A Frameshift Mutation at Gly975 in the Transmembrane Domain of GPIIb Prevents GPIIb-IIIa Expression - Analysis of Two Novel Mutations in a Kindred with Type I Glanzmann Thrombasthenia

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Summary

Two Hispanic siblings presenting with lifelong mucocutaneous bleeding were diagnosed clinically with Glanzmann thrombasthenia on the basis of a normal platelet count, prolonged bleeding time and absent platelet aggregation in response to multiple agonists. Quantitative analysis of the probands’ platelets by flow cytometry showed a complete absence of GPIIb-IIIa, consistent with Type I thrombasthenia. Genetic analysis showed the probands to be compound heterozygotes for two novel mutations of GPIIb: a C1414>G mutation in exon 14, resulting in a premature termination codon replacing residue Tyr440, and the insertion of a G at position 3016 in exon 29, leading to a frameshift affecting the C-terminal half of the transmembrane domain and the cytoplasmic tail. The frameshifted sequence alters residues from Gly975 onwards and is predicted to significantly alter the hydro-pathy and charge profiles of the GPIIb transmembrane domain. The Type I phenotype associated with this mutation suggests that GPIIb residues 975-1008 contain critical structural motifs for heterodimer assembly, membrane retention, export from the ER and surface expression.

Introduction

Glanzmann thrombasthenia (GT) is an autosomal recessive disorder of platelet function resulting from abnormal or absent expression of the platelet fibrinogen receptor, the glycoprotein (GP)IIb-IIIa integrin complex. This condition is characterized by lifelong, mucocutaneous bleeding, a normal platelet count and morphology and a prolonged bleeding time (1). The laboratory diagnosis of GT requires absent platelet aggregation in response to all agonists except ristocetin, as the defective GPIIb-IIIa complex fails to bind fibrinogen. GT can be further categorized into three types, based on the level of GPIIb-IIIa expression on the platelet surface (2). Type I GT is the most common phenotype, with less than 5% of normal levels of GPIIb-IIIa expressed on platelets. In Type II GT, reduced (10-20% normal) but detectable expression on the platelet surface (2). Type I GT is the commonest further categorized into three types, based on the level of GPIIb-IIIa expression. GT can be on the basis of a normal platelet count, prolonged bleeding time and absent platelet aggregation in response to multiple agonists. Quantitative analysis of the probands’ platelets by flow cytometry showed a complete absence of GPIIb-IIIa, consistent with Type I thrombasthenia. Genetic analysis showed the probands to be compound heterozygotes for two novel mutations of GPIIb: a C1414>G mutation in exon 14, resulting in a premature termination codon replacing residue Tyr440, and the insertion of a G at position 3016 in exon 29, leading to a frameshift affecting the C-terminal half of the transmembrane domain and the cytoplasmic tail. The frameshifted sequence alters residues from Gly975 onwards and is predicted to significantly alter the hydro-pathy and charge profiles of the GPIIb transmembrane domain. The Type I phenotype associated with this mutation suggests that GPIIb residues 975-1008 contain critical structural motifs for heterodimer assembly, membrane retention, export from the ER and surface expression.

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Materials and Methods

Platelet Aggregation Studies

In vitro platelet aggregation studies were performed at the Hemostasis laboratory of the Children’s Hospital of Wisconsin. Platelet-rich plasma was obtained from the patients, parents, and a normal control. Washed platelets were prepared and platelet responses to an agonist such as collagen, ADP, and thrombin agonist peptide were measured in a lumi-aggregometer (Chrono Log Corporation, Haverton, PA), using standard methods.

Antibodies

The monoclonal antibody Tab, which binds an epitope on GPIIb, was generously donated by Dr. Roger McEver (University of Oklahoma). The monoclonal antibody AP3, which specifically binds GPIIIa, has been previously described (20). The monoclonal antibodies AP1 and AP2 (21), specific for GPIb and GPIIb-IIIa, respectively, were provided by Dr. Robert Montgomery. (Blood Center of Southeastern Wisconsin, Milwaukee, Wisconsin).

