Glanzmann’s Thrombasthenia Associated With Deletion-Insertion and Alternative Splicing in the Glycoprotein IIb Gene

By Hava Peretz, Nurit Rosenberg, Sali Usher, Eran Graff, Peter J. Newman, Barry S. Coller and Uri Seligsohn

Glanzmann’s thrombasthenia is a bleeding disorder characterized by a decrease or absence of the functional platelet membrane glycoprotein (GP) complex, GPIIb/IIIa (α5β3). We describe a new deletion-insertion mutation in the GPIIb gene causing type I Glanzmann’s thrombasthenia in two siblings of a consanguineous Iranian-Jewish family. The proband’s platelets bound more antibodies against the vitronectin receptor-α5β3 than normal platelets, suggesting a normal GPIIIa (β3) gene and a defect in the GPIIb gene. Sequencing of amplified cDNA and genomic DNA fragments showed a 6-bp deletion and 31-bp insertion in exon 2 of the GPIIb gene. The predominant platelet GPIIb mRNA of the proband was a product of the splicing of exon 24 to a cryptic AG acceptor site in the insertion and encoded for deletion of amino acids Leu817-Asn826 and insertion of eight different amino acids. Cotransfection of COS-7 cells with expression vectors containing wild-type GPIIIa cDNA and the mutated GPIIb cDNA failed to produce detectable amounts of GPIIIa on the surface of the cells. Allele-specific restriction analysis of genomic DNA of family members showed homozygosity for the mutation in the affected siblings, heterozygosity in the parents, and homozygosity for the normal allele in an unaffected sibling. The observed mutation is in a region that is conserved from rodents to humans and has been suggested to be involved in the interaction between GPIIb and GPIIIa when these GPs are complexed in solution.

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The platelet membrane glycoprotein (GP) complex GPIIb/IIIa (α5β3) is a Ca2+-dependent heterodimer that can bind fibrinogen, fibronectin, von Willebrand factor, and vitronectin when platelets are activated. The GPIIb/IIIa complex plays a critical role in platelet aggregation and adhesion, as well as in the trafficking of fibrinogen into megakaryocytes and platelets. Glanzmann’s thrombasthenia stems from an inherited deficiency or dysfunction of the GPIIb/IIIa complex. The disease is broadly classifiable according to the amount of GPIIb/IIIa complex associated with platelets. Type I, 0% to 5% of normal; type II, 6% to 20% of normal; and variant disease, 50% to 100% of normal; type II, 6% to 20% of normal; and variant disease, 50% to 100% of normal. After the characterization of the genes coding for GPIIb and GPIIIa, mutations in either gene were identified in patients with Glanzmann’s thrombasthenia. Seven of the eight mutations that have so far been described in the GPIIb gene result in type I thrombasthenia. These mutations include one major and one minor deletion, one nonsense mutation, two splice site mutations, and two nonsense mutations. The eighth mutation is an nonsense mutation causing type II disease. Of 11 reported mutations in the GPIIIa gene, four nonsense mutations resulted in variant thrombasthenia. Two nonsense mutations caused type II thrombasthenia, and two major rearrangement, a small deletion, and two splice site mutations caused type I thrombasthenia.

We now report a new deletion/insertion mutation in the GPIIb gene associated with type I Glanzmann’s thrombasthenia in an Iranian-Jewish family.

MATERIALS AND METHODS

The proband was a 26-year-old woman of Iranian-Jewish origin who had a lifelong history of moderate mucocutaneous bleeding. Her parents were first cousins, and one of her two brothers was also affected by a bleeding disorder of similar severity. Both affected siblings had no platelet aggregation in response to adenosine diphosphate, collagen, or epinephrine and had a normal response to ristocetin. Their clot retraction was absent and the binding of monoclonal antibody (MoAb) 10E5, which recognizes the GPIIIa/IIIa complex, was minimal. Immunoblotting, conducted as previously described, showed small amounts of GPIIIa and minute amounts of GPIIb. These findings established the diagnosis of type I Glanzmann’s thrombasthenia.

Platelet vitronectin receptor (VnR) quantitation. The surface expression of α5β3 VnR on platelets from both patients was estimated by measuring the binding of a 125I-labeled MoAb specific for α5 (LM142) at a concentration of 58 ng/mL and of a 125I-labeled MoAb specific for α5β3 (LM609) at concentrations of 36 ng/mL and 140 ng/mL, as previously described.

