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Research Article

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Glanzmann Thrombasthenia Resulting from a Single Amino Acid Substitution between the Second and Third Calcium-binding Domains of GPIIb

Role of the GPIIb Amino Terminus in Integrin Subunit Association

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Abstract

To gain insight into regions of the platelet GPIIb-IIIa complex involved in receptor biogenesis and function, we examined the biochemical properties of a defective GPIIb-IIIa complex from patient suffering from type II Glanzmann thrombasthenia. Flow cytometric as well as immunoblot analysis of patient platelets showed significantly reduced levels of GPIIb and GPIIIa compared with a normal control. Patient platelets, however, retained the ability to retract a fibrin clot. Sequence analysis of PCR-amplified platelet GPIIb mRNA revealed an Arg₃₂₇→His amino acid substitution between the second and third calcium-binding domains of the GPIIb heavy chain, a residue that is highly conserved among integrin α -subunits. The recombinant His₃₂₇ form of GPIIb was found to be fully capable of associating with GPIIIa, therefore the role of the calcium-binding domains in intersubunit association was further examined by constructing amino-terminal segments of GPIIb that ended before the first, second, and third calcium-binding domains. All three fragments were found to associate with GPIIIa, demonstrating that the calcium-binding domains of GPIIb are not necessary for initial complex formation. Regions amino-terminal to the calcium-binding domains of GPIIb may play a heretofore unappreciated role in integrin subunit association. (*J. Clin. Invest.* 1995. 95:1553-1560.) **Key words:** integrins • Glanzmann thrombasthenia • calcium-binding domains • protein trafficking • inherited platelet defects

Introduction

In 1918, Glanzmann described a bleeding disorder that he termed "thrombasthenie," meaning "weak platelets," manifested by normal platelet counts but abnormal clot retraction (1). Platelets from thrombasthenic patients could not spread onto a glass surface (2) nor could they aggregate, indicating that the platelets were functionally defective (3, 4). Glanzmann

thrombasthenia (GT)¹ is now recognized as a rare autosomal recessive platelet disorder that is clinically characterized by lifelong mucocutaneous bleeding (5). This disease occurs in a small fraction of the population; however, a high incidence persists in confined geographical areas within certain ethnic groups in which marriage is practiced among descendants of a common ancestor (5). Thrombasthenic platelets fail to bind fibrinogen and aggregate after stimulation by physiologic agonists such as ADP, thrombin, epinephrine, or collagen (6).

GT has been subclassified based upon platelet intracellular fibrinogen content and the ability of platelets to retract a fibrin clot (7). Type I patients lack platelet fibrinogen and clot retraction, whereas type II thrombasthenic platelets contain appreciable levels of platelet fibrinogen and maintain some clot retraction capability. The basis of GT lies within an abnormality of the genes that encode the major receptor for fibrinogen, glycoproteins (GP) IIb-IIIa. GPIIb and IIIa are specifically decreased in thrombasthenic versus normal platelets (8). Type I GT patients lack detectable levels of GPIIb-IIIa, whereas type II GT platelets express moderate (10-25%) levels of GPIIb-IIIa (5). Variant forms of thrombasthenia have also been described with increasing frequency and are characterized as possessing normal to near-normal levels of a dysfunctional form of GPIIb-IIIa present on the cell surface (9-12). Platelets from type I, type II, and variant forms of GT are functionally indistinguishable in that all fail to bind the adhesive ligand fibrinogen and self-associate (aggregate) in response to platelet activation.

GPIIb-IIIa ($\alpha_{IIb}\beta_3$) is a member of the widely known receptor family of integrins, which mediate the adhesion of cells to each other and to the extracellular matrix (13). Glycoproteins IIb (145,000 M_r) and IIIa (95,000 M_r) associate in a noncovalent, calcium-dependent manner to form a complex that is present exclusively on platelets. The complex primarily mediates the interaction of activated platelets with fibrinogen during hemostasis; however, GPIIb-IIIa is also known to associate with other adhesive ligands including von Willebrand factor, fibronectin, and collagen (14, 15). Study of GPIIb-IIIa complex biosynthesis within cultured human leukemic and megakaryocytic cell lines has led to a better understanding of key steps that take place during the synthesis, assembly, and processing of integrins (16-18). GPIIb and GPIIIa subunits are synthesized within the rough endoplasmic reticulum as single-chain molecules and assembled into a pre-GPIIb-IIIa complex. Oligosaccharides, consisting mainly of high-mannose sugar moieties, are added to both GPIIb and GPIIIa within the endoplasmic reticulum, representing ~ 15 and 18% of each subunit's molec-

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1. Abbreviations used in this paper: GT, Glanzmann thrombasthenia; PRP, platelet-rich plasma.

ular mass, respectively (19). Subsequent to complex assembly, pre-GPIIb-IIIa is transported to the Golgi apparatus, where a number of posttranslational modifications occur. Pre-GPIIb is cleaved at amino acid 859 into a heavy (125,000 M_r) and light (23,000 M_r) chain (20) that remains covalently linked by a single disulfide bond formed by residues Cys₈₂₆-Cys₈₈₀ (21). The high-mannose sugars of GPIIb are converted to complex carbohydrates within the Golgi apparatus; however, most of the sugar residues on GPIIIa remain of the high-mannose type. After these processing events within the Golgi apparatus, the mature GPIIb-IIIa complex is then rapidly transported to the cell surface.

