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A 13-bp deletion in α_{IIb} gene is a founder mutation that predominates in Palestinian-Arab patients with Glanzmann thrombasthenia

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Summary

Glanzmann thrombasthenia (GT) is a rare autosomal recessive bleeding disorder caused by lack or dysfunction of $\alpha_{IIb}\beta_3$ in platelets. GT is relatively frequent in highly inbred populations. We previously identified a 13-bp deletion in the α_{IIb} gene that causes in-frame deletion of six amino acids in three Palestinian GT patients. In this study, we determined the molecular basis of GT in all known Palestinian patients, examined whether Jordanian patients harbor the same mutations, analyzed whether there is a founder effect for the 13-bp deletion, and determined the mechanism by which the 13-bp deletion abolishes $\alpha_{IIb}\beta_3$ surface expression. Of 11 unrelated Palestinian patients, eight were homozygous for the 13-bp deletion that displayed common ancestry by haplotype analysis, and was estimated to have occurred 300–600 years ago. Expression studies in baby hamster kidney cells showed that substitution of Cys107 or Trp110 located within the deletion caused defective $\alpha_{IIb}\beta_3$ maturation. Substitution of Trp110, but not of Cys107, prevented fibrinogen binding. The other Palestinian patients harbored three novel mutations: G2374 deletion in α_{IIb} gene, TT1616-7 deletion in β_3 gene, and IVS14:−3C → G in β_3 gene. The latter mutation caused cryptic splicing predicting an extended cytoplasmic tail of β_3 and was expressed as dysfunctional $\alpha_{IIb}\beta_3$. None of 15 unrelated Jordanian patients carried any of the described mutations.

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Author contributions

The roles of each one of the authors of this paper were as follows:

N. Rosenberg, design of the study, performance of most of the experiment, interpretation of data and writing; H. Hauschner, design and performance of mutagenesis, expression of mutations and fibrinogen binding to BHK cells; H. Peretz, design of the study, detection of one mutation and interpretation of data; R. Mor-Cohen, analysis of the founder effect and aging of the mutation; M. Landau, analysis of the crystal structure and modeling of the mutated proteins; B. Shenkman, design and performance the fibrinogen binding assay; G. Kenet, diagnosis of GT patients in four families, compiling clinical data and blood sampling; B.S. Coller, design of the study, interpretation and critical review of the paper; A.A. Awidi, diagnosis of GT patients in 15 families in Jordan, collection of samples and critical review of the paper; U. Seligsohn, design of the study, interpretation of data and writing of the manuscript.

Keywords

founder effect; integrin $\alpha_{IIb}\beta_3$; variant Glanzmann thrombasthenia

Introduction

Glanzmann thrombasthenia (GT) is characterized by a moderate to severe mucocutaneous bleeding tendency, a normal platelet count, absence or reduced clot retraction, and absence or severely reduced platelet aggregation in response to all agonists except for ristocetin. The disease is inherited in an autosomal recessive fashion and is caused by lack or dysfunction of platelet $\alpha_{IIb}\beta_3$ that normally functions as a receptor for fibrinogen and von Willebrand factor and is essential for platelet aggregation. GT is rare worldwide but relatively frequent in inbred populations such as Iraqi-Jews [1], Indians [2], French Gypsies [3], Arabs in Jordan [4], Israel [5] and Saudi Arabia [6]. Many mutations in the α_{IIb} or β_3 genes have been identified in patients with GT including minor or major deletions, insertions, inversions and mostly point mutations located throughout both genes (for further information see database http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu). Founder effects were discerned for mutations identified in Iraqi-Jews [7] and French Gypsies [8]. We have previously identified a 13-bp deletion at the intron 3–exon 4 junction of the α_{IIb} gene in three Palestinian-Arab kindreds with members affected by GT. The deletion was shown to give rise to alternative splicing predicting an in-frame deletion of six amino acids [5]. The six deleted residues (Ala106-Gln111) are located in the second blade of the β-propeller domain, which interfaces with the βA domain of β_3 through interaction of Trp110 of α_{IIb} with Arg261 of β_3 [9,10]. Among the deleted residues, Cys 107 was suspected to be critical for normal folding of α_{IIb} and $\alpha_{IIb}\beta_3$ complex formation [5].

In this study, we examined whether the 13-bp deletion is also present in other unrelated Palestinian-Arab patients residing in Israel and the Palestinian territories or Jordanian-Arabs, and whether the mutation stems from a common founder. We also studied the mechanism by which the 13-bp deletion abolishes $\alpha_{IIb}\beta_3$ surface expression, as well as the molecular basis of GT in Palestinian patients not bearing the 13-bp deletion.

Methods

Patients

Eight Palestinian-Arab patients (four residing in Israel and four in the Palestinian territories) who did not know of any family connection to each other and who had lifelong mucocutaneous bleeding were referred to the Chaim Sheba Medical Center in Israel for evaluation between 1991 and 2004. Six were males and two were females whose age ranged between 3–29 years. Three previously described probands bearing the 13-bp deletion in the α_{IIb} gene were also studied [5]. Ten of the 11 Palestinian probands were offspring of related parents. The diagnosis of GT patients was based on a normal platelet count, absent or minimal clot retraction, absent platelet aggregation in response to adenosine diphosphate (ADP), epinephrine, and collagen, and normal ristocetin-induced platelet aggregation. DNA samples of Jordanian GT patients belonging to 15 families were sent for analysis from the Rawhi Medical Center in Amman or from Children's Mercy Hospital in Kansas City, MI by Dr Michael L. Begleiter. Control Palestinian-Arabs were patients consecutively admitted to the Chaim Sheba Medical Center in Israel. Approval of the study was obtained from the Institutional Review Board of the Chaim Sheba Medical Center.