Immunoblot Analysis of Platelet GPIIb-IIIa

Washed platelets were prepared and fixed in acid citrate/dextrose with prostaglandin E1 (final concentration 50 ng/ml) and centrifuged at 250 g for 15 min at 22 °C. Platelet-rich plasma was collected and centrifuged at 1000 g for 15 min, and the platelet pellet resuspended in Ringer’s citrate/dextrose buffer (108 mM NaCl, 38 mM KCL, 1.7 mM NaHCO3, 21.2 mM sodium citrate, 27.8 mM glucose, 1.1 mM MgCl2-6 H2O, pH 6.5), with 50 ng/ml prostaglandin E1. Aliquots of 5 × 10^6 platelets were incubated with 5 mg/ml of a mouse monoclonal antibody diluted in RCD/0.2% BSA and then washed twice in RCD/0.2% BSA before incubating with 25 mg/ml of FITC labeled goat anti mouse IgG (Jackson Immunoresearch labs, West Grove, PA) in RCD/0.2%BSA for 30 min. Afterwards, samples were washed in RCD/0.2% BSA, and analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Immunoblot Analysis of Platelet GPIIb-IIIa

Washed platelets were lysed in 50 mM Tris, 1% Triton, 10 mM N-ethylmaleimide, 2 mM phenylmethylsulfonlfuryl fluoride (PMSF) and 20 mM leupeptin, and centrifuged at 15000 g before collecting the supernatant. Bicinchoninic acid (BCA) protein assays (Pierce, Rockford, IL) were done to determine the concentrations of proteins in the lysates. Proteins in the thrombasthenic and control lysates were separated by SDS-7% polyacrylamide gel electrophoresis under reducing conditions. Afterwards, proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA), incubated with rabbit polyclonal antibodies directed against the GPIIb and GPIIIa subunits and detected with an alkaline phosphatase conjugated goat anti-rabbit antibody.

Polymerase Chain Reaction Amplification of Genomic DNA

Genomic DNA was isolated from peripheral blood leukocytes. Using oligonucleotides primers flanking the intron sequences, exons of the GPIIb and GPIIIa genes were amplified by PCR. Exon 14 of GPIIIa was amplified using the forward primer 5’- CCATACCACTTGAATGCTCCG -3’ and the reverse primer 5’-CAACACTGGTACGTCGTCGCTCC -3’. Exon 29 of GPIIIa was amplified using the forward primer 5’- CGGGTGTGGGACCTGGAC -3’ and the reverse primer 5’-ACACTTGTGACTGGCACTAACC -3’. Exon 29 of GPIIIa was amplified using the forward primer 5’-CAACACTGGTACGTCGTCGCTCC -3’ and the reverse primer 5’-CAACACTGGTACGTCGTCGCTCC -3’ and 30 cycles of extension (72 °C, 1 min), denaturation (96 °C, 45 s), and annealing (56 °C, 45 s). PCR products were recovered from agarose gel using the Qiaquick Gel Extraction Kit (Qiagen, Chatsworth, CA). Afterwards, DNA segments were analyzed via automated sequencing (Applied Biosystems Incorporated, Foster City, CA). To detect heterozygous alleles, amplified exons were subcloned into the PCR 2.1 vector (Invitrogen, San Diego, CA), and individual clones sequenced.

Results

Platelet Aggregation

Two Hispanic siblings, DV and SV, presented with excessive mucocutaneous bleeding from infancy, suggestive of an inherited defect of hemostasis. There is no family history of consanguinity and no excessive bleeding or bruising in either parent. Bleeding times were prolonged with normal platelet counts and morphology. Platelet function was further assessed by aggregometry. Washed platelets from both patients (DV and SV) failed to aggregate in response to ADP, thrombin agonist peptide or collagen (Fig. 1), consistent with Glanzmann thrombasthenia. Platelets obtained at the same time from the parents, (MV and FV) showed a nonspecific delay in collagen-induced aggregation, most marked in the mother’s sample, but aggregated normally in response to ADP and thrombin peptide.

