RAPID COMMUNICATION

Single-Strand Conformation Polymorphism Analysis Is a Rapid and Effective Method for the Identification of Mutations and Polymorphisms in the Gene for Glycoprotein IIIa

By Ying Jin, Harry C. Dietz, Alan Nurden, and Paul F. Bray

Glanzmann thrombasthenia (GT) is the most common inherited disorder of platelets. Most of the molecular defects previously identified in GT have been caused by point (or other small) mutations in the genes for glycoprotein (GP) IIb or GPIIIa. We have used single-strand conformation polymorphism (SSCP) analysis to rapidly identify single-base changes in the GPIIIa gene. Using genomic DNA from normal individuals and patients with GT, each GPIIIa exon and a short stretch of flanking intronic sequence was amplified, heat-denatured, and separated in nondenaturing acrylamide gels. Only those fragments with an abnormal migration pattern were isolated and the nucleotide sequence determined. Using SSCP, we detected the polymorphism in the HPA-1 (P14) system and all three known silent polymorphisms in the GPIIIa gene. Screening 14 GPIIIa exons from 5 patients with GT, one mutant allele was identified. The nucleotide sequence of the abnormal 240-bp SSCP fragment was determined and a G → A substitution in the splice donor site of exon iv was identified. Analysis of platelet RNA resulting from this mutation showed two mRNA species: one contained a deletion of exon iv, whereas the other had a 27-bp addition to exon iv due to the use of a cryptic splice site in the downstream intron. Single-base substitutions are the most common mutation in GT and often result in abnormal mRNA splicing. SSCP is a rapid and sensitive technique for identifying mutations or polymorphisms in the GPIIIa gene.

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MATERIALS AND METHODS

Subjects. Blood from 15 normal volunteers was used to prepare genomic DNA. We obtained blood from a subject known to be homozygous for HPA-1b (HPA-1a negative) by immunophenotyping. We studied 5 unrelated patients and, in some cases, their family members. All patients had type I GT by standard criteria for the diagnosis of GT.4 In particular, the patient (MH) in whom we identified a mutation has previously been shown to lack GPIIb and GPIIIa by Western immunoblot.5 The abnormality in the mutant allele of the patient’s mother (AH) had not previously been identified.

Oligonucleotide synthesis. All oligonucleotides were designed from the genomic sequence of Zimrin et al.28 In general, oligonucleotides were designed to (1) yield an amplified PCR product of less than 300 bp; (2) be positioned in the intron 15 to 25 bp away from the splice junction; (3) keep the theoretical melting temperatures of the 2 oligonucleotides in a pair within 2°C of one another (most members. All patients had type IIb, radioisotopes were from Amersham (Arlington Heights, IL), and the ds Cycle Sequencing System was from Gibco BRL (Grand Island, NY).

PCR amplification for SSCP analysis. Reactions were performed as described25 and consisted of 100 ng genomic DNA, 10 pmol of each primer, dNTPs at 12.5 μmol/L to 150 μmol/L, 25 μCi 32P-dATP, 25 μCi 32P-dCTP, 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.001% gelatin, and 2.5 U Taq polymerase in a 50 μL volume. Different primers required different dNTP concentrations to minimize background. After 4 minutes at 95°C, 30 cycles of amplification were performed using the following conditions: denaturation for 45 seconds at 94°C, annealing for 30 seconds at 60°C to 62°C, and 45 s cycle for complementarity between two oligonucleotides or within a given oligonucleotide. The oligonucleotides used in this study are listed in Table 1.

Reagents. Taq polymerase was from Perkin Elmer (Norwalk, CT), radioisotopes were from Amersham (Arlington Heights, IL), and the ds Cycle Sequencing System was from Gibco BRL (Grand Island, NY).