Blood sampling and processing

Blood was taken in citrated buffer or in ethylenediaminetetra-acetic acid (EDTA) from the patients and healthy Arab controls. Citrated platelet-rich plasma (PRP) was used for flow cytometry and for immunoblot analysis of lysed platelets. DNA extraction from white blood cells was carried out in blood samples containing EDTA. RNA was extracted from washed platelets prepared from PRP using QuickPrep mRNA purification kit (Pharmacia Biotech, Piscataway, NJ, USA) and first strand cDNA was reverse transcribed using the reverse transcription system (Promega, Madison, WI, USA).

Flow cytometric analysis of platelets

A platelet suspension [20 μL of 200 000 cells/μL in phosphate buffered saline (PBS)] was incubated with fluorescein-isothiocyanate (FITC)-conjugated monoclonal antibodies against glycoprotein (GP) Ibα (CD42b; Dako, Glostrup, Denmark), $\alpha_{IIb}\beta_3$ (CD41/P2; Immunotech, Marseille, France), or $\alpha_v\beta_3$ (LM609; Chemicon, Temecula, CA, USA). Following incubation for 30 min at room temperature, platelets were washed with PBS and analyzed for cell-bound fluorescence in a flow cytometer (Coulter; EPICS, Luton, UK). The background was measured using FITC conjugated anti-mouse IgG.

Fibrinogen binding to resting and activated platelets

Diluted PRP (4 μL in 100 μL HEPES-buffered saline) was incubated for 2 min with 1.25 μM ADP (Dia-Med, Cressier sur Mora, Switzerland), 1.25 μg mL^{-1} collagen (Helena BioScience, Sunderland, UK), or with no agonist, and then 10 μL FITC-conjugated rabbit anti-human fibrinogen (Dako) was added. After 20 min, platelets were washed with HEPES-buffered saline and analyzed for cell-bound fluorescence in the flow cytometer. Non-specific binding was measured using FITC-conjugated anti mouse IgG.

Immunoblot analysis of platelet lysates

PRP was centrifuged at 14 000 g for 10 min and platelet pellets were lysed at 100 °C with loading buffer [40 mM Tris–HCl, pH 6.8, 1.5% sodium dodecyl sulphate (SDS), 8% glycerol, 1 mM 1,4-dithio-threitol (DTT), 0.01% bromophenol blue]. Samples were electrophoresed on NuPAGE Novex 3–8% Tris–Acetate gels (Invitrogen, Paisley, UK) and transferred to a polyvinyl-idenedifluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was immersed in Tris-buffered saline with 0.05% tween-20 (TBST) containing 1:1000 dilution of polyclonal antibody against α_{IIb} [11] and polyclonal antibody against β_3 (Chemicon), and then washed with TBST and immersed in TBST containing 1:2000 horseradish peroxidase conjugated-anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA). Immunoreactive bands on the membrane were detected using an ECL immunoluminescence kit (Amersham, Buckinghamshire, UK) and X-ray film exposure.

Immunoprecipitation of the $\alpha_{IIb}\beta_3$ complex in platelet lysates was carried out by incubation of 10 μg 10E5 monoclonal antibody against the $\alpha_{IIb}\beta_3$ in lysis buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonident P40, 1% deoxycholic acid, and complete TM protease inhibitor mix (Roche Diagnosis, Mannheim, Germany)], followed by goat anti-mouse IgG-Agarose (Sigma-Aldrich, St Louis, MO, USA) as previously described [12]. The samples were electrophoresed in NuPAGE Novex 3–8% Tris–Acetate gels (Invitrogen) under non-reducing conditions; after transfer to PVDF membrane, proteins were detected by immunoblot analysis using monoclonal antibodies against α_{IIb} (SZ22; Immunotech) and β_3 (AP3, GTI, Brookfield, WI, USA) followed by horseradish peroxidase conjugate-anti mouse IgG (Jackson).

Mutation identification and analysis of polymorphisms

DNA samples of each patient were examined for the previously described 13-bp deletion in the α_{IIb} gene [5] using direct polymerized chain reaction (PCR) amplification [13]. DNA fragments containing exons and flanking intronic regions of the α_{IIb} and β_3 genes were prepared by PCR using primers previously described [14,15]. The amplified fragments were directly sequenced by an automatic sequencer. Alternatively, the PCR products were first subjected to single-strand conformation polymorphism (SSCP) analysis, and when the SSCP revealed an abnormal fragment it was sequenced using the fmol DNA sequencing system (Promega).

For determination of a possible founder effect, haplotype analysis was performed using polymorphisms in the region of the α_{IIb} and β_3 genes on chromosome 17. These polymorphisms included microsatelite markers of the THRA1, BRCA1, and D17S579 loci that are adjacent to α_{IIb} and β_3 genes, and polymorphic sites within the α_{IIb} (T2621G; HPA3) and β_3 (CT repeats in IVS6 and A1545G; *Sma*I) genes [16].

