Glanzmann Thrombasthenia Secondary to a Gly273 → Asp Mutation Adjacent to the First Calcium-binding Domain of Platelet Glycoprotein IIb

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Abstract

We studied the defect responsible for Glanzmann thrombasthenia in a patient whose platelets expressed < 5% of the normal amount of GPIIb–IIIa. Genetic and biochemical evidence indicated that the patient's GPIIIa genes were normal. However, DNA analysis revealed the patient homozygous for a G818 → A substitution in her GPIIb genes, resulting in a Gly273 → Asp substitution adjacent to the first GPIIb calcium-binding domain. To determine how this mutation impaired GPIIb–IIIa expression, recombinant GPIIb containing the mutation was coexpressed with GPIIIa in COS-1 cells. The GPIIb mutant formed stable GPIIb–IIIa heterodimers that were not immunoprecipitated by either of two heterodimer-specific monoclonal antibodies, indicating that the mutation disrupted the epitopes for these antibodies. Moreover, the GPIIb in the heterodimers was not cleaved into heavy and light chains, indicating that the heterodimers were not transported from the endoplasmic reticulum to the Golgi complex where GPIIb cleavage occurs, nor were the mutant heterodimers expressed on the cell surface. These studies demonstrate that a Gly273 → Asp mutation in GPIIb does not prevent the assembly of GPIIb–IIIa heterodimers, but alters the conformation of these heterodimers sufficiently to impair their intracellular transport. The impaired GPIIb–IIIa transport is responsible for the thrombasthenia in this patient. (*J. Clin. Invest.* 1994. 93:172–179.) Key words: bleeding disorder • platelet aggregation • glycoprotein IIb–IIIa • in vitro mutagenesis • protein conformation

Introduction

The platelet membrane glycoproteins IIb and IIIa form heterodimers that contain binding sites for fibrinogen, fibronectin, von Willebrand factor, and vitronectin after platelet activation (1). Because a sufficient number of functional GPIIb–IIIa heterodimers on the platelet surface is required for normal platelet aggregation, deficiency or dysfunction of GPIIb–IIIa results in the autosomal recessive bleeding disorder Glanzmann thrombasthenia (2). By electron microscopy, GPIIb–IIIa in detergent solution consists of an 8 × 12–nm globular head with two 18-nm flexible tails extending from one side (3). The globular head of GPIIb–IIIa contains the amino termini of GPIIb and GPIIIa and the ligand binding domain of the heterodimer, while each tail contains the transmembrane domain and carboxyl terminus of one subunit. The GPIIb–IIIa heterodimer is calcium dependent (1), and in the presence of calcium chelators GPIIb–IIIa dissociates into two comma-shaped structures, each containing a single tail and a portion of the nodular head (3). The primary sequences of GPIIb and GPIIIa have been deduced from analyses of cDNA clones, revealing that GPIIb–IIIa is a member of the integrin family of adhesion receptors (4–7). The GPIIb sequence also contains four repeated domains with the consensus sequence Asp-X-Asp-X-Asp-Gly-X-X-Asp-X-X-Val, a sequence resembling calcium-binding domains in many proteins (4, 8). Whether calcium binding to these domains is involved in GPIIb–IIIa heterodimer assembly or other aspects of GPIIb–IIIa function remains to be determined.

The genes for GPIIb and GPIIIa reside in proximity on the long arm of human chromosome 17 at q21 → 22 (9). After gene transcription, nascent GPIIb and GPIIIa are assembled into heterodimers in the rough endoplasmic reticulum (ER)[1] and are transported to the Golgi complex where GPIIb undergoes cleavage into disulfide-linked heavy and light chains (10–12). Studies in vitro indicate that in the absence of heterodimer assembly GPIIb and GPIIIa are retained in the ER and eventually are degraded (13). Nevertheless, heterodimer assembly by itself does not ensure normal GPIIb–IIIa expression since mutations in GPIIb or GPIIIa that alter the quaternary structure of the heterodimer result in retention and degradation of the heterodimers as well (13). In this paper, we report studies of a patient with Glanzmann thrombasthenia whose platelets contained a markedly decreased number of GPIIb–IIIa heterodimers on their surface. We found that the patient's thrombasthenia resulted from a point mutation in the gene for GPIIb. Despite this minimal change, the mutation altered the conformation of the GPIIb–IIIa heterodimer and, as a consequence, impaired the intracellular transport of the mutant heterodimers from the ER to the cell surface.

Methods

Clinical history. The propositus is an Ashkenazi Jewish female child, the product of a consanguineous marriage, who presented at 2 d of age with subdural bleeding and extensive ecchymoses. Although the patient had a normal platelet count, she had a prolonged cutaneous bleed-

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1. *Abbreviation used in this paper:* ER, endoplasmic reticulum.

ing time. Subsequent studies revealed absent ex vivo platelet aggregation in response to ADP (2–10 μM), collagen (0.047–0.19 mg/ml), and epinephrine (2.5–10 μM), but normal platelet agglutination in response to ristocetin. The patient responded well to a platelet transfusion and has had no long-term neurological sequelae. However, the patient has continued to experience extensive ecchymoses and occasional epistaxis, but has not required further hospitalization or platelet transfusion.

Blood samples for the studies described below were obtained from the patient, her parents and other family members, and normal volunteers after informed consent. These studies were approved by the Committee for Protection of Human Subjects Institutional Review Board of the Children's Hospital of Philadelphia.