Analysis of molecular genetic defects present in GPIIb or GPIIIa of patients with GT has previously proved to be beneficial toward defining precise structural domains of integrins that play a role in the biosynthesis, subunit association, maturation, expression, and function. Thus, two regions which play a role in maturation of GPIIb-IIIa have been reported in GPIIb (22, 23), three separate mutations in GPIIIa have helped to further define regions involved in ligand binding and stability of the GPIIb-IIIa complex (24-26), and a single amino acid substitution within the cytoplasmic domain of GPIIIa helped to define a region involved in receptor activation (27). One of the purposes of this investigation was to continue to identify such regions by examining the molecular basis of naturally occurring inherited defects that result in defective surface expression and or function of integrin subunits. In this paper, we will demonstrate that a single amino acid mutation between the second and third calcium-binding domains of the integrin α subunit GPIIb slows intracellular trafficking to the cell surface, resulting in type II GT. Characterization of the biosynthetic blockade induced by this naturally occurring amino acid substitution has revealed regions of GPIIb that appear to play important structural roles in the maturation and subunit association of integrin complexes.

Methods

Patient studies. The propositus is a 14-yr-old German female, the product of a consanguineous marriage, who presented with severe hemorrhage after a tonsillectomy. The patient has a history of frequent bruising and bleeding after the shedding of her deciduous teeth. Although the patient had a normal platelet count, she had a prolonged cutaneous bleeding time > 20 min. Subsequent studies revealed absent *in vivo* platelet aggregation in response to ADP, collagen, epinephrine, and arachidonic acid, but normal platelet aggregation in response to ristocetin, consistent with a diagnosis of GT. Blood samples for the studies described below were obtained from the patient, her mother, other family members, and normal volunteers after informed consent.

Clot retraction. Whole blood was anticoagulated with ACD-A and centrifuged at 200 *g* for 10 min at room temperature to produce platelet-rich plasma (PRP). After centrifugation, only the upper two-thirds of the PRP (to reduce white cell contamination) were transferred to a second polypropylene tube. Platelets were diluted with platelet poor plasma to final concentrations of 2.5, 1.25, and 0.675 $\times 10^8$ platelets/ml. Platelet suspensions were incubated for 5 min at 37°C, then 4 μ l of 1 M CaCl₂ was added to each tube. A clot was allowed to form and contract over 1 h at room temperature. Clot formation and retraction capability of platelets from a normal control were compared with platelets from the patient (KJ) and a previously reported type I Glanzmann thrombasthenic (KW) (28).

Antibodies. The monoclonal antibody Tab, specific for GPIIb (29) was a generous gift from Dr. Rodger McEver (University of Oklahoma). The monoclonal antibody AP3, specific for GPIIIa, has been described

(30). The monoclonal antibody AP2, which recognizes an epitope on the GPIIb-IIIa complex (31), and the monoclonal antibody AP1, which recognizes an epitope on GPIb, was kindly provided by Dr. Robert R. Montgomery (Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, WI).

Flow cytometric analysis of platelet membrane glycoproteins. Whole blood from thrombasthenic patients and normal controls was anticoagulated with ACD-A, and platelets were prepared by differential centrifugation using standard techniques. For the assay, 5×10^6 platelets were aliquoted into polypropylene tubes and then incubated with fluorescein isothiocyanate-conjugated anti-GPIb antibody (AP1) (21 μ g/ml), anti-GPIIIa antibody (AP3) (26 μ g/ml), anti-GPIIb antibody (Tab) (32 μ g/ml), anti-GPIIb/IIIa antibody (AP2) (26 μ g/ml) for 1 h at room temperature in the dark. Samples were analyzed on a FACStar Plus® flow cytometer (Becton Dickinson Mountain View, CA) as previously described (32), and data were analyzed using Becton Dickinson Lysys software.

Platelet membrane glycoprotein analysis. Detergent platelet lysates from both normal and thrombasthenic individuals were prepared as previously described (33), added to the wells of a 5-15% gradient SDS-polyacrylamide gel, and electrophoresed under reducing conditions. Platelet proteins were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA), which was then incubated with rabbit polyclonal antiserum directed against GPIIb (5 μ g/ml) or GPIIIa (20 μ g/ml). Bound antibodies were detected by incubation of the membrane with goat anti-rabbit IgG conjugated to alkaline phosphatase and visualized by development with the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate pair (Sigma Immunochemical, St. Louis, MO).

Amplification of platelet messenger mRNA. Platelet messenger mRNA was isolated from 50 ml of whole blood and reverse transcribed into cDNA with a GPIIb-specific antisense oligonucleotide primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) using previously described methods (32). Overlapping PCR products were directly cloned into pGEM-5zf(+) vector (Promega Corp., Madison, WI) before nucleotide sequence analysis using the Sequenase kit (United States Biochemical Corp., Cleveland, OH).

Site-directed mutagenesis of GPIIb cDNA. The full length cDNA encoding wild-type GPIIb (generous gift of Dr. Morty Poncz, Children's Hospital of Philadelphia, Philadelphia, PA) was cloned into plasmid vector pGEM-7zf(+) (Promega Corp.). The transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA) along with two single-nucleotide mismatched primers was used to construct a GPIIb cDNA which encoded a mutated form of GPIIb. A single-nucleotide mismatched primer (5'-GAAGTGGGGCATGTGTATTTG-3') which abolishes a unique restriction site (XbaI) on pGEM-7zf(+) was used to allow positive selection of mutated clones. Single-nucleotide mismatched primer (5'-GAAGTGGGGCATGTGTATTTG-3') from base 1064 to 1084 of GPIIb was used to construct a mutant form of GPIIb. This construct containing a nucleotide substitution, as well as the wild-type GPIIb cDNA, was treated with the unique restriction enzymes BsmI and AccI (New England Biolabs, Beverly, MA). The restriction-digested mutated cartridge was exchanged for the wild-type sequence to construct the cDNA which encodes the mutated form of GPIIb found in patient KJ. The cDNA was excised from pGEM-7zf(+) with EcoRI and ligated into the mammalian expression vector EMC3 (gift of Dr. Glenn Larsen, Genetics Institute, Boston, MA). cDNAs were subjected to nucleotide sequence analysis to confirm the presence of the mutation and proper insertion of the cartridge into the wild-type GPIIb cDNA.

