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

By Atushi Kato, Koh Yamamoto, Sumio Miyazaki, Stephanie M. Jung, Masaaki Moroi, and Nobuo Aoki

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 GPllb 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 carboxy-terminal of the normal GPllb α-chain. These characteristics are shared by a previously reported defective GPllb molecule, which is neither assembled with GPIIla nor transported to the cellular surface. Despite its normal transcription level, expression of the present defective GPllb molecule was significantly decreased (~8% of the control level). Because the precursor GPIIb molecule is assembled with GPIIla in the endoplasmic reticulum (ER) and its processing, as well as stability, is dependent on the GPIIla subunit, the defective GPllb 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 GPIIla genes of this individual were normal, GPIIla 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 GPllb-IIIa complex.

© 1992 by The American Society of Hematology.

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 Kyoto 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 GPllb 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 GPIIla 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 manufacturers.

Construction and screening of the patient’s genomic library. The patient’s DNA was partially digested by 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 $^{32}$P-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) with 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'PlS: nucleotides (nt) (-)713 to (-)694; EX1-5'P2S: 37 to 56; EX1-5'P3S: (-)143 to (-)134; EX2-3'PlAS: (-)94 to (-)51; EX3-3'P4AS: (-)3824 to (-)3823; EX3-3'P1AS: 4019 to 4000; EX4-3'P5AS: 544 to 535; EX5-3'P6AS: 454 to 453; EX6-5'P7AS: 11035 to 11054; EX7-3'P8AS: 14863 to 14882; EX8-5'P9AS: 5045 to 5046; EX9-3'P10AS: 9095 to 9106; EX10-3'P11AS: 9205 to 9206; EX11-3'P12AS: 13623 to 13624; EX12-3'P13AS: 13841 to 13852; EX13-5'P14AS: 2809 to 2810; EX14-5'P15AS: 5375 to 5376; EX15-5'P16AS: 9702 to 9703; EX16-5'P17AS: 9459 to 9460; EX17-5'P18AS: 16915 to 16934; EX18-5'P19AS: 9799 to 9800; EX19-5'P20AS: 10896 to 10897; EX20-5'P21AS: 11316 to 11317; EX21-5'P22AS: 15015 to 15016; EX22-5'P23AS: 13870 to 13871; EX23-5'P24AS: 14586 to 14587; EX24-5'P25AS: 17262 to 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 electrophoresed on a 5% polyacrylamide gel containing urea. For the sequencing reaction, template fragments were purified with a GeneClean kit (BIO 101, La Jolla, CA).

Nucleotide sequencing. Of the five positive clones derived from the patient's genomic DNA library, template DNA fragments were subcloned into pUC19 and then the nucleotides were sequenced with a Sequanase kit (US Biochemical, Cleveland, OH) using [α-35S]dCTP and primers supplied by the manufacturer or the synthetic oligonucleotide primers described above. Nucleotides on both strands were sequenced. Nucleotides of the PCR-amplified fragments were sequenced either after subcloning into pUC19 or directly using one oligonucleotide primer with a single-stranded template DNA amplified by another primer.

Analysis of platelet mRNA. cDNA-PCR using platelet RNA was performed under the previously described conditions. Briefly, cDNA was synthesized with approximately 50 ng of total platelet RNA using retroviral reverse transcriptase, RAV-2 (Takara Shuzo, Kyoto, Japan), and oligo(dT) or random hexanucleotide primers. Then, two consecutive PCRs were performed using nested primers: with EX25-R1S and EX27-R1AS or oligo(dT) for the first PCR, and then with EX25-R2S and EX27-R2AS for the second PCR.

