Molecular Basis for Glanzmann’s Thrombasthenia (GT) in a Compound Heterozygote With Glycoprotein IIb Gene: A Proposal for the Classification of GT Based on the Biosynthetic Pathway of Glycoprotein IIb-IIIa Complex

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The genetic basis for Glanzmann’s thrombasthenia (GT) was elucidated on a compound heterozygote with glycoprotein (GP)IIb gene: an opal mutation at the end of exon 17 (CGA → TGA) results in only a trace amount of GPIIb mRNA, and a splicing mutation at the acceptor site of exon 28 (CAG → GAG) causes an in-frame, exon skipping process from exon 25 to 27. This aberrant transcript encodes a single-chain polypeptide characterized by a 42-amino acid deletion, which includes the proteolytic cleavage site(s) and a unique, proline-rich region at the location corresponding to the carboxyl-terminal of the normal GPIIb α-chain. These characteristics are shared by a previously reported defective GPIIIa molecule, which is neither assembled with GPIIIa nor transported to the cellular surface. Despite its normal transcription level, expression of the present defective GPIIIa molecule was significantly decreased (~6% of the control level). Because the precursor GPIIIa molecule is assembled with GPIIIa in the endoplasmic reticulum (ER) and its processing, as well as stability, is dependent on the GPIIIa subunit, the defective GPIIIa molecule may be rapidly degraded by the intrinsic quality control system of the ER due to its inability to form a stable heterodimer complex as a consequence of its misfolded structure. Although we did not confirm that the GPIIIa genes of this individual were normal, GPIIIa may be secondarily decreased (~11% of control), because a large part of it could not be complexed, making it vulnerable to proteolysis.

To elucidate the molecular basis for GT, we propose here a classification of GT based on the biosynthetic pathway of the GPIIb-IIIa complex.

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MATERIALS AND METHODS

Southern blot analysis. DNA used for the following experiments was prepared from the Epstein-Barr virus (EBV)-transformed B cells of the family members. The DNA (~3 μg) was digested with 20 to 30 U of each restriction endonuclease under conditions recommended by the suppliers (TaKaRa Shuzo, Osaka, Japan, and Tokyo and Toyobo, Osaka, Japan). Each sample was electrophoresed on a 0.8% agarose gel and then transferred to a Hybond-N membrane (Amersham International, Buckinghamshire, UK). The 2,553-bp fragment of GPIIb cDNA (bases 418 to 2970), which was generously provided by Dr J.C. Loftus (Scripps Institute, La Jolla, CA), and the 2,376-bp fragment of GPIIIa cDNA (bases 51 to 2426) were labeled with [α-32P]dCTP using random hexanucleotide primers and Klenow fragment. Hybridization and washing were performed according to the standard conditions suggested by the manufacturer.

Construction and screening of the patient's genomic library. The patient's DNA was partially digested with Sau3AI and 15- to 25-kb fragments were collected by sucrose gradient fractionation, ligated to EMBL 3A arms, and packaged using the Gigapack Gold Cloning System (Stratagene, La Jolla, CA). Screening of the library was
performed using the 32P-labeled GPIIb cDNA probe described above.