Synthesis of GPIIb amino-terminal fragments. Three amino-terminal fragments of GPIIb were amplified by PCR with a template of full length GPIIb cDNA within plasmid vector pGEM-7zf(+). The previously mentioned primer (5'-GAAGTGGGGCATGTGTATTTG-3') which binds to the multiple cloning region of pGEM-7zf(+) served as the 5' sense primer for each PCR fragment. The antisense primer (5'-CTAGAATTCTCATAACTCGCCACGGCCACCGAGTA-3') from nucleotides 823 to 803 served as the 3' primer for a fragment which

encoded amino acids 1–243 of GPIIb in addition to encoding three stop sequences and an EcoRI site. The antisense primer (5'-CTAGAATTC-TCATAAAGTGACAGCCACTGAATGCC-3') from nucleotides 983 to 963 served as the 3' primer for a fragment which encoded amino acids 1–296 of GPIIb in addition to encoding three stop sequences and an EcoRI site. The antisense primer (5'-CTAGAATTCATAAGCC-CAGGGGTGCGATGGCAGA-3') from nucleotides 1186 to 1166 served as the 3' primer for a fragment which encoded amino acids 1–364 of GPIIb in addition to encoding three stop sequences and an EcoRI site. Three nucleotide strands which encode GPIIb fragments were synthesized by PCR using Vent Polymerase (New England Biolabs) and treated with EcoRI (New England Biolabs) producing EcoRI sticky ends. The PCR products were then isolated with Gene Clean II kit (Bio 101, La Jolla, CA) and ligated into mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) at its EcoRI site. Single clones that encoded GPIIb fragments were treated with restriction enzymes to verify the correct insert size and orientation. Constructs were transfected into Cos-7 cells and analyzed as described below.

Immunoprecipitation analysis of GPIIb-IIIa transfected COS cells.

Cos-7 cells were transfected with GPIIb and GPIIIa expression constructs as previously described (23), cultured for 48–72 h, and then metabolically labeled with [³⁵S]methionine (600 μ Ci/plate) (NEN-DuPont, Boston, MA). Detergent lysates were prepared, precleared with protein A-Sepharose (Sigma Immunochemicals) and incubated with 10 μ g of monoclonal or 25 μ g of polyclonal antibody for 18 h at 4°C with mixing as described (23). 5 μ g of goat anti-mouse IgG was added to samples containing monoclonal antibodies of the IgG₁ subclass and incubated for an additional hour at room temperature. Beads were washed, placed in 50 μ l of 2 \times reducing buffer (4% sodium dodecyl sulfate, 10% β -mercaptoethanol, 100 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.001% bromophenol blue), boiled for 10 min, centrifuged, and the supernatants electrophoresed overnight on a 7% SDS-PAGE gel. Gels were fixed in 45% methanol, 12% acetic acid for 30 min, incubated in Enlightening (DuPont) for 30 min, dried, and exposed to Kodak XRP film for 8–20 h at –80°C.

Biotin labeling and detection of cell surface proteins. Transfected Cos-7 cells were biotin-surface labeled as described previously (23). After biotinylation, cells were solubilized in Triton X-100 and immunoprecipitated as described above. Immunoprecipitates were subjected to SDS-PAGE and transferred onto Immobilon membranes (Millipore Corp.), which were then incubated in a 1:4,000 dilution of streptavidin-horseradish peroxidase (Amersham, Arlington Heights, IL) for 1 h at room temperature. After washing, blots were incubated in detection reagents 1 and 2 (ECG kit; Amersham) for 1 min and exposed to Hyperfilm (Amersham) for up to 3 min.

Results

GPIIb-IIIa content and function of patient KJ's platelets. Platelets from Glanzmann thrombasthenic patient KJ were examined by flow cytometry for the expression of GPIIb-IIIa (see Methods). Platelets were isolated and incubated with FITC-conjugated antibodies specific for GPIb (AP-1), GPIIIa (AP3), GPIIb (Tab), or the GPIIb-IIIa complex (AP2) (Fig. 1). Platelets from all individuals examined expressed similar levels of GPIb (Fig. 1, A and B, upper left); however, antibodies specific for GPIIb, GPIIIa, and the GPIIb-IIIa complex demonstrated that patient KJ expressed only 7–10% of the normal content of GPIIb-IIIa on the platelet surface. Her platelet GPIIb-IIIa surface expression was higher than that of type I GT patient KW (Fig. 1 A), while her mother, EJ, expressed 55% GPIIb-IIIa, consistent with her status as a carrier of thrombasthenia (Fig. 1 B). Based upon these observations, we provisionally classified KJ as a type II thrombasthenic.

To confirm the type II Glanzmann phenotype, KJ's platelets were further examined for their ability to retract a fibrin clot.