Surface expression of platelet membrane glycoproteins was measured by flow cytometry. Platelets isolated from the patients, parents, and a normal control (C) were incubated with FITC-labeled mouse monoclonal antibodies and analyzed as described above (Fig. 1). Platelet binding of AP1, a control antibody directed against platelet GPIb, was normal in both probands and their mother, but reduced to 66% of normal in the father’s sample. However, platelets from both probands showed virtually absent binding of antibodies directed against GPIIb (TAB), GPIIIa (AP3), or the GPIIb-IIIa complex (AP2). The surface expression of GPIIb-IIIa is estimated to be less than 5% that of the normal control in both DV and SV, consistent with a diagnosis of Type I Glanzmann thrombasthenia. The mother’s platelets bound all anti-GPIIb-IIIa antibodies at normal levels, whereas the father’s platelets again showed reduced binding, at 60-70% of the normal control.

To assess total platelet GPIIb-IIIa levels, Triton X-100 detergent lysates were prepared from platelets of the patients, both parents, and a normal control, and analyzed by semiquantitative Western blotting.
Thromb Haemost 1998; 80: 546–50

(Fig. 2). When compared to the normal control, platelets from both patients showed absent (less than 1% of normal) immunostaining of GPIIb and a residual amount (less than 5% normal) of GPIIIa. The preservation of some GPIIIa, expressed here as the αIβ3 subunit at the C-terminal end of the fourth cation binding domain. The residue 440 with a stop codon, and premature truncation of the GPIIb position 1414 of exon 14, resulting in the replacement of tyrosine GPIIb gene (Fig. 3). The first consisted of a C to G base change at the proband, two separate and novel mutations were identified within the individuals clones were analyzed by direct cycle sequencing. In each were subcloned by ligation into a TA cloning vector, and multiple heterozygous mutations or separate alleles, purified PCR products were purified and sequenced directly. To confirm the patient leukocytes and exons of the GPIIb gene amplified by PCR. The thrombasthenia in DV and SV, genomic DNA was recovered from the parents showed that they were each heterozygous for one mutation of the GPIIb gene. The mother was heterozygous for the Tyr440Stop mutation in exon 14, whereas the father was a carrier for the insG3016 frameshift mutation in exon 29. Both GPIIb mutations were present in genomic DNA from the affected children, confirming that they are compound heterozygotes.

To characterize the genetic defects responsible for Glanzmann thrombasthenia in DV and SV, genomic DNA was recovered from patient leukocytes and exons of the GPIIb gene amplified by PCR. The PCR products were purified and sequenced directly. To confirm heterozygous mutations or separate alleles, purified PCR products were subcloned by ligation into a TA cloning vector, and multiple individual clones were analyzed by direct cycle sequencing. In each proband, two separate and novel mutations were identified within the GPIIb gene (Fig. 3). The first consisted of a C to G base change at position 1414 of exon 14, resulting in the replacement of tyrosine residue 440 with a stop codon, and premature truncation of the GPIIb subunit at the C-terminal end of the fourth cation binding domain. The second mutation, found in exon 29 of GPIIb, consisted of the insertion of a G at position 3016, resulting in a frameshift which is predicted to alter the amino acid sequence from residue Gly975 onwards (Fig. 4). This frameshift mutation therefore affects the C-terminal half of the transmembrane domain and the entire cytoplasmic domain of GPIIb. A hydrophathy plot comparing the wild-type and frameshifted sequence demonstrates that the substitution of polar and β-turn inducing residues dramatically reduces the extent of hydrophobic sequence available for insertion into the plasma membrane (Fig. 5). To confirm the association of these two mutations with the thrombasthenia phenotype, multiple individual clones of exons 14 and 29 each mutation were sequenced from the probands and parents. Analysis of genomic DNA isolated from the parents showed that they were each heterozygous for one mutation of the GPIIb gene. The mother was heterozygous for the Tyr440Stop mutation in exon 14, whereas the father was a carrier for the insG3016 frameshift mutation in exon 29. Both GPIIb mutations were present in genomic DNA from the affected children, confirming that they are compound heterozygotes.

