Prothrombin Greenville, Arg\(^{517}\)\(\rightarrow\)Gln, Identified in an Individual Heterozygous for Dysprothrombinemia


A 64-year-old white male was referred for evaluation of prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT) obtained before elective surgery with initial PT and PTT results of 14.9 and 38.4 seconds, respectively, which corrected to normal in 1:1 mixes with normal plasma. Functional prothrombin assay indicated a level of 51% with thromboplastin as an activator. The prothrombin antigen was 102%. This discordance in the functional and immunologic prothrombin levels was evidence for dysprothrombinemia. Western blotting showed that thrombin was formed at a normal rate in diluted plasma consistent with a mutation within the thrombin portion of prothrombin. DNA was isolated from leukocytes and the thrombin exons were amplified by polymerase chain reaction, cloned, and sequenced. For exon 13, eight clones were sequenced with four clones showing a point mutation in the codon for Arg\(^{517}\), which would result in substitution by Gln. Arg\(^{517}\) is part of the Arg-Gly-Asp(RGD) sequence in thrombin and contributes to an ion cluster with aspartic acid residues 552 and 554. Mutation at this residue most probably distorts the structure of the Na\(^+\) binding site in thrombin. This is the first report indicating the critical role of Arg\(^ {517}\) in the normal physiological interaction of thrombin with fibrinogen. This dysprothrombin is designated Prothrombin Greenville.

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PROTHROMBIN, M.W. 72,000, is the plasma glycoprotein zymogen form of the enzyme thrombin, a critical enzyme in the regulation of hemostasis with both procoagulant and anticoagulant activities. In addition, thrombin also stimulates cellular activation through a cell surface thrombin receptor. The gene for prothrombin is located on an autosomal chromosome at 11p11-q12.2 and severe (homozygous) deficiencies with phenotypic manifestations are encountered only rarely. In cases of heterozygosity, functional deficiencies in prothrombin of 50% or less do not result in physiological manifestations and they are not usually detected by the routine screening tests for coagulation factor deficiency, the prothrombin time (PT), or the activated partial thromboplastin time (aPTT). Thus, although mutations resulting in dysprothrombinemia and hypoprothrombinemia may occur with a frequency approximating that of hemophilia, these mutations are not readily identified. Because of its functional importance, mutations in prothrombin that result in alterations of thrombin function are of particular interest. Congenitally mutant thrombins have been identified previously in eight pedigrees with the primary structure defect identified for six distinct mutations.3-11 All of these mutants have been identified because of decreased fibrinogen clotting activity, the thrombin function that is monitored in clinical coagulation tests.

Site-specific mutants of pro tease are of great value in increasing our understanding of structure-function relationship. Because of the several interactions of thrombin with other proteins, there are numerous surface residues that may be implicated in these interactions, the mutation of which may result in decreased function. Both the congenital and other site-specific mutants of thrombin together with knowledge of the thrombin crystal structure12 have contributed to our understanding of critical features required for normal thrombin function. Some features of thrombin that are known to regulate its activity include the catalytic triad residues, primary substrate binding site, anion binding exosite I (which interacts with fibrinogen and the thrombin receptor/substrate), anion binding exosite II, the heparin binding site, the thrombomodulin binding site, and the more recently identified Na\(^+\) binding site.13,14

The studies described here were undertaken to determine an explanation for the observed low functional prothrombin level in a patient who was evaluated for abnormal prothrombin and partial thromboplastin times before elective surgery. DNA sequencing showed that the patient was heterozygous for dysprothrombinemia and normal prothrombin with identification of a G—a transition in exon 13 that predicts a substitution of Gln for Arg\(^ {517}\)(187). (Residues of thrombin are numbered according to the prothrombin sequence15 with the chymotrypsin numbering12 in parentheses). As reported previously,16 this residue forms part of an ionic cluster in thrombin that is adjacent to the Na\(^+\) ion ligands.13

MATERIALS AND METHODS

Reagents. Fibrinogen from KabiVitrum was purchased from Helena Laboratories (Beaumont, TX). Phospholipid was obtained as rabbit brain cephalin from Sigma Chemical Co (St Louis, MO) and a stock solution was prepared according to the manufacturer’s directions. Taipan venom from Oxyuranus scutellatus was obtained from Miami Serpentarium Laboratories (Punta Gorda, FL), and Echis carinatus venom was obtained from Sigma Chemical Co. Dade factor assay reference plasma for preparation of prothrombin standard curves was obtained from Scientific Products (McGaw Park, IL). The chromogenic substrate, tos-Gly-Pro-Arg-pNA (Chromozym TH) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Oligonucleotides were prepared by the DNA core laboratory, East Carolina University (Greenville, NC). The restriction enzyme Mnl I was obtained from Amersham Life Science (Arlington Heights, IL). Other chemicals were obtained from domestic suppliers.