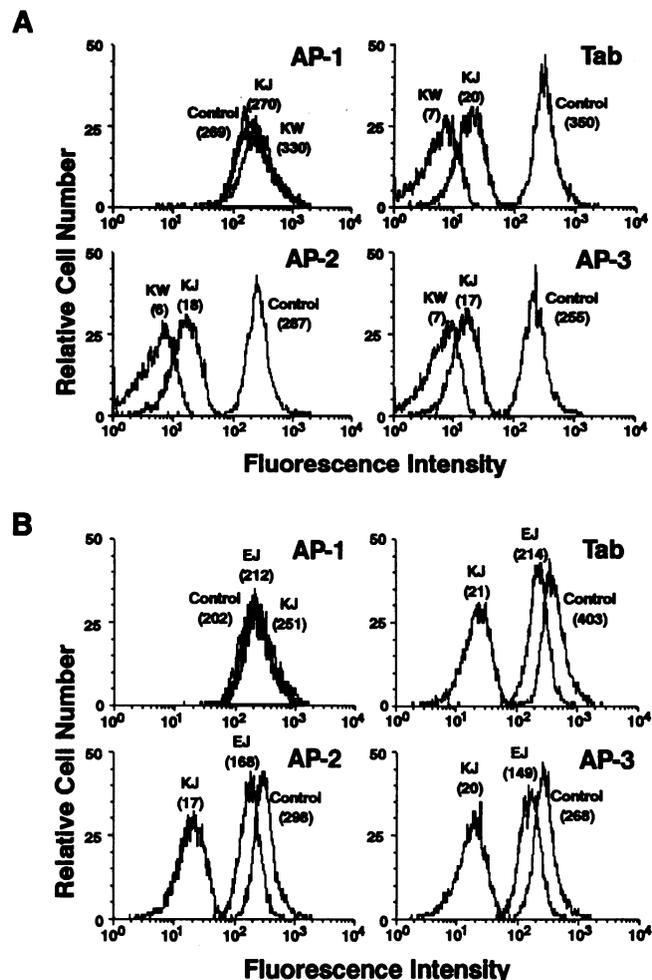


Figure 1. Flow cytometric analysis of platelet membrane glycoproteins. Platelets from Glanzmann thrombasthenic patients KJ and KW, KJ's mother (EJ), and a normal control were resuspended in Ringer's citrate dextrose buffer, pH 6.5, at 5×10^6 platelets per ml, and then incubated with FITC-labeled anti-GPIb antibody (AP1), anti-GPIIIa antibody (AP3), anti-GPIIb antibody (Tab), or anti-GPIIb/IIIa antibody (AP2) as described in Methods. Samples were analyzed on a FACStar Plus® flow cytometer, and the data were analyzed with Becton Dickinson Lysis software. Fluorescence intensities were converted to arbitrary linear units shown above each histogram. A compares the relative level of platelet surface protein from a normal control, type I Glanzmann thrombasthenic patient KW, and type II Glanzmann thrombasthenic patient KJ. B compares the relative level of platelet surface protein from a normal control, patient KJ, and KJ's mother, EJ, an obligate carrier for thrombasthenia.

Platelets were isolated, serially diluted with platelet poor plasma, and treated with calcium chloride at room temperature for 1 h (see Methods). As shown in Fig. 2, platelets from type I patient KW (28) lacked the ability to retract a fibrin clot (Fig. 2, right) compared with the normal control, which retracts the fibrin clot appreciably (Fig. 2, left). Platelets from patient KJ were found to maintain some clot retraction capability (Fig. 2, middle). These data support KJ's classification as a type II Glanzmann thrombasthenic patient resulting from a defective GPIIb or GPIIIa subunit.

To investigate the molecular basis for thrombasthenia in this patient, KJ's platelets were next examined for total GPIIb-

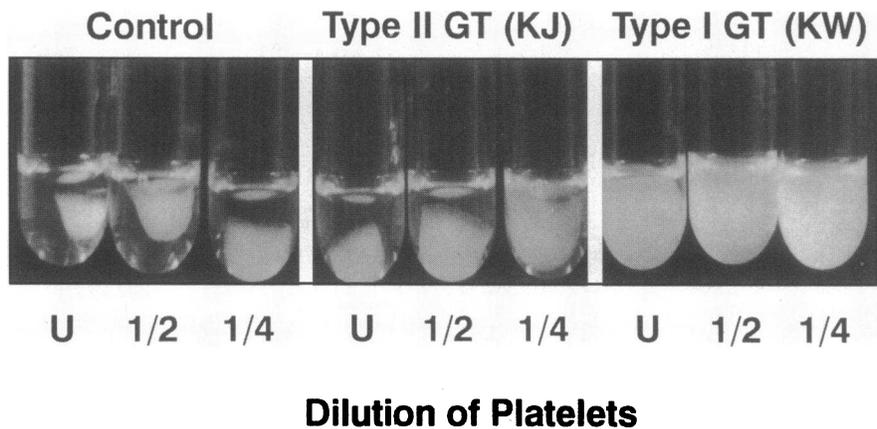


Figure 2. Clot retraction of types I and II thrombasthenic platelets. Whole blood from type I GT patient KW, type II GT patient KJ, and a normal control were anticoagulated with ACD-A and centrifuged at 200 g for 10 min at room temperature to produce PRP. For sample (U), platelets were diluted with platelet poor plasma to a final concentration of 2.5×10^8 platelets/ml. Platelets were further diluted to one-half and one-fourth this concentration as indicated to make the assay semiquantitative. Clots were photographed 1 h after addition of CaCl_2 . Note that whereas type I GT platelets failed to retract the fibrin clot, KJ's platelets were ~50% effective.

