates 37% of the clotting activity relative to normal prothrombin. However, amidolytic activity of the activated product was almost the same as normal. These results suggest that the abnormality of Prothrombin Himi is located in the thrombin portion of the molecule.

Four prothrombin variants, Prothrombin Madrid, Prothrombin Barcelona, Prothrombin Tokushima, and Prothrombin Quick, so far have been characterized by the amino acid substitutions. However, none of the mutations has been determined at the nucleotide sequence level of the prothrombin gene. As Degan and Davie have already reported, the structural organization of the entire human prothrombin gene and the complete nucleotide sequence of cDNA research on the location and nature of the mutation(s) in the prothrombin variant has been facilitated. The genomic structure of the prothrombin gene includes 14 exons and 13 introns, and the thrombin moiety is coded mainly by exons 8 through 14. Because our previous results indicated that the functional defect of Prothrombin Himi is probably caused by an abnormality in the thrombin portion, we presumed that this genomic DNA carries a mutation(s) within exons 8 through 14.

In this study we found that Prothrombin Himi is compound heterozygous for two dysfunctional prothrombin molecules (Met-337 → Thr and Arg-388 → His) after amplifying the genomic DNA regions corresponding to exons 8 through 14 of the proband and her parents’ prothrombin genes, followed by cloning and sequencing.

MATERIALS AND METHODS

Preparation of genomic DNA. DNA was prepared from leukocytes by the method of Kunkel et al. DNA was stored in Tris-EDTA at −20°C before use.

Amplification of genomic DNA. Polymerase chain reaction (PCR) was performed by the method of Saiki et al using an automated thermal cycler (Perkin-Elmer Cetus; Norwalk, CT). Exons 8 to 14 of the prothrombin gene were amplified by PCR using the oligonucleotides listed in Table 1. Five pairs of oligonucleotide primers were synthesized on an automated DNA synthesizer (Cyclone Plus DNA Synthesizer, Millipore, Bedford, MA). The reaction mixture (100 μL) contains 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L MgCl2, 0.01% gelatin, 1 μg of DNA, 200 μmol/L each of four dNTP, 1 μmol/L of oligonucleotide primer, and 0.5 μL of Perfect Match polymerase enhancer (Stratagene; La Jolla, CA). The reaction was initially started at 94°C for 10 minutes to denature the DNA, followed by the addition of 0.5 μL of Taq DNA polymerase (Cetus) and 50 μL of mineral...
After 30 cycles of PCR, the products of the reaction were initially examined by electrophoresis in 1.5% agarose gel and ethidium bromide staining.

Once in this loading oil to prevent evaporation. PCR conditions are given in Table 1.

Identification of single-strand conformation polymorphism (SSCP). PCR-SSCPs were performed according to the method of Hayashi et al15,16. PCRs were performed as mentioned above with 50 ng of genomic DNA, 30 μmol/L each of unlabeled dNTP, 1 pmol/L bromphenol blue, and 0.05% xylene cyanol. Once in this loading solution, the samples were heated at 90°C for 3 minutes to denature the DNA, then loaded onto 5% nondenaturing acrylamide gels (49:1 ratio of acrylamide to methylene-bis-acrylamide) containing 90 mmol/L Tris-borate (pH 8.3), 2.5 mmol/L EDTA, and 5% glycerol. Electrophoresis was performed at room temperature at 40 W for approximately 3 to 5 hours with cooling using a fan.

DNA cloning and sequencing. The DNA fragments amplified by PCR using each 5'-phosphorylated primers were purified by electrophoresis in 0.8% low-melting agarose gel, and cloned onto the Smal site of pUC19 plasmid. The inserts were sequenced by the dideoxy termination method17 and analyzed on 6% polyacrylamide gel containing 8.5 mol/L urea. The sequencing primers were the same as those used for amplification.