RESULTS

Preliminary PCR reactions were performed to optimize the annealing temperatures and the dNTP concentrations with an equal volume of 95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol. This sample was denatured at 95°C for 5 minutes, placed on ice, and promptly loaded onto a 36 cm × 30 cm × 0.4 mm gel composed of 6% acrylamide, 10% glycerol, and 1× TBE (89 mmol/L Tris-borate, pH 8.3, 2 mmol/L EDTA). One normal, non-denatured sample was always included as a control. The gel was run for 16 hours at 10 W at 22°C, dried, and exposed to film for 24 to 48 hours. Glogos II Autorad markers (Stratagene, La Jolla, CA) were used for subsequent alignment of gel and film. Whenever an abnormal fragment was identified, the PCR and SSCP was repeated to be sure the abnormality was not due to a Taq polymerase error.

Recovery of abnormal SSCP fragments. The autoradiogram was carefully aligned on top of the gel and then stapled together. A scalpel was used to cut through both the film and gel, and that portion of the gel containing the abnormal fragment of DNA was recovered. The gel and paper were placed in 100 μL sterile water at 37°C for 3 hours with occasional mixing.

Sequence determination of abnormal SSCP fragment. Ten microliters of the eluted DNA sample was reamplified by PCR in a 50 μL reaction using the same primers that yielded the abnormal SSCP fragment. Two microliters of the PCR reaction was sequenced directly in a reaction that included a [γ-32P]-labeled primer and 2.5 U Taq polymerase (ds Cycle Sequencing System; Gibco BRL). Typically, 20 cycles of amplification were performed using the following conditions: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 58°C, and extension at 72°C for 30 seconds. Products were analyzed on 4% acrylamide/7 mol/L urea gels and subjected to autoradiography.

RNA preparation and sequencing. Total platelet RNA was prepared and reverse transcribed into cDNA with random primers as previously described.33 Using oligonucleotides designed from exon sequence, the platelet cDNA was amplified and analyzed on 3% agarose gels. Abnormal fragments were gel purified and the nucleotide sequence determined as described above.

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## Table 1. GPIIa Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense (5' to 3')</th>
<th>Exon</th>
<th>Product Length (bp)</th>
<th>Antisense (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A</td>
<td>CCAAAATCTGGTCTGTAATGCTT</td>
<td>i</td>
<td>157</td>
<td>GAGAAAGAAAAAAGGTCTGTGAT</td>
</tr>
<tr>
<td>3B</td>
<td>TTCTGATGCTGAGCTTCTT</td>
<td>ii</td>
<td>267</td>
<td>TCCTCCCCCATGGCAAAAGT</td>
</tr>
<tr>
<td>3C</td>
<td>GATAAAAAGTAACTTCTTTCGCT</td>
<td>iii</td>
<td>319</td>
<td>AAAGGTGCTGTCTGGCCTT</td>
</tr>
<tr>
<td>3D</td>
<td>ATGAAAGGTCTGCTTTAAATATT</td>
<td>iv</td>
<td>240</td>
<td>CCAAGCCACTCTGSGAC</td>
</tr>
<tr>
<td>3E</td>
<td>TTTGTTTTGTCCTCCTGCTT</td>
<td>v</td>
<td>242</td>
<td>AGTAAAGATGACCACAGTGGT</td>
</tr>
<tr>
<td>3F</td>
<td>GAGGACTCAATACACCTACAT</td>
<td>vi</td>
<td>165</td>
<td>AGCCAAATGAGTGGACTT</td>
</tr>
<tr>
<td>3G</td>
<td>TTCTGCTGCTGCTGACTT</td>
<td>vii</td>
<td>209</td>
<td>GACCTTGACGAAAGGCCCT</td>
</tr>
<tr>
<td>3H</td>
<td>CAGTCTCAATCTGCTCTTGT</td>
<td>viii</td>
<td>291</td>
<td>GCTCCAGGACAAAGGCCCT</td>
</tr>
<tr>
<td>3I</td>
<td>GGGCACCAAGCTGCTTAAAT</td>
<td>ix</td>
<td>289</td>
<td>AAGGGGATAGTCTCTT</td>
</tr>
<tr>
<td>3J</td>
<td>GTATGGGCGTTTGGGCCTG</td>
<td>ix</td>
<td>289</td>
<td>TATATGGGAGGTTGCTT</td>
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<tr>
<td>3K</td>
<td>CAGGCGGTGCCACCTTCTT</td>
<td>x</td>
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<td>CGAGCCTCCGGCTTCTT</td>
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<tr>
<td>3L</td>
<td>TTGCCTTAAATCAGTGTGCTCT</td>
<td>xi</td>
<td>170</td>
<td>CTTGGTGTGCTGCAACTT</td>
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<td>229</td>
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<td>3N</td>
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<td>3a41</td>
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<td>153</td>
<td>GCCACAGGCTGTATAAGTCTT</td>
</tr>
<tr>
<td>3a44</td>
<td>CAGAACCCTGGGTACCAAGCTGCGC</td>
<td>xi</td>
<td>246</td>
<td>GCCACAGGCTGTATAAGTCTT</td>
</tr>
</tbody>
</table>