IIIa content. Immunoblot analysis (Fig. 3) demonstrated that the patient's mother's (EJ) platelet lysates contained ~50% levels of glycoprotein IIb, while platelets from KJ contained little detectable GPIIb. In contrast the patient (KJ) did express a small amount of GPIIIa at increasing concentrations of platelet protein, presumably as part of the vitronectin receptor ($\alpha_v\beta_3$) complex. Based upon these data, we examined the nucleotide sequence of the GPIIb mRNA transcript from this patient for a molecular genetic defect.

Analysis of (KJ) GPIIb mRNA. Platelet messenger RNA was isolated and reverse transcribed into cDNA using a GPIIb-specific antisense nucleotide primer (see Methods). Four overlapping fragments which spanned the entire length of GPIIb cDNA were amplified by PCR. Secondary PCR products were

synthesized using nested primers, producing a total of 10 fragments of GPIIb cDNA. Examination of the nucleotide sequences of these GPIIb PCR products revealed a single $\text{G}_{1074} \rightarrow \text{A}$ nucleotide substitution in KJ's GPIIb cDNA (Fig. 4). This results in the transformation of Arg_{327} to His in the GPIIb polypeptide chain. Alignment of this region of GPIIb with all other known integrin α -subunits from *Drosophila* to humans revealed the presence of an arginine or glutamine at this relative position between the second and third cation binding domains of integrin α -subunits (Table I). The substitution of histidine conserves a positive charge at this position, however an additional steric constraint is placed upon the GPIIb subunit which may have an effect on the processing or ligand binding capability of the GPIIb-IIIa complex.

Effect of $\text{Arg}_{327} \rightarrow \text{His}$ substitution on biogenesis of the GPIIb-IIIa complex. To determine whether the $\text{Arg}_{327} \rightarrow \text{His}$ mutation of GPIIb represents a neutral polymorphism, or an altered structural region which is responsible for the reduced level of GPIIb-IIIa on the platelet surface in patient KJ, an expression vector encoding the His_{327} form of GPIIb was constructed (see Methods) and cotransfected with a vector encoding wild-type GPIIIa into Cos-7 cells. Transfected Cos-7 cells were surface labeled with biotin 72 h after transfection and then subjected to immunoprecipitation analysis using antibodies directed against glycoproteins IIb and IIIa. As shown in Fig. 5, the His_{327} GPIIb-IIIa complex was detectable on the cell surface, but at greatly reduced levels relative to the wild-type Arg_{327} GPIIb-IIIa com-

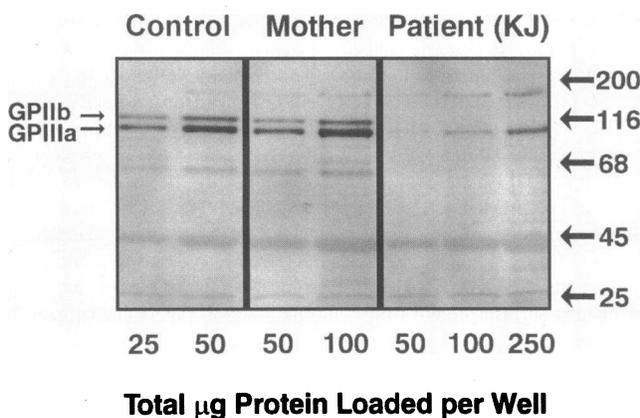


Figure 3. Semiquantitative immunoblot analysis of platelet GPIIb-IIIa content. Detergent lysates of whole platelets were prepared from a normal control, the patient's mother (EJ), and the patient (KJ). 25–250 μg total protein was loaded onto a 5–15% PAGE gel and electrophoresed under reducing conditions. After transfer to Immobilon membranes, the blot was incubated with a mixture of rabbit polyclonal antibodies specific for GPIIb (5 $\mu\text{g}/\text{ml}$) and GPIIIa (20 $\mu\text{g}/\text{ml}$). Immunoreactive bands were detected as described in Methods. The band at 190,000 M, is thought to represent GPIIIa dimer and is most prominent in the patient lysate. From these data, patient KJ appears to express ~10–15% of GPIIIa, while his mother's platelets contain 50–60% levels. The absence of GPIIb after detergent solubilization differs from the results seen by flow cytometric analysis of intact platelets (Fig. 1) and is likely due to an instability in the GPIIb subunit induced by a mutation. Molecular mass markers are to the right in kilodaltons.

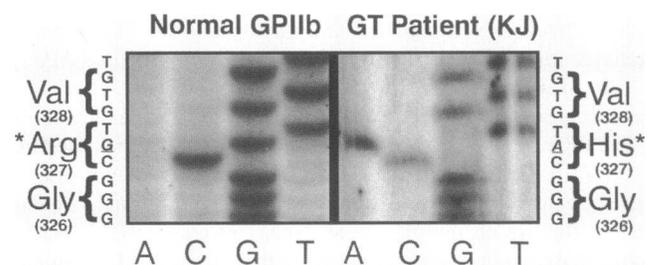


Figure 4. Nucleotide sequence analysis of KJ versus normal GPIIb. Platelet mRNA PCR was used to amplify overlapping segments of the GPIIb mRNA transcript, and the resulting cDNA fragments were subjected to DNA sequence analysis. Nucleotides 1070–1079 of GPIIb, which encode amino acids 326–328, are shown and illustrate the single $\text{G} \rightarrow \text{A}$ point mutation that results from substitution of a His for Arg_{327} .