Construction of expression vectors for mutant cDNAs

cDNAs of α_{IIb} or β_3 in pcDNA3 vector were generously provided by Dr Peter Newman (The Blood Center of Southeastern Wisconsin, Milwaukee, USA). A PCR fragment containing exons 1–7 of α_{IIb} cDNA (nucleotides 4–800) was amplified from the cDNA of an Arab patient who carried the known 13-bp deletion by using a forward primer: 5′-GCCAGAGCTTTGTGTCCACTGC-3′ and a reverse primer: 5′-CCCAGTAGCCGTCGAAGTAC-3′. The PCR product containing the mutation was digested with NotI and SacII restriction enzymes and cloned into pcDNA3/IIb digested with the same restriction enzymes replacing the corresponding wild-type (WT) cDNA fragment. Cys107 or Trp110, located within the six-amino acid deletion, were substituted by Ser and Ala, respectively with QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using pcDNA3/IIb as a template. Two overlapping oligonucleotide primers containing one or two base pair substitutions (underlined) were used: forward primer 5′-GTCATTGTGGCCTCCGCCCCCTGGCAG-3′ and reverse primer 5′-GCTGCCAGGGGGCGGAGGCCACAATGAC-3′ for the Cys107Ser substitution, or forward primer 5′-CCTGCGCCCCCGCGCAGCACTGGAACG-3′ and reverse primer 5′-CGTTCCAGTGCTGCGCGGGGGCGCAGG-3′ for the Trp110Ala substitution. WT β_3 cDNA was excised from pcDNA3/β_3 by *Eco*RI, filled in to create blunt ends, and subcloned into the *Pvu*II site of pCEP4 mammalian expression vector carrying the hygromycin resistance gene as a selection marker (Invitrogen).

Co-transfection of α_{IIb} and β_3 cDNAs

Baby hamster kidney (BHK) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mg mL^{-1} L-glutamine and 5% fetal calf serum (Biological Industries, Beit-Haemek, Israel). The cells were co-transfected with 1 μg of WT or the mutated form of pcDNA3/α_{IIb} and 1 μg of WT pCEP4/β_3 using lipofectamine reagent (Gibco BRL, Paisley, UK). Mock cells were produced by transfecting BHK cells with 1 μg of pCEP4 and 1 μg of pcDNA3 and then subjecting them to selection in medium containing 0.7 mg mL^{-1} G418 (Gibco BRL) and 0.5 mg mL^{-1} hygromycin (Bohringer Gmbh, Mannheim, Germany) for at least 3 weeks before use.

Analysis of surface expression of $\alpha_{IIb}\beta_3$ and fibrinogen binding by flow cytometry

Transfected BHK cells were harvested with PBS/1 mM EDTA, pelleted and incubated in DMEM medium for 30 min at room temperature. Cells were pelleted again, resuspended in PBS, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (10^6 cells/100 μL) and incubated for 30 min at room temperature with a FITC-conjugated monoclonal antibody to the $\alpha_{IIb}\beta_3$ complex (CD41/P2

Immunotech). Subsequently, cells were diluted to 10^6 cells mL^{-1} and analyzed for surface fluorescence by flow cytometery (Coulter).

For fibrinogen binding, the cells were resuspended in TBS, 0.1% BSA (Sigma), 0.25 mM $MgCl_2$ (10^6 cells 100 μL^{-1}) and incubated for 1 h at room temperature with 10 μg/100 μL human fibrinogen (Sigma) and/or 1 μL anti LIBS6 (ligand induced binding site) antibody (kindly provided by Dr Mark Ginsberg, University of California, San Diego). Subsequently, cells were pelleted and resuspended in the same buffer and incubated for 20 min at room temperature with 10 μL FITC-conjugated rabbit anti-human fibrinogen (Dako) diluted to 10^6 cells mL^{-1} and analyzed by flow cytometery (Coulter). Background fluorescence was measured using the same antibodies in mock BHK cells.

Immunoblot analysis of BHK cells

Transfected BHK cells were lysed with lysis buffer and the lysate was electrophoresed on NuPAGE Novex 3–8% Tris–Acetate gels (Invitrogen) with 1 mM DTT. Seperated proteins were then transferred to a PVDF membrane (Millipore). The membrane was immersed in TBST containing a 1:1000 dilution of a monoclonal antibody to α_{IIb} (SZ22; Immunotech), washed with TBST and then immersed in TBST containing 1:2000 horseradish peroxidase conjugated anti-mouse IgG. Immunoreactive bands on the membrane were detected using Super Signal West Pico Chemiluminescent substrate; (Pierce Biotechnology, Rockford, IL, USA) and X-ray film exposure.

Results

Platelet surface expression of $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$

Flow cytometric analysis revealed that all 11 Palestinian-Arab probands expressed normal amount of GPIb/IX complex on their platelet surface (data not shown) and 10 patients had less than 5% of normal $\alpha_{IIb}\beta_3$ surface expression. One patient (proband 11 in Table 1) expressed approximately 50% of normal $\alpha_{IIb}\beta_3$ on his platelets (Fig. 1A), and consequently he was defined as bearing a variant mutation. Integrin $\alpha_v\beta_3$ was expressed on the platelets of probands 1–9 (data not shown), which was consistent with a mutation in the α_{IIb} gene [12,17]. In one patient (proband 10 in Table 1), $\alpha_v\beta_3$ was not expressed on the platelet surface suggesting that the mutation was in the β_3 gene.

Mutation identification

All eight newly examined Palestinian patients, as well as all 15 Jordanian patients, were tested for the presence of the 13-bp deletion in the α_{IIb} gene[13]. Five patients, three residing in Israel and two residing in the Palestinian territories were homozygous for the 13-bp deletion. Thus, of 11 probands identified in this and our previous study[5], eight were homozygous for the 13-bp deletion. Of 158 Palestinian-Arabs controls (316 alleles), none was found to carry the 13-bp deletion in the α_{IIb} gene consistent with a very low allele frequency (97.5%, CI; 0.0000–0.0116). None of the 15 Jordanian-Arab patients carried this mutation.

In each of the three Palestinian probands not bearing the 13-bp deletion, a homozygous novel mutation was identified (Table 1). In proband 9, a one nucleotide deletion (G2374) in exon 24 was found in the α_{IIb} gene. This mutation predicts a shift in the reading frame leading to a stop at codon 764. In proband 10, a dinucleotides deletion (TT1616–7) in exon 10 of the β_3 gene was identified, which also predicts a shift of the reading frame, resulting in a stop at codon 523. Both mutations occurred in homonucleotide sequences, (GG and TTT, respectively). In proband 11, whose platelet expressed approximately 50% of the normal level of $\alpha_{IIb}\beta_3$ (Fig. 1A), a −3C → G point mutation was identified. This mutation is one nucleotide upstream of

the invariant AG of the intron14/exon 15 acceptor splice site of the β_3 gene. This alteration gives rise to a new AG acceptor sequence flanking the natural AG acceptor.