All primers are complementary to intronic sequence and flank the indicated exon, except 3I (antisense), 3M (antisense), 3J (sense), 3a41, and 3a44, which are contained within exon sequence. The PCR template was genomic DNA for all primers except 3a41 and 3a44, which was platelet cDNA. Oligonucleotides are hereafter appended with "$\cdot"S" or "$\cdot"A" for sense or antisense, respectively.
SSCP IDENTIFIES GPIIIA MUTATIONS

for each set of primers. All oligonucleotide primers designed from intronic sequence listed in Table 1 correctly amplify normal genomic DNA (not all data shown). An agarose gel is shown in Fig 1A that contains the PCR products obtained by amplifying the GPIIIa exon xiv from a series of normal genomic DNAs (exon numbering according to Zimrin et al). As expected, a single fragment was obtained. Figure 1B shows the SSCP pattern obtained from the products shown in Fig 1A. Note that there are three fragments in each sample. These represent three different migration patterns from the four possible strands (the 5' to 3' strand and the 3' to 5' strand from each parent). Between 10 and 15 normal genomic DNAs were analyzed by SSCP with all primers (not shown). All normal genomic DNAs yielded banding patterns identical to one another, except with primers amplifying exons ii, viii, and ix. These three exons contain the known polymorphisms in the GPIIIa gene.

To test the ability of the SSCP technique to identify single nucleotide changes in the GPIIIa gene, we analyzed exon ii in the genomic DNA from an individual with the HPA-1b/1b phenotype. Newman et al have shown that a C → T substitution is responsible for the HPA-1 polymorphism, which is contained in GPIIIa exon ii. SSCP analysis of 11 different DNA samples showed three distinct patterns (Fig 2A). The known HPA-1b/1b individual exhibits pattern “Y” (Fig 2A, lane 2). Note that lane 1 shows a distinctly different pattern (“X”), and that pattern “Z” (lanes 4 and 10) is a combination of both of the other patterns. The four different fragments (indicated by arrows) were cut out of the gel and the nucleotide sequence was determined (Fig 2B). The individual known to be HPA-1b/1b is homozygous for a “C” at position 12548 of the GPIIIa gene (lane 4) (SSCP pattern Y). The other two patterns from Fig 2A (Z and X, respectively), are heterozygous C/T (lane 8) or homozygous for “T” (lane 12) at position 12548. Note that although these are small numbers of samples, the frequency of the HPA-1 genotypes are consistent with those reported by immunophenotyping, and are tabulated in Fig 2A.

Different patterns among normal individuals were also observed by SSCP analysis of exons viii and ix (data not shown). As above, polymorphic bands were isolated and sequenced. We were able to detect the previously described C/A polymorphism at position 20508 of exon viii and the two polymorphisms in exon ix (data not shown).