Southern blot analysis. Genomic DNA was prepared from peripheral blood white blood cells by the protein desalting method. The DNA was digested with BglII, BamHI, PstI, and TagI restriction enzymes, blotted onto Hybond N+ membranes (Amersham International, Buckinghamshire, UK) and hybridized sequentially with a full-length GPIIb cDNA probe (3.3-kb EcoRI insert in PMT2), and with six GPIIIa genomic probes obtained by grouped exon amplification (see below). Probes were labeled in the presence of α32P-deoxyctydine triphosphate with a random priming kit (Amersham) according to the manufacturer’s instructions.

Platelet RNA and cDNA preparation. Platelet RNA was prepared from platelet-rich plasma as described except for omission of the final phenol-chloroform extraction. First-strand cDNA was synthesized by using random hexamers or oligo-dT primers (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (RT; Gibco-BRL, Gaithersburg, MD).

Amplification of groups of exons of the GPIIb gene. Amplification of six DNA fragments encompassing exons 2 to 29 of the GPIIb gene was performed with specifically designed primers (Table 1). The primers were designed to permit amplification of cDNA from platelet-derived mRNA as well as genomic DNA, and their length was suitable for screening of point mutations by single-strand con-
formation polymorphism, either directly or after cleavage with restriction enzymes. The primers were synthesized by phosphoramidite and were used unpurified. The amplification reaction was performed according to the instructions of the Taq polymerase manufacturer (Promega). Initial denaturation at 94°C for 5 minutes was performed after the 30 cycles. The amplified products were checked by restriction analysis and 3 minutes at 72°C; a final elongation step at 72°C for 1.5 minutes at 55°C.

The restriction enzymes were purchased from Fermentas (Vilnius, Lithuania) and New England Biolabs (Beverly, MA).

**Nucleotide sequence analysis.** Amplified DNA was purified and recovered from preparative agarose gels by the glass-milk procedure (Bio 101, La Jolla, CA). Direct sequencing was performed with T7 polymerase, with the sequenase version 2.0 kit (US Biochemistry, Cleveland, OH) according to instructions. The same primers used for amplification were used for sequencing. An additional primer (5'-AGGTGAGAGGGAGCAGAACA-3') was used for sequencing of the cDNA fragment encompassing exons 22-26.

**Construction of expression vectors for GPIIb and GPIIIa.** Full-length cDNAs of GPIIb and GPIIIa were inserted into the unique EcoRI site of pGEM-7 (Promega). For cloning of the deletion/insertion mutation (see below), exons 22-26 of the patient's cDNA were amplified. The resultant 520-bp DNA fragment was cleaved with Rsr II and Bgl II restriction enzymes, yielding a 356-bp fragment that contained the deletion/insertion mutation. GPIIb in pGEM-7 was digested with the same enzymes, and the resulting 6-kb fragment was then ligated with the 356-bp Rsr II-Bgl II fragment. The ligated product was transfected into DH5α bacteria, and colonies containing the plasmid were selected on Luria-Bertani (LB)-ampicillin medium. Plasmid DNA was recovered using the minialkaline lysis technique and examined for the presence of the mutation by digestion with *Sac* I, because its cleavage site is abolished by the mutation. The sequence of the ligated polymerase chain reaction (PCR) product was confirmed by direct sequencing. Full-length normal and mutated GPIIb cDNAs were excised from the plasmid pGEM-7 with the restriction enzymes Xho I and *Sma* I and were ligated into the expression vector pSVL (Pharmacia, Uppsala, Sweden) that had been digested with the same enzymes.

Full-length GPIIIa cDNA was excised from the plasmid pGEM-7 with the restriction enzyme *Cla* I (the cohesive ends were filled in with Klenow fragment of *Escherichia coli* DNA polymerase I to create blunt ends) and with the second restriction enzyme *Xba* I. This fragment was ligated with the expression vector pSVL, which had been digested with *Sma* I and *Xba* I.

The resultant plasmids pSVL-GPIIIa, pSVL-GPIIbG2, and pSVL-mutated GPIIb (pSVL-GPIIbmut) were purified using a maxiprep kit (QIAGEN Inc, Chatsworth, CA).