Table 1. Oligonucleotides and Conditions Used to Amplify the Human Prothrombin Gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Oligonucleotides</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-9</td>
<td>5' TGCCTGGGTCCACAAAGGGA 3'</td>
<td>94°C</td>
<td>65°C</td>
<td>72°C</td>
</tr>
<tr>
<td>10</td>
<td>5' AATGGTAGCGAGGCTCCAGGA 3'</td>
<td>60 s</td>
<td>120 s</td>
<td>180 s</td>
</tr>
<tr>
<td>11</td>
<td>5' TACGAATTCTCCTGCTAATGCC 3'</td>
<td>94°C</td>
<td>60°C</td>
<td>72°C</td>
</tr>
<tr>
<td>12</td>
<td>5' AGACACCCACGGGCGAAGTT 3'</td>
<td>60 s</td>
<td>120 s</td>
<td>180 s</td>
</tr>
<tr>
<td>13-14</td>
<td>5' TTTGAGATTTCCAGCGGCTGCT 3'</td>
<td>94°C</td>
<td>60°C</td>
<td>72°C</td>
</tr>
</tbody>
</table>

RESULTS

Identification of nucleotide alterations in Prothrombin Himi. SSCP is a rapid and sensitive assay for nucleotide alterations, including point mutations. DNA segments, approximately 100 to 400 base pairs in length, are amplified by PCR, heat denatured, and electrophoresed on high-resolution, nondenaturing acrylamide gel. Under these conditions each single-stranded DNA fragment assumes a secondary structure determined in part by its nucleotide sequence.

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Five pairs of oligonucleotide primers were used to screen exons 8 to 14 of the prothrombin genes from the proband, four of her family members (Fig 1), and four normal controls. All of the PCR products showed one distinct DNA band of appropriate size on agarose gel electrophoresis, and no differences were detected among the proband, her family, and normal controls (data not shown).

However, on subjecting these PCR products to the SSCP analysis, four unusual alleles specific to the proband and some of her family members were found in exon 10. As shown in Fig 2, all the control samples (lanes 1 through 4) showed only two bands, representing the two complementary single strands of DNA. However, four bands were detected in the DNA from both the father (lane 6) and mother (lane 7). The mobilities of two of the four bands were identical to those of the complementary strands from the controls, indicating that each exon 10 from both the father and mother had two different alleles, one normal and the other mutated. Furthermore, the mobilities of the mutated alleles of the father (named type I mutation) were different from those of the mother (named type II mutation). These two mutated alleles were inherited by the proband (lane 5) and sister II-3 (lane 8). It should be noted that sister II-3 also exhibits lower prothrombin activity. By contrast, sister II-2, who retains the normal level of clotting activity, showed the normal SSCP pattern. In the proband, her parents, and sister II-3 variant alleles were found only in the fragment containing exon 10 (data not shown). No variant allele was observed in the fragment containing exons 8 through 14 of the normal samples (data not shown).

The most likely explanation for these results is that the proband has two different mutations that cause dysfunc-
The substitution of A for G in codon 388, leading to the substitution of a histidine for an arginine residue (Thrombin Himi I). The single-base change at position 8908 is a T to C transition, resulting in a substitution of arginine by histidine in codon 388 (type II mutation; Thrombin Himi II). The single-base change at position 8908 is an A to C transversion, however, the change is not responsible for the substitution of the amino acid sequence.

To confirm that each of these two substitutions was inherited from the parents, we sequenced exon 10 clones from the proband’s parents (Fig 3). Three of five clones of exon 10 from the father show the T to C transition in codon 337 and the others show the normal sequence. On the other hand, two of five clones from the mother show the G to A transition in codon 388. We also detected the single-base change at position 8908 in all clones.

Furthermore, we determined the sequences of the patient’s PCR products containing exons 8 through 14 except exon 10, and confirmed that these sequences were identical to those of normal controls (data not shown).

**DISCUSSION**

In this study, we have undertaken the genetic analysis of a dysfunctional prothrombin molecule, Prothrombin Himi.

To detect single-base substitutions in the proband’s and her parents’ genomic DNA, we first used the PCR-SSCP analysis as described by Hayashi et al. This technique is based on the fact that in nondenaturing polyacrylamide gels, the electrophoretic mobility of single-stranded nucleic acid depends not only on its size but also on its sequence. This strategy made it possible to obtain four variant SSCP bands in the sequence containing exon 10 of the proband, which suggested that Prothrombin Himi might carry two DNA sequence changes within this amplified DNA segment. Since the human genome is believed to contain, on average, one polymorphism every few hundred base pairs, we should also consider the possibility that these two alleles actually represent DNA polymorphisms not associated with Prothrombin Himi. However, neither of these two alleles was found on screening the normal controls, and no variant allele was observed in all other fragments containing exons 8 through 14 except exon 10. Furthermore, mutated alleles observed in the SSCP analysis were co-inherited with lower prothrombin clotting activity by the proband and her sister II-3. These results indicate that it is highly unlikely that both the variants observed in the proband are caused by chance polymorphisms.