Blood specimens for plasma and DNA. Phlebotomy was performed after obtaining informed consent. For coagulation assays, plasma was
Table 1. Oligonucleotide Primers Used in Amplification of Prothrombin Exons

<table>
<thead>
<tr>
<th>Exon</th>
<th>Oligonucleotide</th>
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<tbody>
<tr>
<td>8</td>
<td>5′-TGGCTGGTCCCAAGAGAGGA 3′</td>
</tr>
<tr>
<td></td>
<td>5′-CCTGCGATGCCCAACTCTCA 3′</td>
</tr>
<tr>
<td>9</td>
<td>5′-CCTCAGCTGGGCTCTGTC 3′</td>
</tr>
<tr>
<td></td>
<td>5′-ATGGTACGGACGGCTCAAGGA 3′</td>
</tr>
<tr>
<td>10</td>
<td>5′-TACGAGCTTCTCATGCTGCTG 3′</td>
</tr>
<tr>
<td></td>
<td>5′-AGACCCCAAGGGCAGCAGTT 3′</td>
</tr>
<tr>
<td>11</td>
<td>5′-TTCCTGTCTCCTGTTGGGT 3′</td>
</tr>
<tr>
<td></td>
<td>5′-TCAGCTAAAGACATCTGGTTGCC 3′</td>
</tr>
<tr>
<td>12</td>
<td>5′-TCTCAGGGCCTTCTGTC 3′</td>
</tr>
<tr>
<td></td>
<td>5′-ATCCGATCTGAACCCACAGGCAACAATCTCA 3′</td>
</tr>
<tr>
<td>13</td>
<td>5′-CTTGAGTCTCAACAGCTGTCGTG 3′</td>
</tr>
<tr>
<td></td>
<td>5′-AGGCCCTGCGGGCCTGAGATGC 3′</td>
</tr>
<tr>
<td>14</td>
<td>5′-GCCCTGGCAAGGAATACGTAGT 3′</td>
</tr>
<tr>
<td></td>
<td>5′-TTTGAAGCTTTGAGGAGTCTGGC 3′</td>
</tr>
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obtained from citrated whole blood, subjected to a second centrifugation at 10,000g and stored in aliquots at −80°C. For isolation of DNA, whole blood was anticoagulated with acid citrate dextrose solution (A). Coagulation assays. Prothrombin assays were performed by one stage assay with either taipan venom217 or Echis carinatus venom218 as activators and fibrinogen as substrate. The chromogenic substrate tos-Gly-Pro-Arg-pNA was used in a two-stage assay and prothrombin activation was accomplished with a taipan venom activator reagent consisting of 0.2 mg/mL taipan venom in 0.1 mol/L NaCl, 0.04 mol/L Tris—HCl, pH 7.5. After 1.0 minute incubation at 37°C, 100 µL of the incubation mixture was transferred to 1.1 mL 0.1 mol/L NaCl, 0.05 mol/L Tris—HCl, and pH 7.5. For isolation of DNA, the normal values for the vitamin K-dependent coagulation assays indicates the presence of dysprothrombinemia in this otherwise healthy individual. The dysprothrombin activity was at or near the hemostatic threshold, and aPTT obtained before elective surgery for a right rotator cuff repair. He had no prior history of significant bleeding or bruising and had undergone previous herniorrhaphy, right heel surgery, traumatic amputation of the distal portions of two fingers, and multiple tooth extractions without significant bleeding or the need for blood transfusions. There was no history of liver disease. Diet was normal with only remote use of small amounts of alcohol. No family history of abnormal bleeding was noted. Physical examination was not remarkable for jaundice, hepatosplenomegaly, or signs of chronic liver disease. No echymoses or evidence of recent bleeding was present. No joint deformities or evidence of hemorrhaxis was noted. Limitation of motion of the right shoulder was present because of the rotator cuff injury. Laboratory studies performed by a reference laboratory before referral indicated a low prothrombin activity with normal factor V and X activity levels. The laboratory results of the hemostasis