Table 1. Comparison of α_{IIb} Amino Acid Sequence to Other Integrin α -Subunits

β -Subunit	α -Subunit	α -Subunit amino acid sequence			
β_1	α_2	EEG	R	VYLF	
		EEG	R	VFVY	
	<i>Drosophila</i> α_{PS2}	α_4	EVG	R	VYVY
		α_5	DVG	R	VYIL
		α_L	RGG	R	VFIY
β_2	α_M	RGG	Q	V SVC	
		RGG	Q	V SVC	
	α_X	RGG	Q	V SVC	
β_3	α_V	EVG	Q	V SVS	
	Rodent α_{IIb}	EVG	R	VYLF	
	Human α_{IIb}	EVG	R	VYLF	
	(KJ) α_{IIb}	EVG	H	VYLF	

plex. These data demonstrate that this single amino acid substitution in GPIIb is sufficient to reduce surface expression of GPIIb–IIIa, consistent with the type II Glanzmann phenotype.

To examine the effects of this mutation on the ability of GPIIb and GPIIIa to form a heterodimer complex, both wild-type and mutant forms of GPIIb cDNA were cotransfected with GPIIIa cDNA, pulsed with [³⁵S]methionine for 9 h, and immunoprecipitated using the GPIIIa-specific monoclonal antibody,

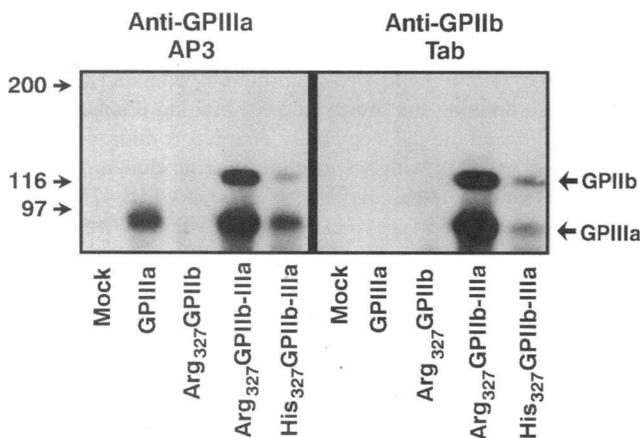


Figure 5. Immunoprecipitation analysis of biotin surface-labeled transfected Cos-7 cells. Cos-7 cells were transfected with the indicated expression plasmid constructs and after a 72-h incubation period were surface labeled with NHS-biotin. Immunoprecipitation using the subunit-specific antibodies, Tab or AP3, revealed that substitution of a single histidine residue for the wild-type arginine at amino acid 327 of GPIIb resulted in a decreased level of surface-expressed GPIIb–IIIa, consistent with the thrombasthenic phenotype of the patient's platelets. Identical results were obtained using polyclonal anti-GPIIb or GPIIIa antibodies (not shown). Molecular mass markers are to the left in kilodaltons. The band at 95 kD immunoprecipitated by AP3 in the GPIIIa transfection likely represents recombinant GPIIIa that became surface expressed as a result of its complexing with endogenous Cos cell vitronectin receptor α -subunit, as previously observed by others (43).

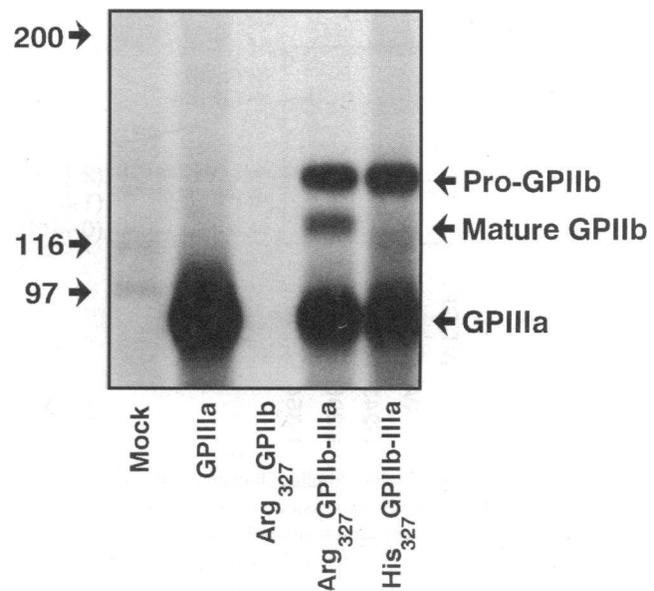


Figure 6. Maturation of wild-type versus His₃₂₇ GPIIb–IIIa complexes. Cos-7 cells were cotransfected with cDNA encoding GPIIIa and either the Arg₃₂₇ (wild-type) or His₃₂₇ (mutant) form of GPIIb. After a 9-h pulse with [³⁵S]methionine, total cellular lysates were prepared, and subunit association was assessed by immunoprecipitation with the anti-GPIIIa-specific monoclonal antibody, AP3. Immunoprecipitates were analyzed by fluorography of a 7% acrylamide gel under reducing conditions. Coprecipitation of either pro-GPIIb or mature GPIIb indicates that complex formation has taken place. Note the ability of the His₃₂₇ form of pro-GPIIb to complex with GPIIIa, but its failure to traffic efficiently to the Golgi apparatus and become cleaved into heavy and light chains. Mock denotes vector without insert as a negative control. Molecular mass markers are to the left in kilodaltons.