For all three novel mutations, PCR-based assays combined with restriction enzyme digestion were designed for detection of carriers among the respective family members. None of the 15 Jordanian GT patients carried any of these three mutations.

Characterization of the novel variant GT mutation

Immunoblot analysis of the platelet lysates from patient 11 revealed apparently normal amounts of α_{IIb} and β_3 (Fig. 1B), and antibody 10E5 immunoprecipitated the $\alpha_{IIb}\beta_3$ complex from the patient's platelet lysate (Fig. 1C).

The possible effects of the − 3C → G substitution at the intron 14/exon 15 junction on the mRNA splicing was analyzed using the Splice Site Prediction Program (http://www.fruitfly.org). In contrast to the score of 0.99 for the normal AG acceptor splice site at intron 14, the score for the − 3C → G mutant was <0.4. The newly created AG in the mutant sequence had a score of 0.93, suggesting that the mutation may result in alternative splicing. This was confirmed by sequencing the cDNA prepared from the patient's platelets, which revealed that the normal AG acceptor was inserted into exon 15 (Fig. 2A, B). This insertion predicts a shift in the reading frame from residue 742 onward, creating a stop at codon 803 instead of codon 763, thereby extending the cytoplasmic tail of β_3 by 40 amino acids (Fig. 2C).

The patient's platelets bound only trace amounts of fibrinogen in response to stimulation by ADP or collagen, in spite of an $\alpha_{IIb}\beta_3$ surface expression of 50% (Fig. 1D).

Characterization of the 13-bp deletion

The 13-bp deletion causes an in-frame deletion of six amino acids (Ala106-Gln111), of which Cys107 was suspected to be critical for normal $\alpha_{IIb}\beta_3$ complex formation [5]. Analysis of the crystal structure of the $\alpha_{IIb}\beta$-propeller in complex with the βA domain of β_3 (PDB: 1TXV) revealed that Trp110 interacts directly with Arg261 of β_3. We evaluated the effects of Cys107Ser substitution (in which the Cys107-Cys130 bond is abolished) and a Trp110Ala substitution (in which its interaction with Arg261 of the β_3 subunit is abrogated). These mutated cDNAs were expressed in BHK cells with WT β_3 cDNA. Flow cytometric analysis with the anti-$\alpha_{IIb}\beta_3$ monoclonal antibody P2 demonstrated lack of surface expression of $\alpha_{IIb}\beta_3$ in cells transfected with the natural 13-bp deletion, and about 30% of WT expression in cells transfected with Cys107Ser or Trp110Ala substitutions (Fig. 3A).

Activation of $\alpha_{IIb}\beta_3$ by anti LIBS6 antibody yielded in cells containing Cys107Ser approximately 50% fibrinogen binding compared with WT cells, but no fibrinogen binding to Trp110Ala containing cells (Fig. 3A).

Immunoblot analysis using an antibody against α_{IIb} (SZ22) showed no α_{IIb} in cells containing the 13-bp deletion and markedly reduced amounts of α_{IIb} in cells expressing either Cys107Ser or Trp110Ala (Fig. 3B). In cells expressing Cys107Ser or Trp110Ala mutations most α_{IIb} appeared as pro-IIb suggesting impaired transport of the pro-$\alpha_{IIb}\beta_3$ complex to the Golgi (Fig. 3B).

Founder effect for the 13-bp deletion and an estimate of the mutation's age

As the 13-bp deletion in α_{IIb} was identified in eight unrelated families residing in different regions of Israel and the Palestinian territories, we assessed whether this mutation resulted from a founder effect or stemmed from several separate mutational events. Haplotype analysis in the eight probands and 50 Palestinian-Arab controls was performed using two polymorphic

markers within the β_3 gene, one polymorphic marker in the α_{IIb} gene and three polymorphic markers (THRA1, BRCA1 and D17S579) located on chromosome 17 in the vicinity of α_{IIb} and β_3 genes. The analysis revealed that all GT patients carrying the 13-bp deletion had the same haplotype for the polymorphic marker within the α_{IIb} gene, and for the D17S579 marker, which is located approximately 350 kb away from the α_{IIb} gene (Table 2). At larger distances from the α_{IIb} gene, homozygosities for polymorphic markers were frequent but some presumed recombination events were apparent. The frequency distribution of each polymorphism examined in control chromosomes and chromosomes bearing the 13-bp deletion differed significantly (Table 3). Collectively, these data are consistent with a founder effect for the mutation.

We estimated the time when the Palestinian 13-bp deletion occurred by analysis of BRCA1 and β_3-CT polymorphisms, which are the closest loci on both sides of the mutation displaying recombination with the mutation. The age estimate was calculated by two methods. One method devised by Risch et al. [18] is based on the recombination fraction between the mutation and the closest marker related to the frequency of the marker on normal chromosomes. By this method, the range of age estimates was 19–31 generations, which translates into 380–620 years, assuming that one generation equals 20 years. The second method [19] was based the age estimate on linkage disequilibrium of multiple genetic markers in normal and affected individuals, and incorporates information about gene location, mutation frequency, and growth rate of the population examined, using a Markov chain Monte Carlo method [20]. Simulations using this program resulted in an estimate of 17 generations (340 years), with 95% credible sets of 14–22 generations, (280–440 years). Thus, the age estimate for the 13-bp deletion ranges between approximately 300 and 600 years.