evaluation were as follows with normal ranges in parenthesis: platelets 220 (150 - 440) × 10^9/L, bleeding time 3.2 (2.5 - 9.5) minutes, PTT 14.9 (11.6 - 13.2) seconds, and for a 1:1 mix with control plasma 12.3 seconds, aPTT 38.8 (22.6 - 33.0) seconds and for a 1:1 mix with control plasma 32.5 seconds, fibrinogen 2.73 (2.00 - 4.00) mg/mL. Values obtained for factor assays with >50% considered normal were as follows: factor V 90%, factor VII 73%, factor IX 64% and factor X 101%. The prothrombin activity assay was 51% (normal >50%) in a one-stage assay with thromboplastin as the activator. In contrast the prothrombin antigen was 102% with a normal range of 75% to 130%. Fibrinogen was the substrate for all factor assays. Dilute Russell’s viper venom time, antinuclear antibody, and anticardiolipin antibody panel testing were normal indicating that a lupus anticoagulant was not present. The normal values for the vitamin K-dependent coagulation factors other than prothrombin indicate that the low prothrombin level was not caused by vitamin K deficiency. Thus, the discordance between the prothrombin antigen and activity assays indicates the presence of dysprothrombinemia in this otherwise healthy individual. The dysprothrombin has been designated Prothrombin Greenville. Because the surgeon did not wish to risk bleeding complications, and because the prothrombin activity was at or near the hemostatic threshold, the patient was given fresh frozen plasma in the perioperative period. The surgery was successfully completed with no unusual bleeding noted. Additional prothrombin assays. To further characterize the dysprothrombin identified in the proband, additional prothrombin assays were performed with venom activators and both fibrinogen and tos-Gly-Pro-Arg-pNA as thrombin substrates.
The results of these assays are presented as mean ± standard deviation for three separate assays: one-stage assay with Echis carinatus venom as activator and fibrinogen as substrate 36% ± 1%, one-stage assay with taipan venom/PL/Ca²⁺ as activator and fibrinogen as substrate 50% ± 2%, and two-stage assay with the latter activator and tos-Gly-Pro-Arg-pNA as substrate 76% ± 4%. The taipan venom assay with fibrinogen as substrate, 50%, was similar to the 51% obtained initially with thromboplastin as activator. If the dysprothrombinemia were the consequence of a mutation in the activation peptide or a defect preventing hydrolysis of the Arg⁵¹⁷(187)-Thr bond of prothrombin, it was predicted that activation by Echis carinatus venom would yield a normal thrombin level. This was not observed, which suggests that the molecular defect is within the thrombin portion of the molecule. When a chromogenic substrate was used in a two-stage assay, the prothrombin activity for the proband was greater than observed with fibrinogen as substrate. This result suggested that the defect in the predicted dysthrombin probably affected thrombin-fibrinogen interaction to a greater extent than the interaction with a low molecular weight substrate. However, there is apparently also some defect in catalysis by the dysthrombin because the activity toward tos-Gly-Pro-Arg-pNA is not equivalent to that expected for the observed antigen level.