Amplification of genomic DNA fragments by polymerase chain reaction. Genomic DNA fragments containing exon(s) of the GPIIb gene were amplified by polymerase chain reaction (PCR),23,25 with approximately 1 μg of DNA using each pair of sense (S) and antisense (AS) oligonucleotide primers synthesized by a DNA synthesizer, model 381A (Applied Biosystems, Foster City, CA) (Fig 1); EX1-5'P1S: nucleotides (nt) (-)713-( -)794; EX1-5'P2S: 37-56; EX1-5'P3S: (-)143-( -)159; EX1-5'P4S: (-)318-( -)329; EX1-5'P5S: (-)493-( -)474; EX1-3'P1AS: 280-261; EX1-3'P2AS: 94-75; EX1-3'P3AS: (-)80-( -)99; EX1-3'P4AS: (-)249-( -)268; EX1-3'P5AS: (-)804-( -)813; EX2-5'P1S: 3609-3628; EX2-3'P2AS: 3833-3841; EX3-5'P1S: 3805-3824; EX3-3'P1AS: 4019-4000; EX4-5'P1S: 4026-4045; EX4-3'P1AS: 4270-4251; EX5-5'P1S: 4379-4398; EX5-3'P1AS: 4544-4525; EX6-5'P1S: 4470-4489; EX6-3'P1AS: 4626-4607; EX7-5'P1S: 4547-4566; EX7-3'P1AS: 4759-4740; EX8-5'P1S: 5045-5064; EX8-3'P1AS: 5194-5175; EX9-5'P1S: 5228-5247; EX9-3'P1AS: 5375-5356; EX10-5'P1S: 5440-5459; EX10-3'P1AS: 5612-5631; EX11-5'P1S: 5636-5655; EX11-3'P1AS: 5789-5770; EX12-5'P1S: 5900-5919; EX12-3'P1AS: 6180-6161; EX13-5'P1S: 8541-8560; EX13-3'P1AS: 8795-8776; EX14-5'P1S: 8935-8977; EX14-3'P1AS: 9095-9076; EX15-5'P1S: 9123-9142; EX15-3'P1AS: 9305-9286; EX16-5'P1S: 9287-9306; EX16-3'P1AS: 9442-9423; EX17-5'P1S: 9459-9478; EX17-3'P1S: 9398-9417; EX17-3'P1AS: 9702-9683; EX17-3'P2AS: 9742-9723; EX18-5'P1S: 9799-9818; EX18-3'P1AS: 10012-9973; EX19-5'P1S: 10896-10915; EX19-3'P1AS: 11054-11035; EX20-5'P1S: 11098-11117; EX20-3'P1AS: 11316-11297; EX21-5'P1S: 11834-11853; EX21-3'P1AS: 11998-11979; EX22-5'P1S: 12536-12555; EX22-3'P1AS: 12688-12669; EX23-5'P1S: 13224-13253; EX23-3'P1AS: 13383-13364; EX24-5'P1S: 13438-13457; EX24-3'P1AS: 13623-13604; EX25-5'P1S: 13636-13656; EX25-3'P1AS: 13750-13769; EX25-3'P2AS: 13877-13806; EX25-3'P3AS: 13858-13839; EX26-5'P1S: 13805-13924; EX26-3'P1AS: 13870-13889; EX26-3'P2AS: 14125-14106; EX26-3'P3AS: 14007-13988; EX27-5'P1S: 14486-14450; EX27-3'P2AS: 14581-14562; EX27-3'P1AS: 14594-14575; EX27-3'P3AS: 14715-14696; EX28-5'P1S: 14863-14882; EX28-3'P1AS: 15034-15015; EX29-5'P1AS: 15140-15159; EX29-3'P1AS: 15359-15340; EX30-5'P1S: 16915-16934; EX30-3'P1AS: 17282-17263.

PCR amplification was performed in an automatic thermocycler (Zymoreactor; Taiyo, Tokyo, Japan) for 35 cycles at 94°C (1 minute), 50 to 60°C (1 minute), and 74°C (1.5 minutes), followed by a 5-minute extension at the end. One tenth of the PCR products were analyzed by electrophoresis of the patient's GPllb gene. Solid boxes represent exons.10 Broken lines (below) indicate the nucleotide regions amplified by PCR and sequenced. The positions of two point mutations (open and closed circles) in the patient's GPIIb gene are indicated.

![Fig 1. Sequence analysis of the PCR-amplified genomic DNA fragments of the patient's GPIIb gene. Solid boxes represent exons.10 Broken lines (below) indicate the nucleotide regions amplified by PCR and sequenced. The positions of two point mutations (open and closed circles) in the patient's GPIIb gene are indicated.](http://www.bloodjournal.org)
cDNA was synthesized on the total RNA extracted from the platelets of the patient and his mother. Then, two consecutive PCRs were performed using nested primers encompassing exon 25 through exon 27. The results indicated the presence of mRNA of two different sizes in their platelets: one is the normally spliced, 202-nt mRNA (exon 25-26-27), and the other is a shorter mRNA of 76-nt (Fig 4).