Flow cytometry. Flow cytometry using a panel of mAbs conjugated with FITC was performed as previously described (14). Monoclonal antibodies used for these studies were A2A9, an antibody that only interacts with intact GPIIb–IIIa heterodimers and recognizes an epitope located in the globular head of GPIIb–IIIa (3, 15); B1B5, an antibody that recognizes an epitope located near the carboxyl terminus of the GPIIb heavy chain (3, 4); SSA6, an antibody that recognizes an epitope located within a 66,000-mol wt chymotrypsin-derived fragment of GPIIIa (3, 16); PAC-1, an antibody specific for the activated conformation of GPIIb–IIIa (17); and AP1, an antibody specific for platelet glycoprotein Ib (18). In the case of PAC-1, platelets were stimulated with 0.2 μM phorbol myristate acetate for 5 min at 25°C before adding the antibody.

Immunoblot analysis of platelet proteins. Immunoblotting of platelet proteins was performed as described previously (13). Briefly, washed platelets were dissolved in 0.01 M Tris-HCl buffer, pH 6.8, containing 3% SDS by heating at 100°C, and 100 μg of soluble platelet protein was electrophoresed in 0.1% SDS–7.5% polyacrylamide gels under reducing conditions. Then the separated proteins were transferred to nitrocellulose paper (0.45 μm; Schleicher & Schuell, Inc., Keene, NH), and the paper was incubated with polyclonal anti-GPIIb or GPIIIa antisera at a concentration of 50 μg/ml. Sites of antibody binding were detected with ^{125}I-labeled staphylococcal Protein A (Boehringer Mannheim Corp., Indianapolis, IN). The labeled blots were dried and exposed to Kodak X-Omat AR film at −70°C.

Quantitation of platelet αv-GPIIIa. The number of αv-GPIIIa complexes per platelet was quantitated as previously described (19). Briefly, blood was obtained from the patient and three controls in Philadelphia, anticoagulated with 0.18 vol acid-citrate-dextran formula A, coded, and sent directly to the State University of New York at Stony Brook. Blood from an additional control was drawn in Stony Brook. Then platelet-rich plasma was either adjusted to a platelet count of ≈3 × 10^5/μl using platelet-poor plasma for binding studies using the anti-GPIbα mAb 6D1 (20) or concentrated to a platelet count of 0.94–1.78 × 10^6/μl for binding studies using the αv-GPIIIa anti-mAb LM609 supplied by Dr. David Cheresh (Scripps Clinic, La Jolla, CA) (21). Aliquots of the platelet-rich plasma (0.2 ml) were incubated with ^{125}I-6D1 or ^{125}I-LM609 at 22°C for 90 or 60 min, respectively, before platelet-bound antibody was separated from free antibody by sedimentation through 30% sucrose. Three subsaturating concentrations of LM609 were used to enhance the ratio of bound to free antibody, improving the reproducibility of the results. At the highest and lowest antibody concentrations, nonspecific LM609 binding was determined by adding 20 μg/ml unlabeled LM609 to the platelet-rich plasma 10 min before adding the ^{125}I-LM609. Nonspecific antibody binding was found to represent 2–9% of specific binding and was substracted from the total antibody binding. However, no adjustments for nonspecific binding were made at the intermediate LM609 concentrations.

Identification of the mutation responsible for thrombasthenia in the propositus. Genomic DNA and total platelet RNA were isolated from the propositus, family members, and controls as previously described (22). Platelet RNA was converted to cDNA using AMV reverse transcriptase (Gibco Laboratories, Grand Island, NY) before analysis. PCR amplification and DNA sequence analysis of the GPIIb coding sequence were accomplished using oligonucleotides based on known cDNA and genomic sequences (4, 23). Oligonucleotides were synthesized using a DNA synthesizer (380B; Applied Biosystems, Inc., Foster City, CA) and were used without further purification. PCR amplification was performed using a thermal cycler (BIOS Labs., New Haven, CT) for 30 cycles under the following conditions: denaturation, 94°C for 30 s; annealing, 60°C for 30 s; and extension, 72°C for 30 s. The reaction volume was 100 μl and consisted of 10 mM Tris-HCl buffer, pH 8.3, containing 10 pmol of each primer, 2.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 0.2 mM dNTP, and 1 U Taq I polymerase (Perkin-Elmer Corp., Norwalk, CT). The PCR products were directly sequenced in both directions using a Taq polymerase chain termination approach and [^{32}P-γ]ATP end-labeled oligonucleotides (24).

Allele-specific analysis by PCR was used to determine whether the propositus was homozygous for GPIIb or GPIIIa polymorphisms (25). PCR amplification of 200 ng of genomic DNA was performed for 30 cycles in a thermal cycler (Perkin-Elmer Corp.) using the following conditions: denaturation at 95°C for 1 min, annealing at 66°C for 2 min, and extension at 72°C for 2 min. 20-μl aliquots of each reaction were then electrophoresed in a 1% agarose gel in the presence of ethidium bromide for 60 min at 50 mA.