Discussion

The data presented in this study, establish the predominance of the 13-bp deletion in the α_{IIb} gene among Palestinian patients with GT. The mutation was detected in 8 of 11 unrelated probands (73%) and in none of 15 unrelated Jordanian-Arab patients. Haplotype analysis was consistent with a founder effect (Table 2, 3), and an age estimate based on two methods revealed that the mutation occurred probably 300–600 years ago. This ‘Palestinian mutation’ is thus the fourth mutation described that has produced a cluster of affected GT patients stemming from a founder effect. The other founder mutations are an 11 bp deletion in β_3 exon 12 and an 11.2 kb β_3 deletion in Iraqi Jews [7], and a splice site mutation IVS15(+1)G > A within the α_{IIb} gene in French Gypsies [8]. The 13 bp Palestinian mutation involves deletion of 3 nt of intron 3 and 10 nt of exon 4, that lead to alternative splicing to a downstream AG acceptor producing an in-frame deletion of six amino acids, Ala106 – Gln111. The deleted residues are located in the second blade of the β-propeller domain of α_{IIb}, which interfaces with the βA domain of β_3. In our previous study we speculated that the absence of Cys107 within the deleted segment might have a critical effect on the folding of α_{IIb} and its ability to form a normal complex with β_3 [5]. Cys107 is completely conserved in 40 integrin β-propellers analyzed [21] and normally forms a disulfide bond with Cys130, maintaining a rigid structure for β-strands 2–3 of blade-2 within the β-propeller (Fig. 4A). Disruption of the Cys107–Cys130 bond by a Cys130Trp substitution was demonstrated to cause GT [22], and here we showed that disruption of the same Cys107-Cys130 bond by a Cys107Ser substitution resulted in impaired α_{IIb} maturation and surface expression of $\alpha_{IIb}\beta_3$ (Fig. 3), but not in impaired fibrinogen binding (Fig. 3A). Another important residue within the Ala106-Gln111 deletion is Trp110 that interacts with Arg261 of the βA domain of β_3 [10] and is also important for fibrinogen binding to $\alpha_{IIb}\beta_3$ as shown by others [23] and in the present study (Fig. 3A). Substitution of Trp110 by the much smaller residue Ala is predicted to disrupt the interaction with β_3-Arg261 as shown in the model (compare Fig. 4A, B). BHK cells transfected with WT β_3 cDNA, and α_{IIb} cDNA in which Cys107 was substituted by Ser or Trp110 was substituted by Ala displayed in both instances

only 30% of normal surface expression of $\alpha_{IIb}\beta_3$ (Fig. 3A). In each of these substitutions, most α_{IIb} appeared as pro-α_{IIb} (Fig. 3B), which suggests that pro-$\alpha_{IIb}\beta_3$ complexes form, but do not mature properly. In contrast to the complete 13-bp deletion, neither Cys107Ser nor Trp110Ala mutations resulted in complete loss of detectable α_{IIb} (Fig. 3A, B). Interestingly, substitution of another residue in the deleted segment, Gln111Ala, did not affect surface expression of $\alpha_{IIb}\beta_3$ in Chinese hamster ovary cells but did reduce fibrinogen binding [24]. Thus, at least three of the six amino acids deleted as a result of the Palestinian mutation affect $\alpha_{IIb}\beta_3$ biogenesis and/or function.

In three other Palestinian patients with GT, three novel mutations were identified, each in the homozygous state. Two of the three novel mutations were small deletions, i.e. G2374 in α_{IIb} gene and TT1616–7 in β_3 gene, respectively. Both mutations cause a shift in the reading frame and create a premature stop codon. The defects conferred by such stop codons can result in accelerated decay of mRNA, or in production of truncated proteins with removal of essential domains necessary for $\alpha_{IIb}\beta_3$ complex formation and its processing [22,25].

The third novel mutation is a point mutation (−3C → G) at the acceptor splice site of intron 14 and exon 15. The mutation creates a tandem repeat of AG at the termination of intron 14, which results in cryptic splicing and insertion of the original AG acceptor into exon 15. Disruption of highly conserved sequences like the intron 14 acceptor site, frequently gives rise to exon skipping unless, as in this case, the alteration produces a new consensus splice site very close to the original AG acceptor [26]. The insertion of AG into exon 15 creates a shift in the reading frame that leads to an aberrant sequence of amino acids from residue 742 and an extension of the β_3 cytoplasmic tail by 40 amino acids. Surface expression of $\alpha_{IIb}\beta_3$ was relatively preserved on the surface of the platelets of the patient, but the patient's variant $\alpha_{IIb}\beta_3$ failed to bind fibrinogen following activation by ADP or collagen (Fig. 1D). These data suggest that the aberrant β_3 cytoplasmic tail disrupted inside out signaling. Of note, the conserved β_3 NPxY sequence (amino acid 744–747) that was implicated in binding talin and β_3-endonexin in the process of inside out signaling [27], was altered by the patient's mutation. Elimination of NPxY motif as a result of truncation at residue 724 found in a patient with variant GT [28] or by the artificial substitution Tyr747Ala were previously shown to impair the normal inside-out signaling pathway [27–29]. Another mutation, a Ser752Pro substitution, was also shown to cause variant GT probably by causing impaired inside-out signaling [30]. It is likely that the aberrant amino acid sequence giving rise to Ser752Arg in the present study was an additional cause for abnormal inside-out signaling.

In conclusion, our data define a new common ancestral cluster of GT families confined to Palestinians and highlight the importance of a region in blade 2 of the β propeller of α_{IIb} that is essential for normal $\alpha_{IIb}\beta_3$ maturation. Three novel mutations causing GT are presented of which one, in the β_3 gene, is a variant related to an extended and aberrant cytoplasmic tail of β_3 that appears to disrupt inside-out signaling.