AP3. As shown in Fig. 6, both the precursor and mature forms of wild-type Arg₃₂₇ GPIIb coprecipitated, confirming their association with GPIIIa. The presence of mature Arg₃₂₇ GPIIb in the immunoprecipitate is indicative that some of the complex had proceeded to the Golgi apparatus, where cleavage of pro-GPIIb into heavy and light chains has taken place. The His₃₂₇ form of pro-GPIIb also associated with GPIIIa, as evidenced by the coimmunoprecipitation of both subunits by AP3, but the immunoprecipitates contained reduced to nondetectable levels of the mature His₃₂₇ GPIIb heavy chain, demonstrating that the Arg₃₂₇→His mutation of GPIIb inhibited the kinetics of processing of this receptor as it traffics to the cell surface.

Role of the calcium-binding domains in GPIIb–IIIa association. Patient KJ represents the third Glanzmann patient to be characterized as having a missense mutation within or immediately proximal to one of the calcium-binding domains of GPIIb (22, 23). Interestingly, despite the importance ascribed to calcium ions in supporting the formation and integrity of the GPIIb–IIIa complex (34–37), subunit association appears to be unaffected by mutations in either the first (22), second/third (this paper), or fourth calcium-binding domains of GPIIb (23). To further examine the role of the GPIIb calcium-binding domains in subunit association, we constructed recombinant GPIIb fragments that contained the signal peptide and amino terminus, but ended before the first (residues 1–243), second (residues 1–296), or third (residues 1–364) calcium-binding domains. After cotransfection with full length GPIIIa into Cos-7 cells,

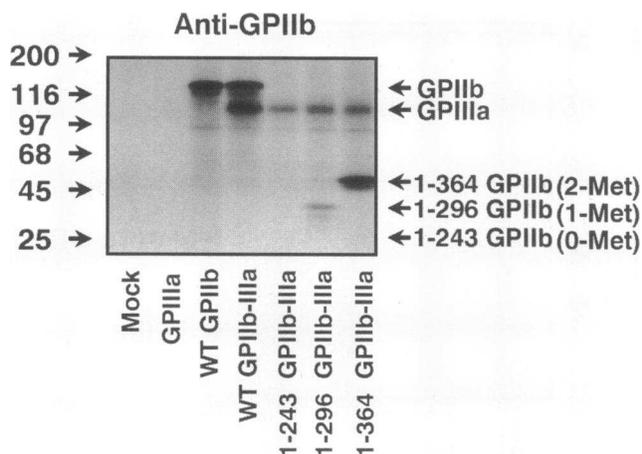


Figure 7. Role of GPIIb calcium-binding domains in the association of GPIIb with GPIIIa. Eukaryotic expression vectors encoding GPIIb fragments ending before the first (amino acids 1–243), second (residues 1–296), and third (residues 1–364) calcium-binding domains were constructed and cotransfected with a vector encoding full length GPIIIa into Cos-7 cells. Newly synthesized proteins were metabolically labeled with [³⁵S]methionine, immunoprecipitated with a polyclonal antibody directed against GPIIb, and analyzed by SDS-PAGE and autoradiography under reducing conditions. Coprecipitation of GPIIIa by the GPIIb-specific polyclonal antibody indicates that association between GPIIb and GPIIIa has taken place. Vector without insert (*Mock*), GPIIIa transfected alone, and full length GPIIb transfected alone served as controls for antibody specificity. Note that GPIIIa was coprecipitated with each fragment of GPIIb (1–243, 1–296, 1–364). Fragments 1–243, 1–296, and 1–364 contain 0, 1, and 2 methionines, respectively, which are available to metabolic label; therefore the GPIIb fragments are detected at different intensities. Coprecipitation of the GPIIb fragments was also found using antibodies directed against GPIIIa (not shown). Molecular mass markers are to the left in kilodaltons.

newly synthesized proteins were metabolically labeled with [³⁵S]methionine for 9 h and immunoprecipitated with a polyclonal antibody directed against GPIIb. As shown in Fig. 7, when full length GPIIb was cotransfected with GPIIIa, both proteins were immunoprecipitated by the GPIIb-specific antibody, indicating that subunit association between GPIIb and GPIIIa had taken place. Somewhat unexpectedly, GPIIIa was also coprecipitated with each of the truncated fragments of GPIIb, including the one that ended before the first calcium-binding domain. Fragments 1–243, 1–296, and 1–364 contain 0, 1, and 2 methionines, respectively, that are available to metabolic label; therefore, the GPIIb fragments are detected at different intensities. Nonetheless, nearly equal amounts of GPIIIa were immunoprecipitated in each instance, indicating that each GPIIb fragment quantitatively associated with GPIIIa. These data indicate that calcium-binding domains may not be necessary for initial subunit association of GPIIb with GPIIIa and may in part explain our observations that a single amino mutation between the second and third calcium-binding domains did not affect formation of the pro-GPIIb–GPIIIa complex.

Discussion

We report the first characterization of a molecular genetic defect resulting in type II GT. Classification as type II GT was a result of detecting > 5% GPIIb–IIIa on KJ's platelets by flow

cytometry (Fig. 1) and due to the ability of KJ's platelets to partially retract a fibrin clot (Fig. 2). Western blot analysis of KJ's platelet protein revealed a substantial reduction in the level of GPIIb and GPIIIa (Fig. 3), with GPIIIa present in slightly greater amounts, presumably due to its additional association with the vitronectin receptor α -subunit ($\alpha_v\beta_3$). Based upon these results, we examined the nucleotide sequence of the mRNA transcript encoding GPIIb and found a single G1074A nucleotide substitution that resulted in an Arg₃₂₇→His amino acid substitution (Fig. 4) between the second and third calcium-binding domains of GPIIb. The conformational constraints placed upon this region of GPIIb by this mutation conceivably could have multiple effects on receptor function in terms of subunit stability, calcium-mediated subunit association, or ligand binding ability. Interestingly, residue 327 is positioned only 13 amino acids away from the region on GPIIb shown to interact with the γ -chain dodecapeptide of fibrinogen (38). Moreover, the presence of an arginine at this location is highly conserved among other integrin α -subunits (Table I), suggesting an important role for this residue in integrin structure or function.