**Transfection of COS cells with plasmids containing GPIIb and GPIIIa cDNAs.** COS-7 cells were grown in media consisting of Dulbecco’s modified Eagle’s medium (high glucose; Biological Industries, Beit-Haemek, Israel) supplemented with 10% heat-inactivated fetal bovine serum, 0.3 mg/mL L-glutamine, and 100 μg/mL streptomycin. Transfection of DNA (3 to 4 μg) into 1 x 10⁶ cells plated in 90-mm petri dishes (Corning, NY) was performed by incubation with diethyl aminoethyl-dextran 400 μg/mL, followed by 100 μmol/L chloroquine diphosphate and 10% dimethyl sulfoxide as described elsewhere. After the transfection, cells were rinsed 3 times with Dulbecco’s phosphate-buffered saline, complete medium

**Table 1. Amplified Exons, Primer Sequences, Length of cDNA and Genomic DNA, and Enzymes Used for Restriction Analysis of the GPIIb Gene**

<table>
<thead>
<tr>
<th>Exons</th>
<th>Primer Sequences</th>
<th>cDNA (bp)</th>
<th>Genomic DNA (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-7</td>
<td>Sense: 5'-GTGGTTCCGTGCCCCCTGG-3'</td>
<td>550</td>
<td>1122</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CAGTCACTGGAAGATC-3'</td>
<td>386</td>
<td>1040</td>
</tr>
<tr>
<td>8-12</td>
<td>Sense: 5'-GGGCTGTCCTGCAGC-3'</td>
<td>169</td>
<td>370</td>
</tr>
<tr>
<td>13-18</td>
<td>Sense: 5'-GGGCTGTCCTGCAGC-3'</td>
<td>169</td>
<td>370</td>
</tr>
<tr>
<td>9-21</td>
<td>Sense: 5'-AGGTGAGAGGGAGCAGAACA-3'</td>
<td>250</td>
<td>1474</td>
</tr>
<tr>
<td>22-26</td>
<td>Sense: 5'-GACGGGGATCTCAACACTACAG-3'</td>
<td>318</td>
<td>741</td>
</tr>
<tr>
<td>27-29</td>
<td>Sense: 5'-CTTCAACATGGCCAGCAG-3'</td>
<td>318</td>
<td>741</td>
</tr>
<tr>
<td>24-25</td>
<td>Sense: 5'-AGGTGAGAGGGAGCAGAACA-3'</td>
<td>169</td>
<td>270</td>
</tr>
</tbody>
</table>

**Fig 1. A missing Ban II site in the GPIIb cDNA of the proband.** (Top) Ban II map of an amplified cDNA fragment encompassing exons 22-26 of a normal subject and the proband. (Bottom) An agarose gel electrophoresis showing the amplified cDNA fragment before (−) and after (+) Ban II digestion in a normal subject (N) and proband (P). In the proband, the Ban II digestion yielded a 479-bp fragment instead of the normal fragments of 240 bp and 245 bp. A constant 35-bp Ban II fragment is not shown.

<table>
<thead>
<tr>
<th>Exon 24</th>
<th>Exon 25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>245</td>
</tr>
<tr>
<td>Patient</td>
<td>479</td>
</tr>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>520 bp</td>
<td></td>
</tr>
<tr>
<td>240 + 245</td>
<td>479 bp</td>
</tr>
</tbody>
</table>
was added, and incubation was continued for 96 hours at 37°C in a 5% carbon dioxide environment.

RT-PCR of mRNA prepared from transfected COS cells. Forty-eight hours after transfection, mRNA was prepared from 10⁶ COS cells by affinity chromatography on oligo dT-cellulose (QuickPrep Micro; Pharmacia Biotech, Piscataway, NJ). First-strand cDNA was synthesized by using oligo dT primer and avian myeloblastosis virus RT (Promega). Amplification of exons 22-26 of the GPlIb cDNA was performed as previously described, and the amplified products were digested with the restriction enzyme BstXI (New England Biolabs).

Identification of GPlIb/IIIa complex on the surface of transfected COS cells. Ninety-six hours after transfection, cells were washed 3 times with Tris-buffered saline (TBS; 20 mmol/L Tris-HCl, pH 7.5, and 150 mmol/L NaCl) and incubated for 30 minutes in TBS containing 0.25% bovine serum albumin (BSA); the cells were then incubated for 60 minutes in 2 mL of TBS containing 0.01% BSA and 10 μg/mL biotinylated MoAb 10E5, which recognizes only the GPlIb/IIIa heterodimer. The MoAb 10E5 was biotinylated using biotin-N-hydroxysuccinimide ester (Sigma Chemical Co, St Louis, MO). After 3 washes with TBS, the cells were incubated with avidin-peroxidase (Vector, Burlingame, CA) for 1 hour, washed 5 times with TBS, and assayed for the presence of the MoAb 10E5 on the surface of the cells with a peroxidase substrate kit containing 2,2-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] and hydrogen peroxide (Bio-Rad, Hercules, CA). At 1-minute intervals, 100 μL of the reaction mixture was transferred to enzyme-linked immunosorbent assay plate wells containing 100 μL 2% oxalic acid to stop the reaction, and the optical density of the wells were then read at 405 nm. The cells were counted, and the optical density per 10⁶ cells was calculated.
RESULTS