We have previously described a kindred with GT in which the affected children had inherited an abnormal allele from the father that contained a complex rearrangement of the GPIIIa gene. The DNA from the mother (AH) was normal by Southern blot analysis using 13 different restriction enzymes (not shown). Using SSCP, we were able to identify an abnormally migrating fragment unique to the mother’s sample (Fig 3A). This fragment and another from a normal individual were isolated and the nucleotide sequence determined for the entire 240 bp. The only difference between the normal individual and the mother of the patient was a G → A substitution in the splice donor site of the exon iv-intron junction (Fig 3B). Because this mutation alters the splice donor site (ATGAT → ATGAT), it is predicted to alter mRNA splicing. PCR amplification of platelet cDNA from the affected child in this kindred (MH) showed two abnormal transcripts (Fig 4A, lane 3). The antisense primer used in this PCR reaction did not amplify the paternal allele because of a previously defined DNA inversion. One abnormal transcript was 163 bp smaller than the normal product, and the other was slightly larger than normal (lanes 3 and 4, Fig 4A). The normal and two mutant PCR products were isolated and sequenced (Fig 4B). The smaller product from the patient was due to the splicing out of exon iv, whereas the larger product contained an additional 27 bp between exons iv and v. The origin of these additional 27 bp appears straightforward: they are the intronic sequence immediately downstream of exon iv in the GPIIIa gene, and...
there is a cryptic splice donor sequence (aggt)\textsuperscript{16} 27 bp downstream of the point mutation. Figure 5 diagrams the presumed mechanism by which the two mutant mRNAs in this patient originated.

DISCUSSION

We have applied the SSCP technique to the characterization of molecular defects in GT. Including the abnormal allele described in this report, 11 of the 15 abnormal alleles described in patients with GT have been caused by point mutations.\textsuperscript{11-23} Thus, any approach to characterizing mutations in the genes for GPIIb and GPIIIa will require the ability to accurately identify single-base changes. Although we have only presented data regarding the analysis of the GPIIIa gene, we have successfully analyzed the GPIIb gene with an identical approach. Although there are other valid methodologies, we argue that SSCP offers at least five distinct advantages. First, there is a minimal amount of DNA sequencing required. The analyses of a given exon can be performed simultaneously on a large number of patient samples. Only when an abnormal fragment is identified is the nucleotide sequence determined. Because the fragments are generally less than 300 bp, all sequencing can be accomplished in a single reaction. Second, because the template for the PCR reaction is genomic DNA, the analyzed products contain sequences that participate in mRNA splicing. This advantage was shown in the analysis of the mutation in “AH,” in which we found a G → A substitution in the splice
Fig 3. SSCP identifies a mutant allele in the mother (AH) of the patient. (A) PCR-amplified genomic DNA from 13 different normal individuals (lanes 1 through 14) and individuals from 5 different kindreds with GT (lanes 15 through 22) using primers 3D.S and 3D.A that amplify exon iv. The "patient" lanes include both clinically affected individuals and other family members. Arrow indicates the abnormal fragment in AH (lane 17). (B) Nucleotide sequence of different SSCP fragments isolated from the gel in (A). The abnormal fragment from lane 17 and a normal fragment from lane 2 were isolated as described in the Materials and Methods and the nucleotide sequence determined. Lanes 1 through 4, fragment from the normal individual; lanes 5 through 8, fragment from AH. As indicated, sequence was performed in both sense and antisense orientations with primers 3D.S and 3D.A. Position of the nucleotide substitution is indicated by the arrowhead.

Donor site of the exon iv-intron junction. There are several examples of point mutations causing splicing abnormalities in GT.17,20 Therefore, analysis of the splice sites should be considered an important aspect of these sorts of studies. Because it is difficult to resolve data immediately downstream of the sequencing primer, oligonucleotides for PCR amplification and sequencing should be designed at least 15 bp from the splice junctions. Third, with SSCP there is immediate information to suggest whether or not the nucleotide change is a polymorphism or a mutation. Because numerous DNA samples are processed at a given time, only when a sample displays a completely unique SSCP pattern would one pursue this fragment as possibly containing a mutation. Fourth, SSCP allows the detection of mutant alleles associated with reduced transcript levels, such as those containing nonsense or frameshift mutations. Finally, patient DNA is generally easier to obtain, handle, and transport, and is much more resistant to degradation than platelet RNA.