We determined that the Arg₃₂₇→His amino acid substitution in GPIIb was not a naturally occurring neutral polymorphism of GPIIb, but instead was the defect that resulted in GT in patient KJ. Mammalian expression vectors encoding GPIIIa and the His₃₂₇ form of GPIIb were constructed and cotransfected into Cos-7 cells. Biotin-labeling of cell surface proteins followed by immunoprecipitation analysis demonstrated that substitution of a single histidine residue resulted in a highly reduced level of GPIIb–IIIa on the Cos-7 cell surface (Fig. 5), consistent with the thrombasthenic phenotype resulting in defective platelet aggregation.

Recently, two other thrombasthenic patients have been described that harbor single amino acid substitutions within calcium-binding domains of GPIIb. Interestingly, both mutations (Gly₂₇₃→Asp within the first calcium-binding domain [22], and Gly₄₁₈→Asp flanking the fourth calcium-binding domain [23]) introduced an additional negatively charged residue proximal to aspartate residues within a calcium-binding domain, and in each case resulted in the complete absence of GPIIb–IIIa from the cell surface, i.e., type I GT. In contrast, the Arg₃₂₇→His mutation in type II GT patient KJ preserved an existing positive charge and resulted in only a partial biosynthetic blockade in the maturation of the integrin GPIIb–IIIa complex. Ligand binding functions of the residual complex that reaches the cell surface appear to be relatively unaffected by the nature of this mutation, as evidenced by the ability of KJ's platelets to retract a fibrin clot, which is preceded by fibrin interactions with GPIIb–IIIa (39). Residual $\alpha_v\beta_3$ does not appear to be capable of mediating clot retraction, since platelets from type I patient KW, who also has a normal complement of $\alpha_v\beta_3$, failed to retract a fibrin clot (Fig. 2).

It has been recognized for more than 10 yr that the GPIIb–IIIa complex requires the presence of calcium to maintain subunit association (34–37), and it has recently been shown that optimal fibrinogen binding occurs when each of the four calcium-binding sites are occupied (40). The precise relationship, if any, between the role of calcium in maintaining subunit association versus its role in mediating ligand binding to the complex is as yet unclear. The opportunity to analyze the effects of the three unique mutations described above that result in GT, each proximal to one of the calcium-binding domains of GPIIb,

has enabled us to begin to make some observations regarding the role of these domains in integrin biogenesis and function. In each case, substitution for a highly conserved amino acid within or proximal to a calcium-binding domain did not affect association of pro-GPIIb with GPIIIa, though downstream effects on maturation of the GPIIb-IIIa complex ultimately led to the thrombasthenic phenotype. These data suggest that a region(s) *outside* the calcium-binding domain may participate, at least early on, in subunit association. Interestingly Rosa and McEver (18) have already shown that the pro-GPIIb-IIIa complex is less easily dissociable by EDTA treatment than is mature GPIIb-IIIa, supporting the notion that the association of pro-GPIIb with GPIIIa that occurs during early biogenesis may be calcium independent.

To test this hypothesis, we constructed three overlapping amino terminal fragments of GPIIb containing none, one, or two calcium-binding domains and examined their interaction and association with GPIIIa. All three fragments, including one that contained no calcium-binding domains (residues 1-243), were able to associate with GPIIIa (Fig. 7). These data demonstrate that the calcium-binding domains of GPIIb are not necessary for initial association of GPIIb with GPIIIa and provide a possible explanation for the observation that amino acid substitutions at or near calcium-binding domains of GPIIb do not affect formation of the pro-GPIIb-GPIIIa complex.

Several other investigators have examined the structural requirements for integrin subunit association. Lam et al. (41) used mild reduction to dissociate the heavy chain of GPIIb from the light chain and showed that the heavy chain was still complexed with GPIIIa. Calvete and colleagues (42) used limited proteolysis of isolated heterodimers followed by protein-chemical analysis of the resulting peptide fragments to identify a number of putative contact sites between GPIIb and GPIIIa. Interestingly, these authors were able to deduce that three regions of the GPIIb heavy chain were involved in intersubunit interactions: amino acids 486-553, 696-734, and 780-814. They also proposed that residues 901-946, located within the GPIIb light chain, formed a contact site with GPIIIa. They found no evidence that the amino terminus of GPIIb interacted with GPIIIa, however, most probably due to the fact that this region is highly susceptible to proteolytic degradation, and is therefore not amenable to analysis using their approach. Finally, Wippler et al. (43) constructed and expressed in baculovirus truncated subunits consisting of GPIIb 1-874 and GPIIIa 1-469 and showed that these residues were sufficient to form a heterodimer complex and bind ligand. Our studies further showing that subunit association may require only residues 1-243 help to further define the domains involved in noncovalent intersubunit interactions and provide an additional example of the unique contributions that can be provided through detailed examination of the biochemical consequences of naturally occurring inherited platelet defects.

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