Heterologous expression of recombinant GPIIb–IIIa. The effect of mutations on GPIIb–IIIa expression was examined by introducing the mutation into the sequences of wild-type cDNAs by site-directed mutagenesis as described previously (12). After confirmation of the site-directed mutation by DNA sequencing, the mutant cDNA was shuttled into the expression vector pMT2ADA (26) and was introduced into COS-1 cells using the DEAE-dextran method as described previously (12). To avoid flask-to-flask variation in transfection efficiency, cells were harvested at 24 h, pooled, and replated, and incubation was continued for an additional 24 h. 48 h after transfection, the COS-1 cells were labeled with [^{35}S]methionine (Dupont NEN Research Products, Boston, MA) at 200 μCi/ml for 60 min before harvesting. For pulse–chase studies, media containing labeled methionine were removed and replaced with media containing unlabeled methionine for various periods of time before the cells were harvested.

At 48 h, labeled cells were disrupted with a plastic cell scraper (Fisher Scientific Co., Pittsburgh, PA) and were extracted overnight at 4°C with 0.02 M Tris-HCl buffer, pH 7.8, containing 1% Triton X-100 (Sigma Immunochemicals, St. Louis, MO). Then the extracts were cleared by incubation with 4 μl of nonimmune mouse serum for 15 min at 4°C, followed by an incubation with 100 μl of a 10% suspension of fixed *Staphylococci* (Pansorbin; Calbiochem Corp., La Jolla, CA) for 30 min at 4°C. Recombinant GPIIb, GPIIIa, or GPIIb–IIIa were immunoprecipitated by adding 25 μg of a murine mAb to 500-μl aliquots of the cleared cell extracts. The monoclonal antibodies used for these immunoprecipitations were B1B5, SSA6, and A2A9 (described above), and a second heterodimer-specific antibody 10E5 (27). After incubation for 1 h at 4°C, immune complexes were collected using Protein A agarose (GIBCO BRL, Gaithersburg, MD), eluted at 100°C with 0.01 M Tris-HCl buffer, pH 6.8, containing 3% SDS, and electrophoresed on 0.1% SDS–7.5% polyacrylamide slab gels. Gels were fixed with 10% acetic acid, treated with Autofluor (National Diagnostics Inc., Manville, NJ), dried, and exposed to Kodak X-Omat AR film at −70°C. Apparent molecular weights of the immunoprecipitated proteins were determined from the mobilities of prestained protein molecular weight markers obtained from Bio-Rad Laboratories (Richmond, CA).

Recombinant GPIIb–IIIa heterodimers were detected on the surface of cotransfected COS-1 cells after labeling the cell surface with ^{125}I using lactoperoxidase/H_2O_2 (13). Surface-labeled cells were extracted with 1% Triton X-100, and proteins were immunoprecipitated from the extracts using a polyclonal anti-GPIIb antisera that interacts with determinants on both the heavy and light chains of GPIIb.

Results

Quantitation of GPIIb–IIIa in propositus platelets. The complete absence of agonist-stimulated platelet aggregation sug-

gested that our patient suffered from Glanzmann thrombasthenia (2). To confirm this diagnosis, we measured GPIIb–IIIa expression on the surface of the patient's platelets using flow cytometry and a panel of GPIIb–IIIa-specific monoclonal antibodies. As seen in Fig. 1, < 5% of the normal amount of GPIIb–IIIa was detected on the surface of the patient's platelets after reacting the platelets with the GPIIb-specific mAb B1B5 or the GPIIIa-specific mAb SSA6. Somewhat less GPIIb–IIIa (< 2%) was measured after reacting resting platelets with the heterodimer-specific mAb A2A9 or after activating the platelets with phorbol myristate acetate and reacting with the activation-dependent anti-GPIIb–IIIa mAb PAC-1. These values are near the limits of sensitivity of the flow cytometer and indicate that little, if any, GPIIb–IIIa was present on the surface of the patient's platelets. On the other hand, the platelets bound a normal amount of the anti-GPIb mAb AP-1, verifying that the abnormality in the platelets was limited to the GPIIb–IIIa complex.

Despite the marked reduction in GPIIb–IIIa antigen on the surface of the patient's platelets, it was possible that sufficient GPIIb–IIIa remained to be detected by immunoblotting. To test this possibility, equal quantities of protein from patient and control platelets were subjected to SDS-PAGE under reducing conditions and were immunoblotted using either polyclonal GPIIb or GPIIIa antisera. The results of these experiments are shown in Fig. 2. Immunoblotting of protein from control platelets revealed prominent bands corresponding to GPIIIa and to the GPIIb heavy chain (GPIIbα) and GPIIb light chain (GPIIbβ). In contrast, immunoblotting of protein from patient platelets revealed a faint GPIIIa band when anti-GPIIIa antisera was used and a faint band corresponding to a protein with a molecular weight of ≈140,000 when anti-GPIIb antisera was used. Thus, these immunoblots indicate that a small amount of normal size GPIIIa remained in the patient's platelets. The blots also suggest that the residual GPIIb in the patient's platelets consisted primarily of the uncleaved 140,000-mol wt GPIIb precursor pro-GPIIb (4, 13). Several additional bands were also present on the control and patient immunoblots when the GPIIb antisera was used and likely resulted from the presence of antibodies with other specificities in the antisera.