Discussion

This paper describes the molecular characterization of two Hispanic siblings with Glanzmann thrombasthenia. The probands, DV and SV, presented with symptomatic, chronic mucocutaneous bleeding, consistent with a platelet or vascular defect. The clinical diagnosis of thrombasthenia was made on the basis of a normal platelet count and morphology, coupled with absent platelet aggregation in response to

**Fig. 2** Western blot analysis of total platelet GPIIIa-Ila. For a semiquantitative analysis, the indicated amounts of Triton X-100 platelet lysates from a normal control, the parents (FV and MV) and the probands (DV and SV) were separated by SDS-7% polyacrylamide gel electrophoresis, transferred to PVDF membrane and detected with rabbit polyclonal antibodies against GPIIb and GPIIIa as described in Materials and Methods. The position of GPIIb and GPIIIa is indicated. Compared to control, FV and MV show reduced levels of GPIIb-Illa, whereas the probands show absence of GPIIb and residual GPIIIa only

**Fig. 3** DV and SV are compound heterozygotes for two GPIIb mutations associated with Type I Glanzmann thrombasthenia. The family tree is shown, with two affected children (DV and EV) and their asymptomatic parents (MV and FV). Sequencing of PCR-amplified genomic DNA showed a missense C1414G mutation (Tyr440Stop) in exon 14 of GPIIb (left panel) in MV and in both children. An insertion G3016 mutation (frameshift Gly975-Glu1008) in exon 29 of GPIIb (right panel) was present in FV and both children. As this exon was sequenced using a reverse primer, the inserted nucleotide is shown as a C

**Fig. 4** An insertion mutation in exon 29 of GPIIb results in a frameshift of the transmembrane and cytoplasmic domains. The predicted amino acid sequence for wild-type (WT) and insG3016 (DV) exons 29 and 30 is shown, with the transmembrane domain of WT GPIIb underlined. Note that the insertion of a single nucleotide at 3016 alters the amino acid sequence C-terminal to residue Gly 975 onwards and produces a cytoplasmic domain only six residues shorter than WT GPIIb. X = stop codon

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548

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multiple agonists. We have used two methods to define the level of platelet GPIIb-IIIa expression; both flow cytometry of washed platelets and immunoblotting of platelet lysates showed less than 5% of normal GPIIb-IIIa expression in the probands’ platelets. The low to absent expression of the receptor defines this kindred’s defect as Type I thrombasthenia. Although DV or SV platelets showed no binding of subunit-specific monoclonal antibodies, a trace amount of the GPIIIa (β3) subunit was identified by immunoblotting (Fig. 2). Persistent GPIIIa expression has been previously described in a GPIIb mutation, due to a compensatory increase in platelet levels of the vitronectin receptor, αvβ3 (22). This prompted us to search initially for mutations in the GPIIb gene, by PCR-amplification of the coding sequence from leukocyte genomic DNA. Genetic analysis showed that the affected siblings were compound heterozygotes, having inherited a mutant GPIIb allele from each parent (Fig. 3). These mutations, in exons 14 and 29 of the GPIIb gene, have not been previously described.

The first novel GPIIb mutation identified in this kindred was a C1414G substitution in exon 14, resulting in the substitution of a terminal codon for Tyr440, at the C-terminal end of the fourth cation-binding domain. This mutation is predicted to produce a truncated form of GPIIb comprising the N-terminal region and all four cation-binding domains. A recent theoretical analysis of GPIIb and other integrin α-subunits has predicted that their N-terminal, ligand-binding regions fold into a β-propeller structure (23), a torus-like structure with seven “blades”. Based on this model, truncation of GPIIb at residue 440 would delete half of the seventh “propeller blade”, possibly altering the conformation of the upper ligand-binding surface and/or the lower calcium-binding surface. The association of the Tyr440Stop mutation leads to intracellular trapping of the GPIIb-IIIa complex. Surface expression of GPIIb-IIIa receptor is inversely correlated with this mutant complex. Asp426 (6) within the cation-binding domains has been described, affecting the GPIb subunit of the platelet GPIb-V-IX receptor complex (33, 34). This patient’s platelets showed high expression of GPIIb-IIIa receptor; three point mutations, Gly242Asp (11), Arg327His (28), Gly418Asp (12) and a two-residue deletion, ΔVal425-Asp426 (6) within the cation-binding domains of GPIIb all resulted in a Type I phenotype.