Prothrombin activation and Western blotting. To further examine the nature of the defect in the dysprothrombin, the time course of prothrombin cleavage was determined by Western blotting. The results following activation of diluted whole plasma by taipan venom and electrophoresis on a nonreducing gel shown in Fig 1, indicate that prothrombin is cleaved at a similar rate for both the proband and the control and that the products for both have similar molecular weights. A mutation at Arg⁵¹⁷ would result in an activation defect and approximately half of the total prothrombin in this apparently heterozygous individual should have remained as the higher molecular weight proteins, meizothrombin (with the same molecular weight as prothrombin), or prethrombin 1 at the end of the incubation period. A mutation at Arg²¹⁰ would result in the accumulation of prethrombin 2 (uncleaved thrombin A and B chains). Under reducing conditions, prethrombin 2 should appear at a higher molecular weight than the thrombin heavy chain. Electrophoresis performed under reducing conditions indicated conversion of all prothrombin to thrombin (results not shown). Thus, the results are consistent with the presence of a dysthrombin. This dysthrombin was predicted to be the consequence of a point mutation because of the similar molecular weights for prothrombin and its hydrolysis products obtained from plasma of the control and the proband.

DNA sequencing. Attempted screening of PCR products for the thrombin exons was not successful in identifying a point mutation. Because the proband was expected to be heterozygous for a dysprothrombin, at least six clones were sequenced for each exon to verify the presence of a unique point mutation. The following list gives the number of the prothrombin exon with the number of clones sequenced indicated in parenthesis: 8 (7), 9 (6), 10 (14), 11 (12), 12 (11), 13 (8), and 14 (8). The nucleotide at position 8908 in exon 10 was C in all the clones that were sequenced as originally reported for the human prothrombin cDNA sequence. This does not result in an amino acid substitution. For exon 13, 4 of 8 clones sequenced yielded a transitional mutation at nucleotide 19777 corresponding to a codon change from CGA to CAA at amino acid residue 517(187), which results in an Arg—Gln mutation. The finding of 50% normal sequences confirms that the proband is heterozygous for the mutation. The results for sequencing two clones for exon 13 amplified from DNA of the proband are shown in Fig 2. A mutation at Arg⁵¹⁷(187), a surface residue that forms part of an ionic cluster in thrombin, has not been previously reported. This region of thrombin, which is near the base of the primary substrate binding pocket, has not been specifically associated with the interaction of thrombin with fibrinogen.

Structural consequences of the Arg⁵¹⁷(187)—Gln mutation. The mutated arginine residue in this dysprothrombin is a part of an RGD sequence in thrombin. However, the aspartic acid residue of this sequence is located at the base of the primary substrate binding pocket and neither this residue nor the adjacent glycine are accessible to the molecular surface, which makes it unlikely that this sequence contributes to binding interactions in native thrombin. The ionic cluster formed by Arg⁵¹⁷ and Asp⁵⁵₂(221) and Asp⁵⁵₄(222) is shown schematically in Fig 3. A Na⁺ binding site that contributes to optimal coagulant activity and involves an adjacent region of thrombin has been identified in which one of the Na⁺ ligands is provided by the carboxyl oxygen of Arg⁵₅₂(221). Examination of the thrombin structure indicates that the correct positioning of this Na⁺ ligand must depend on formation of the ionic cluster between the two adjacent aspartic acid residues and Arg⁵¹⁷.

Thus, in Thrombin Greenville, where this ionic interaction is lost because of the substitution of the positively charged...
arginine residue by the neutral residue glutamine, there is most probably a significant perturbation of the structure of the Na\textsuperscript{+} binding site, which appears to result in an enzyme lacking significant fibrinogen clotting activity as indicated by the functional prothrombin assays. Previous results from Di Cera’s laboratory\textsuperscript{26,27} indicate that in the absence of Na\textsuperscript{+} the rate of release of fibrinopeptide A by thrombin is decreased to a greater extent than is the hydrolysis of the low molecular weight substrate H-D-Phe-pipecolyl-Arg-p-nitroanilide. Similarly, the results obtained here for the prothrombin assays suggest that structural features associated with the ionic cluster around Arg\textsuperscript{517(187)} are apparently less critical for the normal hydrolysis by thrombin of the low molecular weight substrate tos-Gly-Pro-Arg-pNA than for the release of fibrinopeptide A from fibrinogen.

Restriction enzyme digestion. Sequence analysis of the normal and Prothrombin Greenville nucleotide sequences identified an \textit{Mnl I} restriction site in the normal sequence that was lost from Prothrombin Greenville. The recognition sequence for \textit{Mnl I} is CCTC. The recognition sequence on the complementary strand, GAGG corresponds to the prothrombin gene coding sequence for nucleotides 19777 to 19780 in exon 13. The Prothrombin Greenville mutation converts this sequence to AAGG resulting in the loss of the \textit{Mnl I} restriction cleavage site. The PCR amplified exon 13 for the proband and several family members were subjected to \textit{Mnl I} digestion. The results shown in Fig 4 indicate that in the proband and one son, digestion of this fragment is incomplete, which is consistent with the loss of the \textit{Mnl I} restriction site as predicted by the observed mutation and the expected heterozygosity for this mutation. Figure 5 shows the pedigree for the proband with prothrombin assays for several family members. These results are consistent with the findings obtained by \textit{Mnl I} digestion.

DISCUSSION

The dysprothrombin identified in these studies has been designated Prothrombin Greenville for the North Carolina location where this mutant protein was characterized. It was anticipated that a dysthrombin with decreased fibrinogen clotting activity would have an altered residue associated with either the catalytic triad residues, anion binding exosite I, or the primary substrate binding pocket as has been observed with other dysthrombins.\textsuperscript{2} This did not appear to be the case for the

Fig 2. Results of DNA sequencing. Nucleotide sequence for a portion of two clones obtained for prothrombin exon 13, which was amplified by PCR from DNA of the proband. Sequencing was by the dideoxy method of Sanger.\textsuperscript{21} The asterisk (*) indicates the mutated amino acid residue.