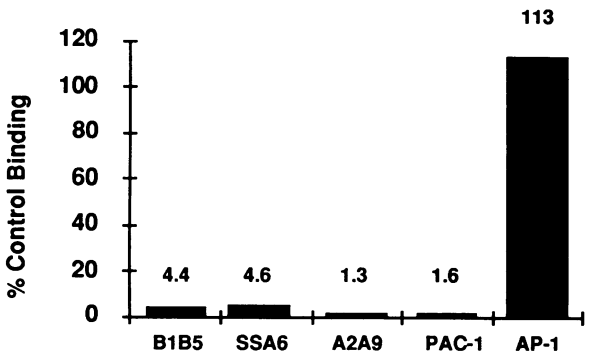

Figure 1. Measurement of the GPIIb–IIIa content of the patient's platelets using flow cytometry. Patient and control platelets were reacted with saturating amounts of the anti-GPIIb mAb B1B5, the anti-GPIIIa mAb SSA6, the GPIIb–IIIa heterodimer-specific mAb A2A9, and the activation-dependent GPIIb–IIIa heterodimer-specific mAb PAC-1. The anti-GPIb mAb AP-1 was used as a positive control. Each mAb was directly conjugated with FITC, and the extent of antibody binding was expressed as mean fluorescence intensity. The data in the figure represent the percentage of antibody binding to patient compared with control platelets. The number above each bar is the mean of two experiments.

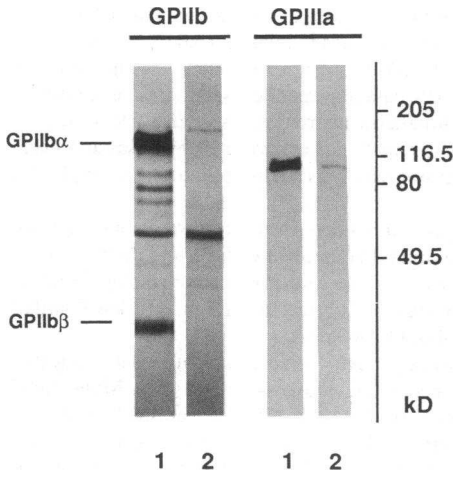

Figure 2. Immunoblot analysis of residual GPIIb and GPIIIa in the patient's platelets. 100 μg of control and patient platelet protein, dissolved in SDS, was electrophoresed in 0.1% SDS–7.5% polyacrylamide gels under reducing conditions, transferred to nitrocellulose paper, and blotted with either rabbit polyclonal anti-GPIIb or anti-GPIIIa antisera. Sites of antibody binding were detected using ^{125}I-labeled staphylococcal protein A. (Lane *1*): control platelets; (lane *2*): patient platelets.

Identification of the mutation responsible for thrombasthenia in the propositus. Because it was not possible to determine from the immunoblots whether a mutation in GPIIb or in GPIIIa was responsible for the patient's thrombasthenia, we took advantage of the observation that the number of vitronectin receptors (αv-GPIIIa) may be increased in the platelets of thrombasthenic patients with GPIIb abnormalities and decreased in the platelets of thrombasthenic patients with GPIIIa abnormalities (19, 28). To determine the number of αv-GPIIIa heterodimers expressed on the patient's platelets, we measured the binding of the anti-vitronectin receptor mAb LM609 to patient and control platelets at a series of subsaturating concentrations. The patient's platelets bound 173–227% as much antibody as the controls (Table I). From these data, we concluded that it is likely that the genetic abnormality responsible for the patient's thrombasthenia was present in her genes for GPIIb. In support of this conclusion, we also observed that the patient was heterozygous for a polymorphism in the GPIIIa intron xi sequence 5′-TTCCC(C/T)GG-3′ that is located 85 bp upstream from exon xii (data not shown). Accordingly, because the patient is the offspring of a consanguineous marriage, we would have anticipated her to be homozygous, rather than heterozygous, for this polymorphism if her genetic abnormality had resided in her GPIIIa genes.

Because our preliminary data suggested that a GPIIb mutation was responsible for the patient's thrombasthenia, the nucleotide sequence for approximately two thirds of the coding region of her GPIIb genes was determined using PCR-amplified genomic and platelet-derived cDNA. Using the sense primer 5′-GTGGTCACTCAGGCCGGAGAG-3′ and the antisense primer 5′-CCATATACAGTGGAGCGCCCACC-3′, corresponding to nucleotides 613–633 and 1,068–1,046 of the published GPIIb cDNA sequence, respectively, we detected a

Table I. Measurement of Platelet αvGPIIIa

	Antibody LM609 concentration			
	ng/ml			
	38	63	154	288
	No. of LM609 molecules bound per platelet			
Patient	64	94	166	149
Control 1	8	15	32	48
Control 2	43	58	83	93
Control 3	44	69	103	94
Control 4	38	65	103	94
Control mean	33	41	79	86
Patient as percentage of the control mean	192	227	210	173

Blood was obtained from the patient and controls 1–3 concurrently in Philadelphia. After transport of the blood to Stony Brook, blood was obtained from control 4 in Stony Brook before the αvGPIIIa measurements were made.

base substitution of G → A at position 818 of the GPIIb reading frame (4) (Fig. 3 *A*). This substitution results in a Gly273 → Asp amino acid substitution within the first of the four repeats in the GPIIb protein that contain its putative calcium-binding domains (4).