Surface expression of \( \alpha_\beta \) VnR in patients’ platelets. The proband’s platelets bound 24 molecules per platelet of the anti-\( \alpha_\beta \) antibody, whereas control platelets tested at the same time bound 15 molecules per platelet. This 60% increase in binding of the antibody to the patient’s platelets is similar to that observed with platelets from Arab patients with Glanzmann’s thrombasthenia who have a defect in GPIIb, but is dramatically different from the virtual absence of binding of the same antibody to platelets of Iraqi-Jewish patients with Glanzmann’s thrombasthenia who have a defect in GPIIIa. Platelets from the proband’s brother bound with a higher concentration of 149 ng/mL and 3 molecules per platelet at a concentration of 36 ng/mL. The mean increase in antibody binding of 113% is also similar to that found with Arab patient’s platelets. These data suggested that the mutation responsible for the disease in this family resides in the GPIIb gene.

Identification of an aberrant Ban II fragment in the GPIIb cDNA. Southern blot analysis with restriction enzymes for which maps were available failed to detect major changes in fragment lengths in the genomic DNA of the proband. Therefore, a more refined restriction analysis was performed on the six amplified cDNA segments and the six amplified genomic DNA segments encompassing exons 2 to 29 (Table 1). The lengths and restriction patterns of all amplified segments appeared identical in control and patient samples (data not shown), except for the cDNA fragment of exons 22-26 in which a Ban II site at the junction of exons 24 and 25 was missing in the proband. Therefore, the proband’s cDNA yielded a 479-bp fragment instead of the 240- and 245-bp products obtained in the control (Fig 1).

Nucleotide sequence of the mutated genomic DNA and cDNA. Sequence analysis of an amplified fragment from the patient’s genomic DNA containing part of exon 24, the whole intron 24, and part of exon 25 showed a deletion of 6 bases and an insertion of 31 bases in the patient (Fig 2). Sequence analysis of amplified cDNA encompassing exons 24 and 25 showed that the first 30 bases of exon 25 were replaced by an altered sequence abolishing the Ban II site at the junction of exons 24 and 25 (Fig 1). This change, which was confirmed by sequencing the antisense strand, indicated that the mutated cDNA sequence stems from alternative splicing at an AG acceptor in the insertion (Fig 2). Nucleotide sequence analysis of the rest of the amplified GPIIb-cDNA segments disclosed no additional abnormalities.

Detection of small amounts of mutant cDNA spliced at the normal AG acceptor site. The predominance of the aberrantly spliced cDNA in the proband was surprising in view of the preservation of the normal sequences of intron 24 and the 5’ end of exon 25. To further explore this finding, we took advantage of two restriction sites, Ban II and BstXI, that are present in the normally spliced cDNA but absent in the cryptically spliced cDNA (see bottom of Fig 2). The amplified control cDNA cleaved by BstXI yielded two fragments of 71 bp and 98 bp (Fig 3, lane 2), whereas cleavage with Ban II yielded 62-bp and 107-bp fragments (Fig 3, lane 3). In the proband, most of the cDNA remained uncleaved (Fig 3, lane 4), consistent with loss of BstXI and Ban II sites. The small amounts of BstXI and Ban II cleavage products (lanes 5 and 6, Fig 3) suggested that only a minute amount of the normally spliced mRNA was present in the proband’s platelets.

Genotype analysis in family members. The inheritance of the mutation in members of the affected family was studied by amplification of exons 24-25 of the genomic DNA and BstXI restriction analysis. The normal allele was characterized by a 100-bp fragment, and the defective allele by a 125-bp fragment. The affected siblings were homozygous for the mutant allele (125 bp), the parents were heterozygous carriers (100 bp + 125 bp), and the unaffected brother was homozygous for the normal allele (100 bp) (Fig 4).