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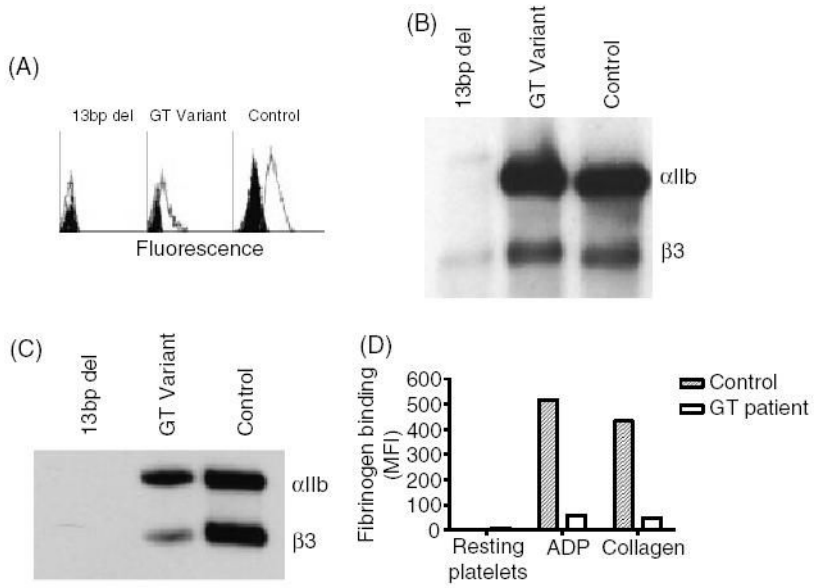

Fig. 1.

Platelet abnormalities in patients with Glanzmann thrombasthenia (GT). (A) Flow cytometric analysis of $\alpha_{IIb}\beta_3$ in platelets of a GT patient carrying the 13-bp deletion in the α_{IIb} gene, a patient with a −3C → G mutation in intron 14–exon 15 junction of the β_3 gene which causes variant GT, and a healthy control. fluorescein-isothiocyanate (FITC)-conjugated P2 monoclonal antibody against the $\alpha_{IIb}\beta_3$ complex was used. Background staining with FITC-conjugated mouse IgG is shown in black. (B) Immunoblotting under reduced conditions of sodium dodecyl sulphate-solubilized platelets using polyclonal antibodies against α_{IIb} and β_3. Notable is the trace amount of pro-α_{IIb} and β_3 in solubilized platelets of a patient with the 13-bp deletion and the normal amount of α_{IIb} and β_3 detectable in the patient with the variant mutation. (C) Immunoblot analysis under non-reducing conditions of $\alpha_{IIb}\beta_3$ immuno-precipitated by 10E5 monoclonal antibody, using SZ22 and AP3 monoclonal antibodies against α_{IIb} and β_3, respectively. Notable is the absence of α_{IIb} and β_3 in the patient with 13-bp deletion, and the reduced amounts of α_{IIb} and β_3 in the patient with the variant mutation compared with control. (D) Fibrinogen binding to resting platelets and platelets activated by 1.25 μ_M adenosine diphosphate or 1.25 μg mL^{-1} collagen. Note the impaired binding of fibrinogen to platelets of the patient with the variant mutation compared with control platelets.