To establish that the patient was homozygous for this DNA substitution, allele-specific PCR analysis was performed using genomic DNA, an antisense oligonucleotide primer 5′-CCTGAGAACTGGGATAAGG-3′ derived from intron 9 of the GPIIb gene (24), and either of two sense oligonucleotide primers, 5′-GGTACTCGGTGGCCGTGGG-3′ or 5′-GGTACTCGGTGGCCGTGGA-3′, corresponding to nucleotides 800–818 of the wild-type or mutant GPIIb coding sequence, respectively (4). As seen in Fig. 3 *B*, a DNA fragment of the expected size was generated by PCR using wild-type genomic DNA and the wild-type, but not the mutant, sense primer. Conversely, an identically sized DNA fragment was generated using patient DNA and the mutant, but not the wild-type, sense primer. However, using genomic DNA obtained from the patient's mother, DNA fragments of identical size were generated using either the wild-type or the mutant primer, confirming with the mother's status as an obligate heterozygote for the mutation. Identical results were obtained using paternal DNA (data not shown). To further examine the pattern of inheritance of the GPIIb mutation, allele-specific PCR analysis and measurements of platelet-surface GPIIb–IIIa using flow cytometry were performed using DNA and platelets obtained from other members of the patient's family (Fig. 4). There was complete concordance between the PCR analysis and the GPIIb–IIIa measurements, such that ≈50% levels of GPIIb–IIIa were present on the platelets of obligate carriers and ≈100% levels were present on the platelets of nonaffected family members. These data confirm the autosomal recessive pattern of inheritance of the G818 → A base substitution and verify that the patient is homozygous at this position.

Expression of GPIIb containing Gly273 → Asp in COS-1 cells. To verify that the glycine → aspartic acid substitution resulting from the G818 → A mutation actually interferes with GPIIb–IIIa expression, we introduced the mutation into the sequence of a wild-type GPIIb cDNA by site-directed mutagen-

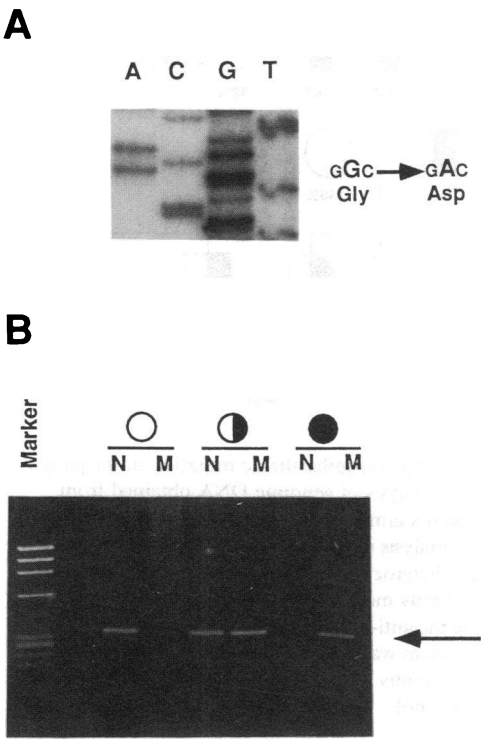

Figure 3. Identification of the mutation responsible for the patient's thrombasthenia. (*A*) Nucleotide sequence analysis of the patient's gene for GPIIb. Direct sequencing of the patient's GPIIb gene was performed using cDNA derived from reverse-transcribed total platelet RNA by PCR. The analysis revealed a single G → A base substitution at nucleotide 818 of the GPIIb reading frame. This substitution converts the codon for amino acid residue 273 from GGC encoding glycine to GAC encoding aspartic acid. (*B*) Allele-specific analysis of the GPIIb genes. Genomic DNA, isolated from a normal control, the patient's mother, and the patient was amplified by PCR using a common 3′ oligonucleotide derived from intron 9 of the GPIIb gene and either of two 5′ oligonucleotides derived from exon 8, one ending in the normal G or one ending in the A mutation. The PCR products were then examined by agarose gel electrophoresis and ethidium bromide staining. A single normal (*N*) DNA fragment is present in the control lanes (○); a single mutant (*M*) fragment of identical size is present in the patient lanes (●); and two fragments identical to *N* and *M* are present in the mother's lanes (◑).

esis and expressed the mutant protein, either alone or with wild-type GPIIIa, in COS-1 cells. Recombinant proteins were then immunoprecipitated from detergent extracts of cells that had been labeled with [^{35}S]methionine and were visualized by SDS-PAGE and autofluorography. Initially, we expressed mutant and wild-type GPIIb alone in COS-1 cells and immunoprecipitated the recombinant proteins using the anti-GPIIb mAb B1B5. As shown in Fig. 5 *A*, wild-type and mutant GPIIb were each present in extracts of transfected cells, indicating that the amino acid substitution by itself does not impair GPIIb biosynthesis.

Next, we coexpressed mutant and wild-type GPIIb with GPIIIa and immunoprecipitated GPIIb–IIIa heterodimers using the heterodimer-specific mAb A2A9. Whereas GPIIb–IIIa heterodimers were immunoprecipitated from extracts of cells coexpressing GPIIIa and wild-type GPIIb, heterodimers were not immunoprecipitated from extracts of cells coexpressing GPIIIa and the GPIIb mutant (Fig. 5 *B*). An identical result