The RNase protection assay was performed essentially as described. The 394-bp SacI-Stali GPIIb cDNA fragment was ligated into the SacI-Smal site of the pSPT18 vector (Boehringer-Mannheim, Germany). After cleavage with EcoRI, an antisense RNA probe was synthesized with SP6 RNA polymerase (Boehringer-Mannheim) and [α-32P]CTP. Approximately 50 ng of platelet RNA was hybridized overnight with 5 × 10⁶ cpm of the labeled probe, followed by digestion with RNase A and T1, and then electrophoresed on a 5% polyacrylamide gel containing urea.

RESULTS

Analysis of the GPIIb and IIIa genes. To elucidate the genetic defects of the patient, Southern blot analysis was performed with the patient's DNA using GPIIb or GPIIIa cDNA probes. However, after digestion of the patient's DNA by the following restriction enzymes: BamHI, Bgl II, Dra I, EcoRI, EcoRV, HindIII, Hpa I, Kpn I, Not I, Sac I, Stu I, Xba I, and Xho I, neither of the probes detected any differences from control DNA (data not shown). Since it was suggested from Western blot analysis that the patient was a compound heterozygote of the GPIIb gene, the nucleotide sequences of all 30 exons and the exon-intron boundaries of the GPIIb gene were determined. The results showed two independent mutations: anopal mutation at the end of exon 17 (CGA → TGA, Arg 384 → stop) (Fig 2A) and another one abolishing the consensus splice acceptor site (−3) at exon 26 (CAG → G AG) (Fig 2B).

To determine the origin of these two mutant alleles, exons 17 and 26 with their flanking regions were amplified by PCR, followed by digestion with Tag I or Mva I, as the restriction sites of these enzymes were abolished by each of the mutations, respectively. The agarose gel electrophoresis indicated that theopal mutation was derived from the patient's father (Fig 3A) and the splicing mutation from his mother (Fig 3B). These results were concordant with the phenotypic analysis by Western blotting of the whole platelet protein of the family members.

cDNA-PCR with platelet RNA and the nucleotide sequence of aberrant cDNA. Previous studies on various hereditary disorders and the in vitro mutagenesis model indicate that a nonsense mutation often decreases the mRNA level of the GPIIb gene and that a mutation in the splice acceptor site leads to either activation of the cryptic acceptor sites or an exon skipping process.

To determine which splicing pattern works in this case,
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
MOLECULAR BASIS AND CLASSIFICATION OF GT

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.

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 α-chain of GPIIb and is abundant in proline residues that prevent α-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).

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. 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). 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.

Although classification of GT based on the amount of platelet GPIIIa 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. 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. 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.

A GPIIb gene deletion (~4.5 kb) reported by Burk et al, as well as two types of deletion in the GPIIIa or IIIa gene reported by Newman et al, 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 GPIIIa gene reported by Bray and Shumansky may also belong to the pretranslation 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 GPIb-IIIa may be useful. Precise analyses of molecular defects caused by various mutations will elucidate the biological importance of each structural domain of GPIb-IIIa in maturation, the intracellular sorting process, and ligand-binding activity.

ACKNOWLEDGMENT

The authors thank Dr K. Yamamoto for supplying the EBV stock and help in establishing cell lines, Dr S. Hiroswa for useful discussions, and H. Maeda for typing the manuscript.

REFERENCES


38. Aebi M, Hornig H, Padgett RA, Reiser J, Weissmann C: Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. Cell 47:555, 1986


41. Kieffer N, McEver RP: Residual amounts of glycoproteins IIb and IIIa may be present in the platelets of most patients with Glanzmann’s thrombasthenia. Blood 65:1021, 1985


49. Kunicki TJ (chairman): The report of the Platelet Subcommittee on the Classification of Glanzmann’s thrombasthenia. Thirty-Sixth Annual Meeting of the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis, Barcelona, Spain, 1990


54. Nurden AT, Didry D, Kieffer N, McEver RP: Residual amounts of glycoproteins IIb and IIIa may be present in the platelets of most patients with Glanzmann’s thrombasthenia. Blood 65:1021, 1985


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

A Kato, K Yamamoto, S Miyazaki, SM Jung, M Moroi and N Aoki