To determine the nucleotide sequences of variant alleles detected by the SSCP analysis, we first cloned exon 10 from the proband onto pUC 19. Approximately equal amounts of exon 10 fragments amplified by 10 independent PCRs were mixed and then ligated to the dephosphorylated Smal I site of pUC 19. Six clones isolated independently were purified and sequenced as described in Materials and Methods. All sequences of the cloned exon 10 segments were different from the previously published sequence for the human prothrombin gene, either at positions 8751 and 8908 or at 8904 and 8908 (Fig 3). The single-base change at position 8751 is a T to C transition, resulting in a substitution of methionine by threonine in codon 337 (type I mutation; Thrombin Himi I). The single-base change at position 8904 is a G to A transition, resulting in a substitution of arginine by histidine in codon 388 (type II mutation; Thrombin Himi II). The single-base change at position 8908 is an A to C transversion, however, the change is not responsible for the substitution of the amino acid sequence.

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demonstrated for Prothrombin Tokushima, Prothrombin Quick, Prothrombin Salakta, Prothrombin Metz, and Prothrombin Molis. Two of these prothrombin variants have so far been characterized by amino acid substitutions. In Prothrombin Tokushima, Arg-418 is replaced by Trp. Miyata et al suggested that Arg-418 in the thrombin molecule is important for binding to fibrinogen. On the other hand, Prothrombin Quick is the only other compound heterozygous dysprothrombin for which a primary structural defect has been identified. On activation, Prothrombin Quick gives rise to two dysfunctional thrombins, Thrombin Quick I (Arg-382 → Cys) and Thrombin Quick II (Gly-588 → Val). Henriksen et al concluded that Arg-382 is a critical residue in determining the specificity of thrombin toward fibrinogen, and that Gly-588 forms part of the primary substrate-binding pocket and is critical for controlling the primary substrate specificity in thrombin.

In the present study, two replacements, Met-337 → Thr and Arg-388 → His, are identified in Prothrombin Himi. Arg-388 (equivalent to Asp-72 in α-chymotrypsin) in the thrombin molecule is not conserved in other serine proteases, including the blood coagulation factors. Bode et al have determined the spatial structure of human α-thrombin. Using Bode’s model, Arg-388 would form part of the arginine-rich surface of the 70-80 loop, which probably represents part of the anion-binding exosite. A variety of experiments suggest that anion-binding exosite, which is a positively charged surface region located some distance from the active site, contributes to the remarkable specificity of thrombin interaction with many substrates, cofactors, and inhibitors. Fibrinogen, thrombomodulin, and hirudin all seem to bind competitively to this exosite. Recently, Wu et al prepared some mutant thrombins with single amino acid substitutions, and showed that the mutant thrombin (Arg-73 → Glu substitution) had markedly reduced ability to clot fibrinogen and to activate protein C, but had amidolytic activity similar to the recombinant normal thrombin. They also showed that decreased activa-
tion of protein C correlated with decreased binding affinity for thrombomodulin. These results suggest that residue Arg-73 (equivalent to Arg-388 in prothrombin) is required for recognition of both fibrinogen and protein C and, furthermore, required for binding to thrombomodulin. Prothrombin Himi\(^6\) shows a unique enzymatic form having reduced fibrinogen clotting activity, while retaining amidolytic activities. These properties may be explained by the fact that the site for interacting with fibrinogen in Thrombin Himi is impaired by the Arg-388 \(\rightarrow\) His replacement, resulting in the partial loss of the clotting activity. Moreover, the interesting question is why the proband has no history of excessive bleeding. Because the variant thrombin (Arg-37 \(\rightarrow\) Glu) exhibits markedly decreased procoagulant and anticoagulant activities, Thrombin Himi\(^7\) (Arg-37 \(\rightarrow\) His) might cause a less bleeding phenotype relative to a variant thrombin that retained anticoagulant function. This may not be the full explanation, but at least it must be a partial one.

**REFERENCES**

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Little is known about the function of the other mutant residue in thrombin, Met-337. However, the decrease in prothrombin activity in the father's plasma suggests that Met-337 \(\rightarrow\) Thr replacement seems to reduce its interaction with substrates including fibrinogen.

The results presented here confirm that Arg-388 in human prothrombin is important for binding to fibrinogen. To elucidate the function of the residue Met-337 (Thrombin Himi I), additional investigations will be required. Gene expression experiments using cDNA, which contains this mutation, should be conducted to examine the effect of amino acid substitution on the function of Prothrombin Himi.

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