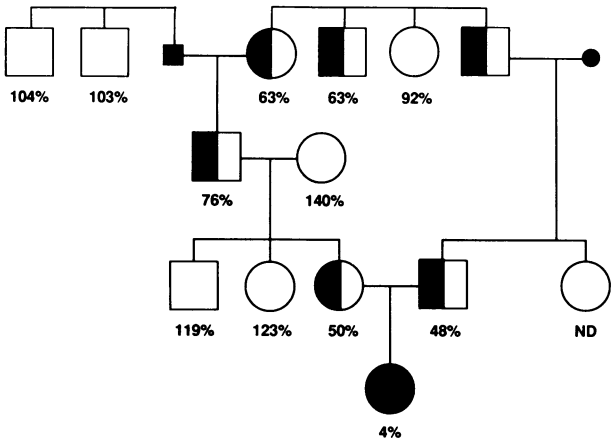

Figure 4. Inheritance of the thrombasthenic mutation in the patient's family. Allele-specific analysis of genomic DNA obtained from members of the patient's family was performed as described in Fig. 3. The results of the analysis are indicated by the shading of the symbols: ○, unaffected; ◐, heterozygote; and ●, homozygote. The GPIIb–IIIa content of each family member's platelets was also determined by flow cytometry using the anti-GPIIb mAb B1B5 as described in Fig. 1. The GPIIb–IIIa content was then compared with that of a normal control studied concurrently and is shown as a percentage of the control below each symbol.

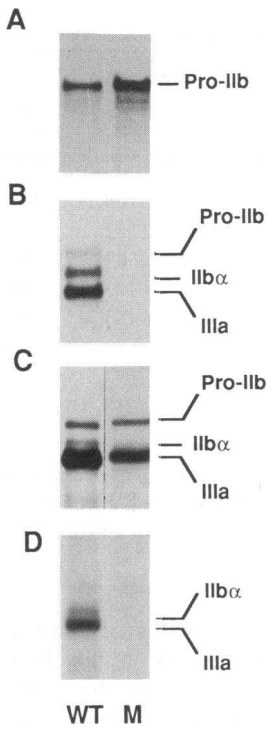

Figure 5. Expression of GPIIb containing the Gly273 → Asp mutation in COS-1 cells. cDNA for the wild-type GPIIb or for GPIIb containing the G818 → A base substitution were introduced into COS-1 cells, either alone or with a cDNA for GPIIIa. 48 h later, the cells were either metabolically labeled with [^{35}S]methionine for 60 min or surface labeled with ^{125}I. Then GPIIb or GPIIb–IIIa were immunoprecipitated from cell extracts using one of a panel of anti-GPIIb, anti-GPIIIa, or anti-GPIIb–IIIa antibodies. (*A*) Immunoprecipitate of GPIIb from metabolically labeled cells expressing wild-type or mutant GPIIb alone using the GPIIb-specific mAb B1B5. (*B*) Immunoprecipitate of GPIIb–IIIa heterodimers from metabolically labeled cells coexpressing GPIIIa and with either wild-type or mutant GPIIb using the GPIIb–IIIa-specific mAb A2A9. (*C*) Immunoprecipitate of GPIIb–IIIa heterodimers from metabolically labeled cells coexpressing GPIIIa and with either wild-type or mutant GPIIb using the GPIIIa-specific mAb SSA6. (*D*) Immunoprecipitation of GPIIb–IIIa heterodimers from surface labeled cells coexpressing GPIIIa and with either wild-type or mutant GPIIb using GPIIb-specific polyclonal antisera. Pro-IIb is the single-chain GPIIb precursor; IIbα is the GPIIb heavy chain that appears after GPIIb cleavage in the Golgi complex.

was seen when the immunoprecipitations were performed using another heterodimer-specific mAb, 10E5 (data not shown). There are three possible explanations for this result: (*a*) the GPIIb mutant may be unable to associate with GPIIIa to form heterodimers; (*b*) heterodimers do form, but are unstable and do not persist for a sufficient time to be recognized by A2A9 or 10E5; and (*c*) the GPIIb mutation alters the conformation of GPIIb–IIIa and, in the process, disrupts the epitopes for these complex-specific antibodies.

To test whether the GPIIb mutant is unable to associate with GPIIIa to form heterodimers, we repeated the cotransfections, but performed the immunoprecipitations using the anti-GPIIIa mAb SSA6 (Fig. 5 *C*). As expected, SSA6 immunoprecipitated GPIIb–IIIa heterodimers from extracts of cells coexpressing wild-type GPIIb and GPIIIa. Furthermore, a portion of these heterodimers contained GPIIb that had undergone cleavage into heavy and light chains, indicating that these heterodimers had been transported out of the ER and into the Golgi complex where GPIIb cleavage occurs (12, 13). SSA6 also immunoprecipitated GPIIb–IIIa heterodimers from extracts of cells that were coexpressing GPIIIa and the GPIIb mutant. Thus, the GPIIb mutant is able to associate with GPIIIa. However, the GPIIb in these heterodimers had not been cleaved into heavy and light chains, implying that the mutant heterodimers had not been transported from the ER to the Golgi complex. This result also implies that mutant complexes would not be present on the cell surface. To test the latter conclusion, we labeled the surface of the cotransfected cells with ^{125}I and immunoprecipitated labeled heterodimers with the polyclonal anti-GPIIb antisera. As seen in Fig. 5 *D*, labeled heterodimers were detected on the surface of cotransfected cells containing wild-type GPIIb, but not on the surface of cells containing the GPIIb mutant. Thus, the presence of a Gly273 → Asp mutation in GPIIb clearly inhibits the transport of heterodimers containing the mutant to the cell surface. This result also verifies that our in vitro expression system recapitulates the consequences of the naturally occurring GPIIb mutation.