We cannot make firm conclusions about the sensitivity of SSCP to identify all thrombasthenic mutations. We found one mutation in the five kindreds examined, and cannot rule out the possibility that some were missed. Of course, the GPIIb gene may have been affected in any or all of the other families. We did not make rigorous attempts to vary the SSCP conditions to maximize our chances of detecting all GPIIb abnormalities, although all known polymorphisms were identified using the conditions described. The earliest descriptions of the SSCP technique were able to identify all mutations in fragments up to 200 bp and 83% of mutations in fragments up to 400 bp.20 The accuracy of the technique was shown by its ability to detect 20 of 20 point mutations in PCR products sized 181 to 336 bp in the δ-aminotransferase gene.21 With respect to sensitivity, the upper size limit of the PCR product has not been determined. Nevertheless, we chose to amplify the smallest possible exon-containing genomic fragment. One must balance the need to identify 100% of all mutations with the number of PCR reactions (and hence the effort) one is willing to make. Some investigators are amplifying 400 to 500 bp and restriction enzyme digesting these into fragments of ~200 bp before analysis on an SSCP gel.23 We have attempted to keep the fragment length less than 300 bp, but the length of a given exon affects the design of the oligonucleotide. In cases in which two small exons are separated by a small intron, one set of primers can be used to amplify two exons at a

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time. Such a situation occurs at least four times in the GPIIb gene.

Denaturing gradient gel electrophoresis requires much longer oligonucleotides (due to the “GC clamp”), equipment that is not standard in most laboratories, and specific and complicated PCR and electrophoresis conditions for each primer pair. Allele-specific oligonucleotide hybridization is only useful for identifying known mutations. Chemical cleavage requires the use of some hazardous chemicals. Finally, sequencing patient platelet cDNA requires a great deal of unnecessary sequence determination and still may miss point mutations in intron splice sites. In addition, sequencing is not 100% sensitive because of stable secondary structures of template DNA, compression on the gel, etc. With SSCP, once a variation has been found in a given region, one can relentlessly pursue an accurate determination of the sequence knowing that a nucleotide change will be present. The major disadvantage to SSCP is the cost of synthesizing oligonucleotides and the use of radioactivity. However, most other approaches to these studies will require an equivalent number of oligonucleotides and frequently use radioisotopes such as $^{32}$P. Ainsworth et al. have
used silver stains of the SSCP gels to avoid the use of radioisotopes. A modification of the SSCP technique using biotinylated primers and restriction enzyme digests of PCR products before SSCP electrophoresis has been used to identify mutations in the GP IX gene.40 SSCP identified all known polymorphisms in the GPIIIa gene except for the HPA-4 (Pen) system in exon iii that we did not test. Because the HPA-4/4b phenotype is quite rare, we had no template DNA to analyze. The SSCP methodology could be applied to the genotyping of individuals in certain clinical situations (eg, neonatal alloimmune thrombocytopenia), because characteristic banding patterns are seen for both the heterozygous and homozygous states. Other techniques have been successfully used for determining platelet antigen genotypes,35,41 and we are not advocating SSCP as the technique of choice for this genotyping. However, SSCP does identify the known polymorphisms, will likely find others, and, for laboratories using SSCP, would be an effective approach to determining an individual's genotype.

One point mutation was identified in the 5 kindreds with GT we studied, and it has some interesting features. The G → A substitution at the splice donor site of the exon iv-intron junction resulted in the abnormal splicing of GPIIIa mRNA. Visual examination of the PCR-amplified platelet cDNA from the patient's maternal allele suggests that about half of the time the splicing machinery removes exon iv rather than using the cryptic site 27 bp downstream in the intron. The addition of 27 bp to the larger abnormal mRNA species does not create a frameshift and would potentially add 9 amino acids to the protein. There was no detectable protein on Western analysis of platelet proteins in the affected individuals in this kindred.11 A very long exposure of a Northern blot of patient platelet total RNA hybridized with a GPIIIa cDNA showed a faint signal at the expected position for GPIIIa mRNA (not shown). We postulate that a small amount of unstable protein is synthesized and subsequently degraded.

In summary, we have reported a new point mutation responsible for the GT phenotype that was easily and rapidly identified by the SSCP technique. A wide spectrum of mutational mechanisms cause GT. It is therefore important to characterize these defects with a technique that is likely to identify the most number of mutations. SSCP is very well suited for the identification of single-base changes, including splice site abnormalities, and, hence, is well suited for the characterization of defects causing the thrombasthenic phenotype. This technique meets several desirable criteria for a screening test: it is both simple and effective.

ACKNOWLEDGMENT

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