It was conceivable from its size and the restriction enzyme analysis (data not shown) that the latter aberrant mRNA was generated through exon skipping; the donor site of exon 25 was joined to the acceptor site of exon 27. To confirm this putative, exon skipping process, nucleotides of this aberrant cDNA were sequenced. The results proved that the whole nucleotide sequence of exon 26 was deleted and indicated that the 3' terminal base of exon 25 was directly joined to the 5' terminal base of exon 27 (Fig 5).

RNase protection assay. To estimate the amount of mRNA transcribed from these two mutant alleles, an

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**Fig 2.** Direct sequencing of the nucleotides encompassing exons 17 and 26. Exons 17 and 26 with flanking regions were amplified by PCR with approximately 1 μg of the patient’s DNA and oligonucleotide primer pairs of either EX17-5’S and EX17-3’ P1AS, or EX26-5’S and EX26-3’P1AS, respectively. Nucleotides were sequenced directly as described in the Materials and Methods. The base changed by each mutation is indicated by a letter in a box (right side): (A) CGA → TGA at the end of exon 17, and (B) CAG → GAG at the splice acceptor site (−3) of exon 26. Presence of both the normal and mutated bases was confirmed by sequencing nucleotides of the subcloned fragments of PCR-amplified regions and of five positive clones from the patient’s genomic library.

**Fig 3.** Agarose gel electrophoresis of PCR products of exons 17 and 26, followed by digestion with Taq I (exon 17) and Mva I (exon 26), respectively. PCR was performed using the same primer sets described in Fig 2. (A) Taq I digestion of exon 17 with flanking regions yields 185-bp and 59-bp fragments in the normal allele, but only a 244-bp fragment is observed in the mutant allele. (B) Mva I digestion of exon 26 with flanking regions yields 187-bp and 27-bp fragments in the normal allele, but only a 214-bp fragment is observed in the mutant allele. C, P, F, and M denote DNA from the control, the patient, the patient’s father, and the patient’s mother, respectively. The 59-bp and 27-bp fragments are not shown. Size markers are depicted on the right.
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Fig 4. cDNA-PCR from platelet RNA. After cDNA synthesis, half of the reaction mixture was subjected to PCR using nested primers as described in the Materials and Methods. One tenth of the final products was electrophoresed on a 2% agarose gel. C, P, and M denote platelet RNA of the control, the patient, and the patient’s mother, respectively. The 202-bp fragment represents the cDNA of normally spliced, exon 25-26-27 mRNA, and the 76-bp fragment (arrow) observed in lanes P and M is derived from the mutant mRNA, where exon 26 is skipped. The relative amounts of these two cDNA fragments were not constant and either one of them was amplified in the other tubes under the same conditions (data not shown). Size markers are on the right.

RNase protection assay was performed on the platelet RNA (Fig 6). After RNase digestion, the normally spliced, 394-nt fragment, as well as 151- and 117-nt fragments derived from the aberrant mRNA, in which exon 26 was skipped, were protected in the patient’s mother’s platelets (Fig 6, lane 4). Densitometric analysis of these fragments indicated that the overall amount of the aberrant mRNA was comparable to that of its normal counterpart, after correction for the difference in length (data not shown). Although both the abnormal 151- and 117-nt fragments in the patient’s platelets exhibited even higher density than those of the mother’s, 394-nt fragment could not be observed (Fig 6, lane 3). These results indicate that in the patient’s platelets, mRNA transcribed from the allele with an opal mutation was significantly decreased and at most below the level detectable by the RNase protection assay.

Fig 5. Nucleotide sequence of the aberrant cDNA. The 76-bp cDNA fragment in lane P of Fig 4 was subcloned into pUC 19, and then the nucleotides were sequenced. The 3' end of exon 25 is directly joined to the 5' end of exon 27.

DISCUSSION

We analyzed the genetic basis for GT in a family and found two independent mutations in the GPIIb gene. An opal mutation results in the significant decrease in mRNA. A splicing mutation causing an in-frame exon skip results in a normal amount of the aberrant mRNA encoding a truncated GPIIb molecule with a 42-amino acid deletion.

Nonsense mutations have been reported to decrease mRNA levels of various mammalian genes. However, it is controversial whether the primary causative defect resides in the intranuclear metabolism, nuclear-cytoplasmic transport, or the cytoplasmic instability of mRNA.