Because wild-type GPIIb–IIIa, but not GPIIb or GPIIIa alone, is transported out of the ER (10, 11), it is possible that heterodimers containing the GPIIb mutant are unstable and dissociate before they can undergo transport. To test this possibility, we performed a pulse–chase analysis of cells coexpressing wild-type GPIIIa and either wild-type or mutant GPIIb. Transfected cells were pulse-labeled with [^{35}S]methionine for 1 h and were chased with media containing unlabeled methionine for various periods of time before GPIIb–IIIa heterodimers were immunoprecipitated using SSA6. As seen in Fig. 6, there was essentially no difference in the stability of GPIIb–IIIa heterodimers containing either the wild-type or mutant GPIIb over the course of the 8-h chase. However, as expected from results illustrated in Fig. 5, a fraction of the wild-type, but not the mutant, GPIIb in the heterodimers was cleaved into heavy and light chains. Thus, these experiments exclude heterodimer instability as an explanation for the inability of heterodimers containing the GPIIb mutant to be transported out of the ER. Consequently, they suggest that it is the altered GPIIb–IIIa conformation, induced by the GPIIb mutation, that inhibits the egress of GPIIb–IIIa from the ER to the Golgi complex.

Discussion

Glanzmann thrombasthenia results from quantitative and/or qualitative abnormalities in the platelet-membrane GPIIb–IIIa

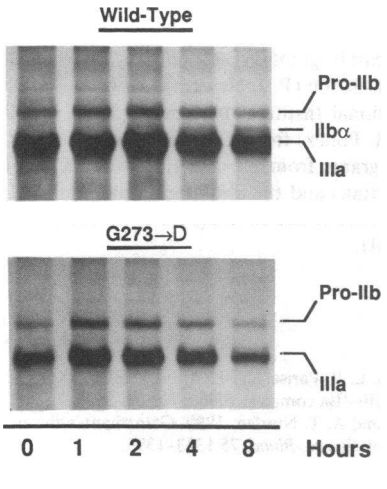

Figure 6. Pulse–chase analysis of the stability of GPIIb–IIIa heterodimers containing the GPIIb mutation G273 → D. Cotransfections of COS-1 cells were performed with a cDNA for GPIIIa and a cDNA for either the wild-type or the mutant GPIIb, as described in Fig. 5. Then the cells were pulse-labeled with [^{35}S]-methionine for 60 min and chased with media containing unlabeled methionine for various periods of time before GPIIb–IIIa heterodimers were immunoprecipitated using the GPIIIa-specific mAb SSA6.

heterodimer that preclude platelet aggregation and prevent normal primary hemostasis (2). The patient studied presented with a clinical picture consistent with thrombasthenia, a diagnosis we confirmed by finding < 5% of the normal amount of GPIIb–IIIa on the surface of her platelets. To identify the mutation or mutations responsible for the patient's disease, we focused our attention on her genes for GPIIb because of genetic and biochemical evidence that her genes for GPIIIa were normal. This evidence included the observation that the patient, the product of a consanguineous marriage, was heterozygous, rather than homozygous, for a polymorphism in intron xi of the GPIIIa gene. Thus, had she inherited the same GPIIIa mutant allele from each parent, we would have expected her to be homozygous for the polymorphism. In addition, we found that the patient's platelets expressed an increased number of αv-GPIIIa complexes on the surface of her platelets. This observation proved to be particularly useful in localizing the patient's mutation because mutations that decrease GPIIIa expression also decrease the number of αv-GPIIIa complexes on the platelet surface (19, 28). Thus, the presence of increased numbers of αv-GPIIIa complexes is incompatible with this type of GPIIIa gene abnormality.

Sequence analysis of two thirds of the coding region of the patient's GPIIb genes revealed a G → A substitution at base 818 in exon 8 (23). This base substitution converted amino acid residue 273 from glycine to aspartic acid. Applying reasoning identical to that used to eliminate GPIIIa mutations as responsible for her disease, we studied the inheritance of the G818 → A base substitution in the patient's immediate family using allele-specific PCR. These studies established that the patient was homozygous at this position and increased our confidence that G818 → A substitution was indeed pathogenic.

To confirm our presumption that the substitution of Gly273 with Asp actually impairs GPIIb–IIIa expression and therefore was responsible for the patient's thrombasthenia, we introduced the G → A base substitution into the wild-type GPIIb sequence by site-directed mutagenesis and expressed the GPIIb mutant, with and without wild-type GPIIIa, in COS-1 cells. Although the mutation did not appear to impair the biosynthesis of recombinant GPIIb, we were unable to immunoprecipitate recombinant heterodimers containing the GPIIb mutant with A2A9, a heterodimer-specific mAb that readily immunoprecipitates wild-type GPIIb–IIIa complexes (13). Because neither GPIIb nor GPIIIa appear on the platelet surface in the absence of heterodimer formation, this result suggested that the mutation might produce thrombasthenia by interfering with the process of heterodimer assembly.