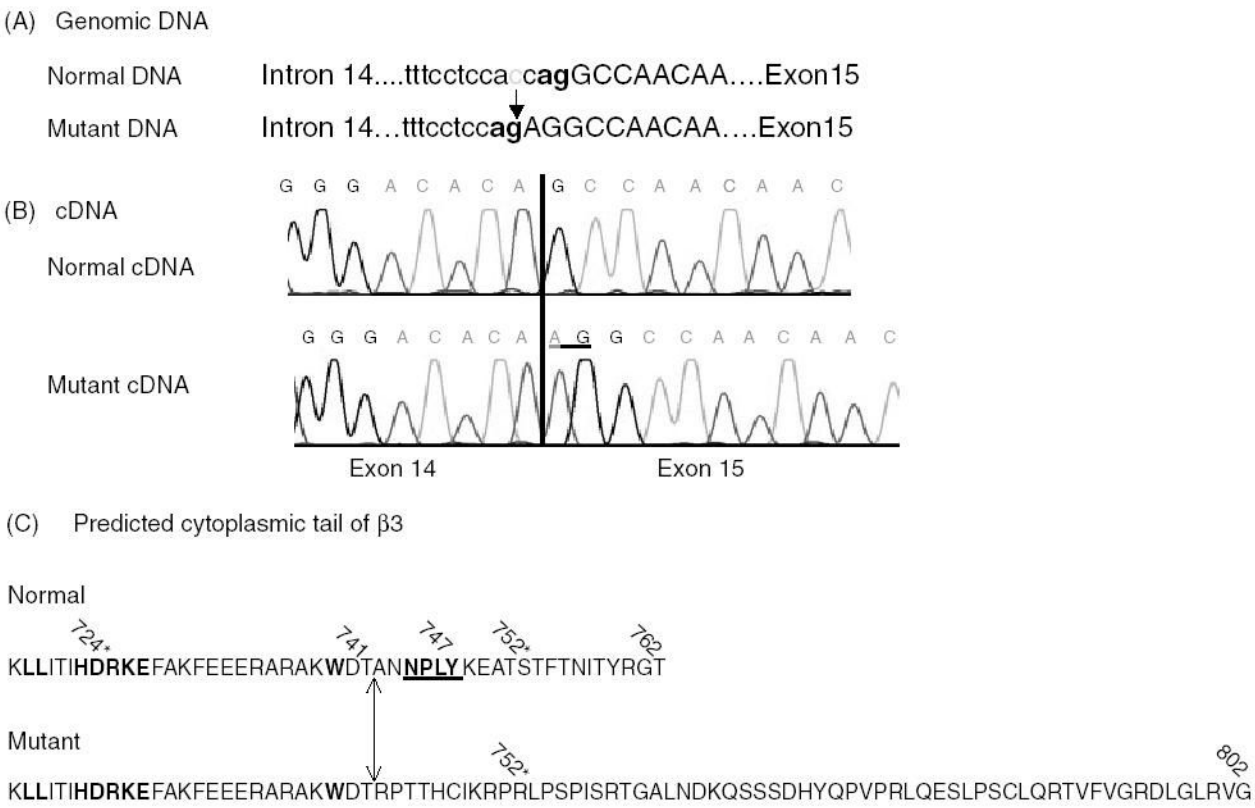

Fig. 2.

Alterations in DNA, cDNA and β_3 protein in the variant −3C → G mutation (A) In the mutated gene, the depicted −3C → G alteration creates a new AG splice site for the intron 14/exon 15 junction, predicting a change in the reading frame. (B) Sequence of the cDNA from the normal and the patient's platelets. Note the insertion of AG into exon 15 in the mutant. (C) The normal and the mutated β_3 cytoplasmic tail. Because of the AG insertion into exon 15, the reading frame in the mutant protein is changed after residue 741, resulting in a shift of the stop codon from 763 to 803. The β_3 tail is therefore extended by 40 amino acids. The conserved sequence LLxxxHDRKE (in bold), which has been implicated in the interaction with the α_{IIb} tail is not changed in the mutated protein, whereas the NPxY motif (in bold and underlined), which interacts with talin, is missing in the mutant tail. Naturally accruing mutations causing variant GT patients are depicted by an asterisk.

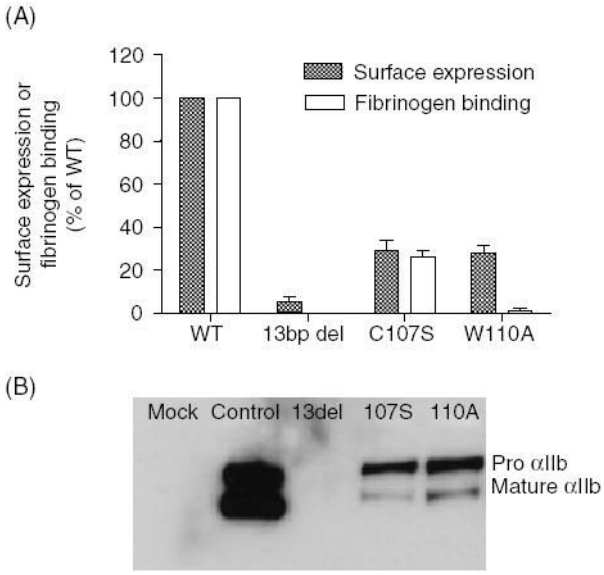

Fig. 3.

Expression of the natural 13-bp deletion and Cys107Ser or Trp110Ala artificial mutations. (A) Flow cytometric analysis of $\alpha_{IIb}\beta_3$ surface expression and fibrinogen binding to transfected baby hamster kidney (BHK) cells. BHK cells were transfected with normal β_3 cDNA and either normal α_{IIb} cDNA or mutated cDNAs (Cys107Ser, Trp110Ala or the 13-bp deletion). Cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated P2 monoclonal antibody against the $\alpha_{IIb}\beta_3$ complex or with FITC-conjugated rabbit anti-human fibrinogen antibody following activation by anti LIBS6 antibody in the presence of human fibrinogen. Surface expression of $\alpha_{IIb}\beta_3$ or fibrinogen binding were normalized to 100% for control $\alpha_{IIb}\beta_3$ expression or fibrinogen binding, respectively, and is presented as mean ± SE of three experiments. Note the substantially reduced amount of $\alpha_{IIb}\beta_3$ expression in the Cys107Ser and Trp110Ala mutants and the lack of fibrinogen binding to cells containing Trp110Ala substitution. (B) Immunoblots of $\alpha_{IIb}\beta_3$ expressed in transfected BHK cells. BHK cells transfected with normal β_3 and normal or mutated α_{IIb} or pcDNA3 and pCEP4 plasmids without the inserts (mock) were lysed and subjected to immunoblot analysis under reducing conditions. In cells expressing the 13-bp deletion, no α_{IIb} was detected, and in cells expressing Cys107Ser or Trp110Ala, most α_{IIb} appeared as pro-α_{IIb}.