Surface expression of the GPIIb/IIIa heterodimer in transfected COS-7 cells. The major mutant mRNA encodes...
Fig 4. Genotype analysis in family members. (A) BstXI cleavage sites of normal and mutated genomic DNA fragments encompassing exons 24-25. (B) Agarose gel electrophoresis of amplified genomic DNA after BstXI digestion. The affected homozygous siblings (closed symbols) show a 125-bp fragment in contrast to a 100-bp normal fragment in a healthy sibling. The heterozygous parents have both 100- and 125-bp segments, representing the normal and mutant alleles, respectively. All individuals have a constant 170-bp fragment.

DISCUSSION

The platelets of the proband and her sibling affected with Glanzmann’s thrombasthenia contained small amounts of GPIIIa(β3) and proGPIIb and normal to increased amounts of α1β3, VnR, suggesting that the genetic defect in this family resides in the GPIIb gene. Indeed, a homozygous 6-bp deletion and 31-bp insertion in exon 25 of the GPIIb gene was found in both patients but not in a normal sibling.

Notwithstanding the preservation of the sequence of intron 24 and the 5’ end of exon 25, the predominant mRNA found in the platelets of the patients resulted from alternative splicing at an AG site in the inserted sequence (Fig 2), with the correctly spliced mutant mRNA found only in very small amounts (Fig 3). Figure 6 is a schematic representation of the platelet GPIIb/IIIa complex and the altered mRNA splice pattern described here.
the mutant and normal genes, the normal mRNA, and the major mRNA stemming from alternative splicing. Several explanations may account for this finding. Enhanced splicing at the cryptic AG acceptor site may have occurred because of a higher pyrimidine content of its polypyrrimidine tract (77% for the 20-bp polypyrrimidine tract upstream to the cryptic AG site and 55% for the 22-bp polypyrrimidine tract upstream to the normal AG site). Alternatively, because correct splicing of the mutant gene creates a premature stop codon (see below), and because premature termination of translation may result in mRNA instability, the relative increase in the mRNA using the cryptic site may be caused by excessive destruction of the correctly spliced mRNA. Other examples of nonsense mutations being associated with reduced relative levels of mRNA have been reported.7,8,11,16,38

Finally, it has been suggested that nonsense mutations may affect splice site selection,36,38 but the mechanism by which this might occur has not been defined

Both the scarce and abundant mRNA species found in the patient’s platelets encode for defective proteins. The scarce but correctly spliced mRNA would cause premature termination with an altered C-terminal sequence of 105 amino acids starting at Val-825. In this truncated protein, the site of cleavage of proGPIIb that yields α and β chains of GPIIb would be altered, and the transmembrane and the cytoplasmatic domains would be missing. The abundant, alternatively spliced mRNA encodes for a substitution of 10 amino acids Leu817–Asn826 by an altered sequence of 8 amino acids, followed by normal translation of the rest of the protein (Fig 2). This substitution is not within a known functional domain of the GPIIb subunit and does not affect cysteine residues or known sites of glycosylation,39 but it has been suggested to be involved in the heterodimer interaction between GPIIb and GPIIIa.40 In support of the importance of this region is the observation that it is fully conserved from rodent to human.41

To confirm the importance of the observed abnormality and to define better the biosynthetic abnormality, we have coexpressed the mutated GPIIb cDNA and the normal GPIIIa cDNA in COS-7 cells. Although transcription of both normal and mutated mRNAs was shown in the cells by RT-PCR experiments, only the normal heterodimer complex was detectable on the surface of the cells (Fig 5). These observations suggest that the substituted amino acids are essential for surface expression of the GPIIb/IIIa complex. Further experiments will be necessary to elucidate the intracellular fate of GPIIbαε.

Specific DNA sequences such as direct repeats, inverted repeats, and symmetric elements predispose to small deletions and insertions.42,43 In the deletion/insertion mutation described here, the breakpoints were flanked by the symmetric elements CTGG/GGTC, TCAG/GACT and the direct repeats AATGG. Apparently, the inserted DNA fragment (see Fig 2) was derived from sequences flanking the break points of the deletion. Thus, the sequence CCAGGGCCCTT of the insertion is complementary to AATGGCCCTG located upstream to the deletion, and the sequence CACCT in the insertion is symmetric to TCCAC present both at the 5′ end of exon 25 and at the 3′ end of the insertion.

The described patients belong to a family of Iranian-Jewish origin. The Iranian Jews lived in isolation for about 2.5 millennia, a length of time similar to that of the Iraqi Jews among whom Glanzmann’s thrombasthenia caused by a mutation in GPIIb is observed quite frequently.8,44 The availability of an assay to detect the carriers of the newly described mutation (Fig 4) will enable the screening of the Iranian-Jewish population to discern whether the mutation is frequent or limited to the described family.

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