A characteristic feature of integrin α subunits like GPIIb is the presence of three or four homologous repeats that are similar to domains in calcium-binding proteins such as calmodulin and troponin C and whose negatively charged amino acids are thought to be responsible for calcium binding (1). Recently, a GPIIb fragment containing its four calcium-binding domains was expressed as a bacterial fusion protein and was shown to contain two classes of calcium-binding sites with dissociation constants of 30 and 120 μM (29). Nevertheless, the role these domains play in GPIIb–IIIa structure and function is not known. Gly273 in GPIIb is located at the carboxyl-terminal end of a stretch of uncharged residues that has been highly conserved among integrin α subunits and is immediately adjacent to the first calcium-binding domain (7). Because the GPIIb–IIIa heterodimer is calcium dependent (1) and a chymotrypsin-derived amino-terminal fragment of GPIIb–IIIa containing the calcium-binding domains dissociates in the presence of calcium chelators (30), we postulated that a Gly273 → Asp mutation might interfere with heterodimer assembly by disrupting the function of the first calcium-binding domain. However, when immunoprecipitations were performed with an antibody directed against an epitope present in the carboxyl-terminal tail of GPIIIa (3), we found that the GPIIb mutant readily formed stable heterodimers with GPIIIa. This result suggests that the first calcium-binding domain, by itself, is not responsible for heterodimer formation. Moreover, in preliminary experiments, we found that removal of each of the four calcium-binding domains in GPIIb by "loop-out" mutagenesis did not prevent heterodimer assembly; although like the heterodimer containing the Gly273 → Asp mutation, the heterodimers were not transported to the cell surface (M. Kolodziej, M. Poncz, and J. S. Bennett, unpublished observations).

The inability of GPIIb–IIIa heterodimers containing the GPIIb mutant to be immunoprecipitated by either of two heterodimer-specific monoclonal antibodies suggests that the conformation of these heterodimers was altered by the point mutation in GPIIb. Although the epitopes recognized by these antibodies have not been identified, it is clear that the epitopes require the presence of an intact heterodimer (15). Furthermore, ultrastructural studies indicate that one of the antibodies, A2A9, binds to the globular head of GPIIb–IIIa composed of the amino-terminal portions of both GPIIb and GPIIIa (3). Thus, it is likely that the mutation altered the conformation of the amino terminus of GPIIb–IIIa sufficiently to impair its interaction with these antibodies.

The ER contains mechanisms that retain malfolded or improperly assembled proteins and protein complexes (31). Because we found that recombinant heterodimers containing the GPIIb mutant did not contain cleaved GPIIb, a process that occurs in a Golgi or *trans*-Golgi compartment (12, 13), it is likely that these heterodimers were retained by the ER. On the other hand, a small quantity of GPIIb–IIIa appeared to be present on the surface of the patient's platelets, suggesting that the retention mechanism for this malfolded protein in the ER may

not be absolute. The retention mechanism involves, at least in part, resident proteins termed chaperones that transiently associate with normal proteins as they acquire their correctly folded and assembled configurations (31). Chaperones form more stable associations with malfolded and incorrectly assembled proteins and consequently may be involved in their intracellular retention. One chaperone found in the ER, the heat-shock protein BiP (GRP78), associates with forms of GPIIb and GPIIIa that have been truncated just proximal to their transmembrane domains, but does not associate with the heterodimers formed by the truncated proteins (32). This suggests that either heterodimer formation occurs after GPIIb or GPIIIa have folded sufficiently to preclude their association with BiP or that heterodimer formation abrogates BiP binding. Because the Gly273 → Asp mutation appears to impair the transport of heterodimers containing the GPIIb mutant out of the ER, BiP alone is likely not responsible for their ER retention. Nevertheless, our experiments indicate that the intracellular retention of the bulk of the mutant heterodimers, a direct consequence of the GPIIb mutation, is responsible for the decreased GPIIb–IIIa expression on the surface of the patient's platelets and for her thrombasthenia. The presence of a small amount of protein corresponding to pro-GPIIb, but not GPIIbα, on immunoblots of patient platelets is consistent with this conclusion.

The identity of the GPIIb–IIIa mutations resulting in thrombasthenia has been determined in a limited number of kindreds. Six other mutations have been detected in GPIIb, five of which result in gross changes in the size of the GPIIb precursor. One mutation consists of a deletion of $\approx$ 4.5 kb of the GPIIb gene containing exons 2–9 (22), two mutations result in the deletion of mRNA splice acceptor sites and aberrant mRNA splicing (28, 33), and two mutations produce a premature termination codon in the GPIIb reading frame (33, 34). The sixth mutation, described in a preliminary report (35), is Gly418 → Asp substitution adjacent to the fourth calcium-binding domain of GPIIb. This mutation, like the mutation we have described, appears to impair the intracellular trafficking of GPIIb–IIIa. Seven different mutations have been detected in GPIIIa. One mutation results from the insertion of at least 7.2 kb into the GPIIIa gene (36), a second results from an 11-base deletion producing a frameshift and a premature termination codon (28), and a third results from the loss of a splice donor site, aberrant mRNA splicing, and a shift in the reading frame, producing a premature termination codon (37). The four remaining GPIIIa mutations consist of single amino acid substitutions and produce variant thrombasthenia in which normal or nearly normal numbers of nonfunctional GPIIb–IIIa heterodimers are expressed on the platelet surface. In one mutant, a Ser752 → Pro substitution in the cytoplasmic domain of GPIIIa prevents activation of the heterodimer (38). The three other point mutations involve the extracellular domain of GPIIIa and are the result of the replacement of a charged amino acid with an uncharged residue (Asp119 → Tyr; Arg214 → Trp/Gln) in regions of GPIIIa thought to be involved in ligand binding (39–41). The reason point mutations in GPIIIa permit normal or nearly normal GPIIb–IIIa expression while the point mutation we detected in GPIIb does not is not obvious. It is possible, however, that the substitution of a charged by an uncharged amino acid has a less deleterious effect on the overall GPIIb–IIIa conformation than does the addition of additional charge to the region of the protein likely to have a well defined secondary structure.

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