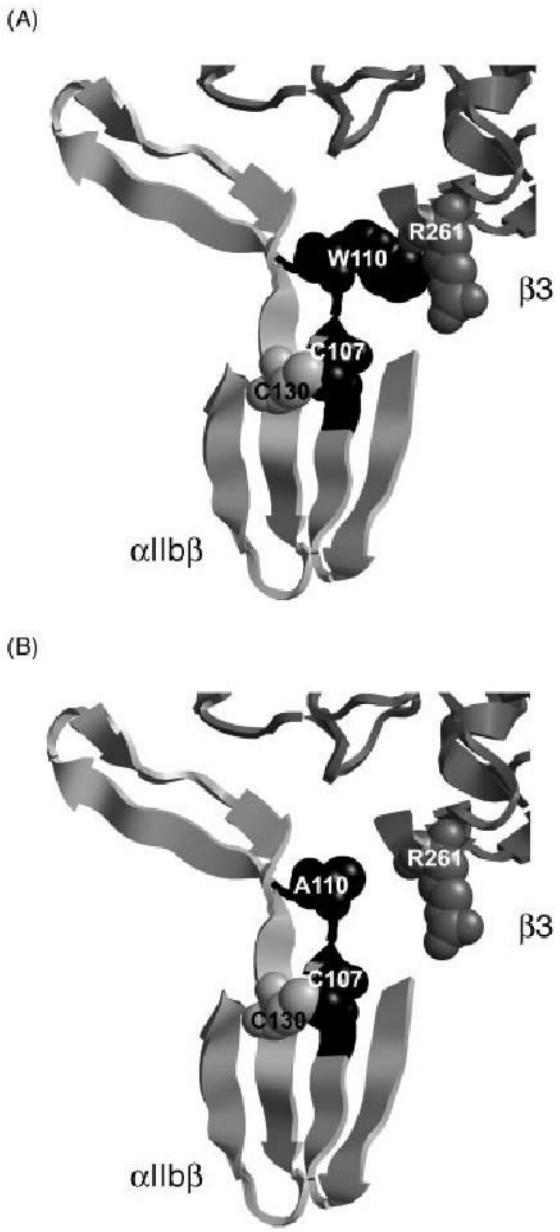

Fig. 4.

Model of the interface between blade 2 of the $\alpha_{IIb}\beta$-propeller and βA domain of β_3. Blade 2 of the $\alpha_{IIb}\beta$-propeller (PDB 1TXV) shown in black are the six amino acids that are deleted in the Palestinian mutation. Trp110 of α_{IIb} and Arg261 of β_3 are shown as balls (A). Also depicted are Cys107 and Cys130 that are normally juxtaposed by disulfide bond and predictably are disrupted by replacing Cys107 by Ser. Note that the contact between Trp110 and Arg261 is disrupted when Trp110 is replaced by Ala (B). The figure was made using RasMol program and was based on the crystal structure of $\alpha_{IIb}\beta_3$ [10].

Table 1

Mutations identified in 11 Palestinian-Arab probands with Glanzmann thrombasthenia

Unrelated probands	Mutated gene	DNA and mRNA alterations†	Predicted changes in protein†	Polymerized chain reaction primers and restriction enzyme for detection
1–8*	α_{IIb}	IVS4: −3, 13-bp deletion with cryptic splicing of exon 5	In-frame deletion of Ala106-Gln111	F: 5′-AGGAGGAGCCCAAGTCTCGCGC-3′ R: 5′-TCCCCATCGTGGCCCTTTC-3′ No restriction enzyme
9	α_{IIb}	Exon 24: deletion of G2374	761–763 altered amino acids with a 764 stop	F: 5′-CCTTTCCAGCCTCCATGGTG-3′‡ R: 5′-CCAGGGACGCGAGGCTCC-3′ *BstX*I
10	β_3	Exon 10: deletion of TT1616–7	513–522 altered amino acids with a 523 stop	F: 5′-GGGCCCAACTGTGTCTAAAT-3′ R: 5′-TATATGAGGGGGTGTGGGTT-3′ *BseN*I
11	β_3	IVS14: −3C → G with cryptic splicing of exon 15	742–802 altered and extended amino acids with a 803 stop	F: 5′-AGGAAGTCACTGTAAGATGCT-3′ R: 5′-GGCACAGGCTGATTATGATCT-3′ *Bpm*I

*Including three probands previously described [5].

†Nucleotide numbering starts with the first ATG of the cDNA, amino acids numbering starts with the first amino acid of the mature protein.

‡One nucleotide mismatched in forward primer (underlined) was designed to create the recognition site in normal allele.

Table 2

Haplotypes of eight unrelated probands carrying the 13-bp deletion in the α_{IIb} gene*

Distance from α_{IIb} gene (kb)†	Polymorphic sites‡	Probands							
		1	2	3	4	5	6	7	8
4324	THRA1	6 6	4 6	4 4	4 4	4 6	4 4	3 3	2 2
1234	BRCA1	3 3	3 3	3 3	3 3	2 1	3 3	2 2	3 3
0	α_{IIb}-HPA3	2 2	2 2	2 2	2 2	2 2	2 2	2 2	2 2
c349	D17S579	5 5	5 5	5 5	5 5	5 5	5 5	5 5	5 5
3233	β_3: (CT)n	8 4	8 8	8 8	8 8	4 4	6 8	4 5	8 8
	*Sma*I	1 2	1 1	1 1	1 1	1 1	2 1	1 1	1 1

* The founder haplotype is boxed.

† The distance from α_{IIb} gene was calculated according to http://www.ncbi.nlm.nih.gov/mapview.

‡ The allele designation is according to French et al. [16].

Table 3

Comparison of allele frequencies of polymorphic markers in chromosome 17 in unrelated probands with Glanzmann thrombasthenia (GT) bearing the 13-bp deletion in the α_{IIb} gene and Palestinian-Arab controls

Polymorphic site	Allele	Controls (*n* = 100)	GT probands (*n* = 16)	χ^2 analysis (*P*-value)
THRA1	2	0.03	0.125	<0.0001
	3	0.14	0.125	
	4	0.30	0.50	
	5	0.28	0	
	6	0.01	0.25	
	Others	0.24	0	
BRCA1	1	0.13	0.06	<0.0001
	2	0.15	0.19	
	3	0.19	0.75	
	5	0.10	0	
	Others	0.43	0	
α_{IIb}-HPA3	1	0.67	0	<0.0001
	2	0.33	1.00	
D17S579	5	0.18	0	<0.0001
	8	0.04	1.00	
	Others	0.78	0	
β_3-(CT) *n*	3	0.43	0	<0.0001
	4	0.01	0.25	
	5	0.03	0.06	
	6	0.07	0.06	
	8	0.20	0.63	
	Others	0.26	0	
β_3- *sma*I	1	0.63	0.13	0.0002
	2	0.37	0.87	

n, number of alleles.