Experimental studies suggest that all of these mutations block receptor expression by leading to intracellular retention, and degradation of the mutant complex. Surface expression of GPIIb-IIIa receptor is known to require the formation of an intracellular heterodimer (14, 15). PreGPIIb is synthesized in excess in the ER, and is degraded unless it can form a complex with GPIIIa subunits (14, 16). In the mutants listed above, the initial steps of subunit synthesis and heterodimer formation may, in fact, still occur. GPIIb truncated at residues 243, 296 and 364 (28), or at residue 870 (10) was shown in co-transfection experiments to associate within the endoplasmic reticulum with wild-type GPIIIa, although none of these complexes were expressed. Similarly, co-transfection experiments with GPIIb carrying the Gly242Asp (11) and ΔVal425-Asp426 mutations (6) formed heterodimers with GPIIIa, but did not undergo further processing, indicating that the mutant complexes were retained in the ER. These, and other studies (29) demonstrate that the first 200 residues of GPIIb alone are sufficient to bind GPIIIa, but that additional elements of the heterodimer are necessary for surface expression. These elements may include a specific conformation; substitutions of Gly242 with Glu, Lys, Pro or Asn caused intracellular retention of the complex, whereas the more conservative substitutions of Ala or Val allowed expression (30). Alternatively, the transmembrane and cytoplasmic domains of GPIIb may contain structural signals for export, as discussed below. Therefore, based on data from other truncation mutants of GPIIb, it is likely that the Tyr440Stop mutation leads to intracellular trapping of the GPIIb-IIIa complex.

The second GPIIb mutation identified in this kindred is the insertion of a G at base 3016 of exon 29. This results in a frameshift of the transmembrane and cytoplasmic domains from residue Gly975 onwards. The overall polypeptide is predicted to be only six residues shorter than wild-type, but the sequence of the C-terminal half of the transmembrane and putative cytoplasmic tail is radically altered (Fig. 4). The frameshifted transmembrane sequence would include multiple polar histidine and β-turn-inducing proline residues, while the mutated cytoplasmic sequence loses essential structural features such as a basic residue immediately C-terminal to the membrane, and the GFFKR motif necessary for heterodimer formation (31) and implicated in modulating integrin affinity (32). In this family, the insG3016 mutation was associated with a Type I GT phenotype in the compound heterozygotes, and with a 50% reduction of GPIIb-IIIa expression in the father who carries this allele. This study therefore implies that GPIIb residues 975-1008 are required for intracellular trafficking and integrin expression.

The insG3016 mutation is the first description of a GT mutation which specifically alters the sequence of the GPIIb transmembrane domain. A preliminary report of two cases with an in-frame deletion of exon 29, encoding the entire GPIIb transmembrane domain, has also been published (7). There is no comparable mutation to insG3016 in the GT literature, but in the analogous condition of Bernard-Soulier syndrome, a very similar frameshift of the transmembrane/cytoplasmic domains has been described, affecting the GPIbα subunit of the platelet GPIb-V-IX receptor complex (33, 34). This patient’s platelets showed little or no expression of GPIbα, but soluble GPIbα was detected in plasma. This apparent secretion may be specific to GPIbα and reflect its biosynthetic pathway and relative sensitivity to proteases; we have not examined the plasma of our GT patients for soluble GPIIb. However, in a GPIIIa mutation common among Iraqi Jews, where the GPIIIa...
subunit is truncated just before the transmembrane domain, no soluble GPIIIa could be detected in patient plasma (5).

In summary, we have genotyped siblings with a defect in GPIIb-IIIa expression and found two novel mutations in the GPIIb gene: a premature truncation at Tyr440 and an insertion/framing shift mutation affecting the C-terminal half of the transmembrane domain and cytoplasmic tail (residues 975-1008). The deletion or scrambling of GPIIb residues 975-1008 in both alleles is likely to be due to intracellular trapping of the mutant GPIIb-IIIa complex. Further studies of naturally occurring mutations within integrin subunits should continue to provide novel insights into the role these interesting molecules play in regulating platelet adherence, aggregation, and signaling.

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