Fig 3. Structure of thrombin showing location of mutated ionic cluster. Ribbon structure for thrombin is shown as produced by the RasMol modeling program with coordinates obtained from the Brookhaven database. Side chains are shown at the bottom left for residues Arg\textsuperscript{517(187)} marked by the arrow head, Asp\textsuperscript{552(221)}, and Asp\textsuperscript{554(222)}. In this view the active site serine residue is located at the center, anion binding exosite I extends across the center right, and anion binding exosite II is along the upper left edge.

Fig 4. Restriction digest with \textit{Mnl I}. The mutation identified in Prothrombin Greenville predicts the loss of an \textit{Mnl I} restriction site where the sequence GAGG in the normal is the enzyme recognition site. This site is converted to AAGG in the mutant. Exon 13 for the proband and five family members was amplified by PCR and subjected to digestion with \textit{Mnl I}. Hydrolysis was continued for 7 hours at 37°C. Additional enzyme was added to samples for lanes 3, 4, and 6 after 6 hours of hydrolysis to ensure complete hydrolysis. Shown are the final hydrolysis products after agarose gel electrophoresis and ethidium bromide staining. Lane numbers correspond to samples obtained from individuals shown in the pedigree (Fig 5). Shown in the lane at left is a 100-base pair ladder.
mutation that we identified, so other structural features were examined in an attempt to relate the identified mutation to defective fibrinogen cloting activity. Examination of the results of structural studies of thrombin\textsuperscript{13,25} suggests that this mutation most probably affects the Na\textsuperscript{+} binding site. Binding of Na\textsuperscript{+} at this site is proposed to participate in the regulation of the procoagulant and anticoagulant forms of thrombin in which fibrinogen or protein C are the respective thrombin substrates.\textsuperscript{14} This is the first report of a dysthrombin in which the Na\textsuperscript{+} binding site appears to be the locus of the mutation. These findings are also consistent with a critical structural role for the ionic cluster involving residues 517 (187), 552 (221), and 554 (222) located on the surface of thrombin. From comparison of the prethrombin 2 and thrombin structures, it was proposed\textsuperscript{25} that this ionic cluster is a crucial driving force for the structural rearrangement that occurs in this region of thrombin on cleavage of the thrombin A and B chains to yield the active enzyme thrombin. This structural rearrangement results not only in formation of the Na\textsuperscript{+} binding site by positioning one of the Na\textsuperscript{+} ligands, R553 (221A), but also results in a shift of the position of D519 (189) at the base of the primary substrate (arginine) binding pocket. It is probable that improper positioning of D519 (189) also contributes to the functional defect observed in Thrombin Greenville. This ionic cluster is not conserved in the primary structure of other serine proteases,\textsuperscript{13} although it is conserved in bovine thrombin as is the Arg that provides a Na\textsuperscript{+} ligand corresponding to Arg 553(221A) of human thrombin.\textsuperscript{28} A related mutant thrombin with the sequence Ala\textsuperscript{552(221A)}ArgLys has been prepared by recombinant DNA methods. In this mutant, the allosteric effect of Na\textsuperscript{+} is lost with the result that the kinetics for release of fibrinopeptide A are similar in the presence and absence of Na\textsuperscript{+}.\textsuperscript{13} It appears that this latter mutant has a relatively greater activity in the release of fibrinopeptide A than does Thrombin Greenville. As with other dysprothrombins that have been reported, this dysprothrombin was identified because of its decreased fibrinogen clotting activity. This heterozygous defect does not appear to have resulted in any phenotypic manifestation in the proband. There is no history of either excessive bleeding or thrombosis in the proband.

The G→A transitional mutation has most probably arisen on the noncoding DNA strand where the sequence CpG occurs, a sequence that has been identified as a mutational “hot spot.” These mutations are thought to arise when a methylcytosine is deaminated and then recognized as a T with the result that A is incorporated on the complementary strand during DNA replication. Other dysthrombins that have arisen by a similar mechanism are Tokushima\textsuperscript{3} and Quick I.\textsuperscript{13}

The identification of individuals heterozygous for dysprothrombinemia is somewhat fortuitous because they are not generally manifested by prolonged coagulation screening tests. For Prothrombin Greenville, the dysthrombin may have been inhibitory contributing to the prolonged PT and aPTT tests or the mildly decreased factor VII and factor IX levels in combination with the low prothrombin level may have resulted in the prolonged times.\textsuperscript{29}

Further characterization of the enzymatic activity of this mutant thrombin will require isolation of Prothrombin Greenville from plasma or the expression of the mutant protein by recombinant DNA techniques. These studies are currently planned and will permit a more definitive analysis of the role of the ionic cluster formed around Arg\textsuperscript{517(187)} in normal thrombin function.

**ACKNOWLEDGMENT**

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