The deleted 42-amino acid region contains the proteolytic cleavage site(s) required for conversion of the GPIIb precursor to the mature form. It is part of the unique carboxyl-terminal region of the a-chain of GPIIb and is abundant in proline residues that prevent a-helix formation. Therefore, the exon skipping process from exon 25 to
inability to form a stable heterodimer complex as a consequence of its misfolded structure.

On the other hand, the amount of GPIIIa, which is indistinguishable from the control GPIIIa by Western blot analysis, is also significantly decreased (~11% of control).17 Although it has not been confirmed whether the GPIIIa genes of the patient are normal, most of GPIIIa may be secondarily decreased without forming the heterodimer complex, because only a defective GPIIb molecule is present.

Before discovery of the GPIIb-IIIa abnormality in GT, Caen et al proposed the classification based on the platelet fibrinogen content and clot retraction.25,26 Recently, George et al classified GT based on the platelet GPIIb-IIIa content: type I (<5% of normal), type II (10% to 20% of normal), and variants (half-normal to normal amount of functionally defective molecules).9 In addition, a classification into + and 0 variants according to the presence or absence of GPIIb-IIIa complex on the platelet surface has been proposed.40 Although classification of GT based on the amount of platelet GPIIb-IIIa is convenient, it does not accurately reflect the functional abnormality on the molecular level.

At present, only seven different types of mutations in the GPIIb or IIIa gene, including two types of the present case, have been characterized in GT patients.12,13,16-33 However, for a concise correlation between mutations in these genes and molecular defects leading to the thrombasthenic phenotype, another classification of GT may be useful. In other words, GT can be classified in terms of the biosynthetic stages of the GPIIb-IIIa complex predominantly impaired by mutation(s) in the GPIIb gene or GPIIIa gene. In essence, the classification is based on whether these two molecules have been assembled to form a stable heterodimer complex (postassembly stage) or not (preassembly stage).

The rationale for this classification is that the bleeding tendency in GT seems to be largely dependent on the amount of the "functional" GPIIb-IIIa complex on the platelet surface, and for expression of the complex, assembly between pro-GPIIb and IIIa in the ER is prerequisite.13-16 If a mutation in GPIIb or IIIa gene does not prohibit heterodimer formation, a large portion of even an aberrant complex would be expressed on the platelet surface; such a defect can be classified as a mutation impairing predominantly the postassembly stage.

In contrast, any mutation that causes misfolding of the molecule itself or prevents assembly would significantly decrease the amount of both molecules; such a defect can be classified as a mutation impairing predominantly the preassembly stage. Misfolded molecule may be sorted to the degradation pathway either after binding to such luminal ER proteins as Bip (binding protein) or by forming insoluble aggregates.42-47 A GPIIb gene deletion (~4.5 kb) reported by Burk et al,12 as well as two types of deletion in the GPIIb or IIIa gene reported by Newman et al,22 may belong to this
category. In general, most of the so-called type I and type II GTs seem to belong to this category.

These preassembly stage defects may be subdivided into two categories on the mRNA level, pretranslation and posttranslation defects, where the decrease of each subunit is attributed to either a decreased level of mRNA or an increased degradation of the unstable molecule despite the presence of a normal amount of mRNA, respectively. From this point of view, the opal mutation in exon 17 of the present case belongs to the pretranslation defect, and the splicing mutation of exon 26, to the posttranslation defect. A large insertion (~7 kb) in the GPIIia gene reported by Bray and Shuman may also belong to the pretranslational defect.

In the postassembly category of mutations, defects in the receptor function of the complex, rather than its quantity, are the major determinant of the severity of the bleeding tendency. Most of the “variant” types, including the point mutation in the GPIIia gene reported by Loftus et al. and some of the “type II” GTs, may belong to this postassembly stage.

Thus the classification proposed should more concisely describe the molecular basis for each case of GT. To begin to categorize the thrombasthenic phenotype for further molecular studies, the platelet flow cytometry using monoclonal antibodies for GPIIb-IIIa may be useful. Precise analyses of molecular defects caused by various mutations will elucidate the biological importance of each structural domain of GPIIb-IIIa in maturation, the intracellular sorting process, and ligand-binding activity.

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