

Three novel mutations in the glycoprotein IIb gene in a patient with type II Glanzmann thrombasthenia

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ABSTRACT

In the platelets of a type II Glanzmann thrombasthenia patient, the amount of glycoprotein (GP) IIb and IIIa was significantly reduced. Three novel mutations were identified in the GPIIb gene (c.440C→G/p.Leu116Val, c.1772_1773insG/p.Asp560 GlyfsX16 and c.2438C→A/p.His782Asn). p.Leu116Val did not represent a causative mutation. The c.1772_1773insG mutation resulted in an early stop codon and nonsense mediated decay of mRNA. When expressed in transfected BHK cells, the truncated protein was unable to form complex with GPIIIa. The p.His782Asn mutation compromised transport of the pro-GPIIb/IIIa complex from the endoplasmic reticulum to the Golgi, hindering its maturation and surface expression.

Key words: fibrinogen receptor, Glanzmann thrombasthenia, glycoprotein IIb mutations.

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The fibrinogen receptor (GPIIb/IIIa complex, $\alpha_{IIb}\beta_3$ integrin; CD41a) is synthesized in megakaryocytes and expressed on the platelet surface.¹ In the endoplasmic reticulum, pro-GPIIb forms heterodimer with GPIIIa, a prerequisite for surface expression. In the Golgi, pro-GPIIb is cleaved into heavy and light chains, linked through a disulfide bridge (mature GPIIb). GPIIb consists of four major domains, β -propeller, thigh, calf-1 and calf-2 domains. The main contact site with GPIIIa is located in the β -propeller, while calf-1 and calf-2 domains contribute minor interfaces. Glanzmann thrombasthenia (GT) is an autosomal recessive bleeding disorder characterized by quantitative or qualitative defect of the fibrinogen receptor and the inability of platelets to aggregate.² The GT database (<http://sinaicentral.mssm.edu/intranet/research/glanzmann>) lists 62 and 41 mutations in the GPIIb and GPIIIa genes respectively. In type I disease no fibrinogen receptors or only a very low number can be detected on the platelet surface, while in type II GT their number, although reduced (below 15%), is measurable. This report discusses a type II GT patient with three novel heterozygous mutations in the GPIIb gene and analyzes their consequences at protein level.

Design and Methods

A 52-year-old male patient, of non-consanguineous parents, was investigated because of recurrent hemorrhagic symptoms. Family members showed no sign of bleeding diathesis. His bleeding time was >20 min and PFA-100 closure times were >300 sec. The patient's platelets failed to aggregate in response to ADP, arachidonic acid, epinephrine and collagen. Clot retraction was partially retained (45% versus 30% in normal controls). The patient and family members gave informed consent. Ethical approval for the study was obtained from the Ethics Committee of the University of Debrecen. Preparation of platelet specimens, flow cytometric analysis, SDS-PAGE of platelet lysate, immunodetection of GPs by Western blotting, isolation of genomic DNA, PCR amplification and sequencing of exons of GPIIb gene, isolation and reverse transcription of platelet RNA have all been previously described.³

Baby Hamster Kidney (BHK) cells were transiently transfected with wild type (WT) GPIIIa and one of the mutant GPIIb containing plasmids.^{3,4} The surface appearance of GPIIb in transfected BHK cells was investigat-

ed by confocal laser scanning microscopy (CSLM), while its expression in whole cells was studied by immunoprecipitation of GPIIb/IIIa or GPIIIa and Western blotting.^{3,4}

Fibrinogen binding to transfected BHK cells was assessed by flow cytometry. Cells expressing p.Leu116Val or WT GPIIb and WT GPIIIa were incubated for 1 hour in Tris buffered saline (TBS) containing 0.1% bovine serum albumin (BSA), 0.25 mM MgCl₂ and 0.1 mg/mL human fibrinogen (Sigma) in the presence or absence of 10 µg/mL PT25-2 monoclonal antibody (Takara, Shiga, Japan) known to activate GPIIb/IIIa. Cells were washed and incubated for 20 min with 10 µL FITC-conjugated rabbit anti-human fibrinogen (Dako) and analyzed by flow cytometry.

The intracellular fate of mutant GPs expressed in BHK cells was studied by pulse-chase analysis.⁴ After chase, cells were lysed, and GPIIb and IIIa were immunoprecipitated by anti-GPIIb-IIIa polyclonal antibody (Affinity Biologicals). The immunoprecipitate was analyzed by SDS-PAGE and fluorography. Relative amounts of radio-labelled proteins were estimated by quantitative densitometry. For the intracellular localization of GPIIb, transfected cells were washed in PBS, fixed and permeabilized for 2 min in -20°C methanol, washed in PBS containing 0.05% Triton X-100 and 0.5% BSA (TX-BSA-PBS), and then incubated for 40 mins at room temperature with the following antibodies: monoclonal mouse antibody against protein disulfide isomerase (PDI) (ascites, 1:200 dilution) (Affinity Bioreagents, Golden, CO), 1 µg/mL anti-Golgin-97 mouse monoclonal antibody (Invitrogen) and 4 µg/mL phycoerythrin (PE) labelled anti-GPIIb antibody (DAKO). The anti-PDI antibody and the anti-Golgin-97 antibody were previously complexed with AlexaFluor 488 and AlexaFluor 647 conjugated goat anti-mouse IgG, respectively, using the Zenon labelling and blocking kit (Invitrogen). Cells were then washed 3 times in TX-BSA-PBS, mounted in 5 µL Mowiol, and examined by CLSM. Laser scanning cytometry (LSC) was performed using an iCys instrument (CompuCyte, Cambridge, MA, USA) based on an Olympus IX-71 inverted microscope. As well as the immunostainings described above, nuclei were also stained with 4',6-diamidino-2-phenylindole (DAPI). Quantitative digital image processing tasks were performed using the C programming environment of SCIL-Image (TNO, Delft, The Netherlands). Average GPIIb labelling intensities were determined for both the ER and Golgi regions and the ratio of the two was calculated for each cell.

Results and Discussion

Flow cytometric analysis showed a clearly detectable but strongly reduced amount of surface expressed GPIIb (Figure 1A,a) and GPIIIa (Figure 1A,b) on patient's platelets. Permeabilization increased the labelling, but there was still a difference between patient and control

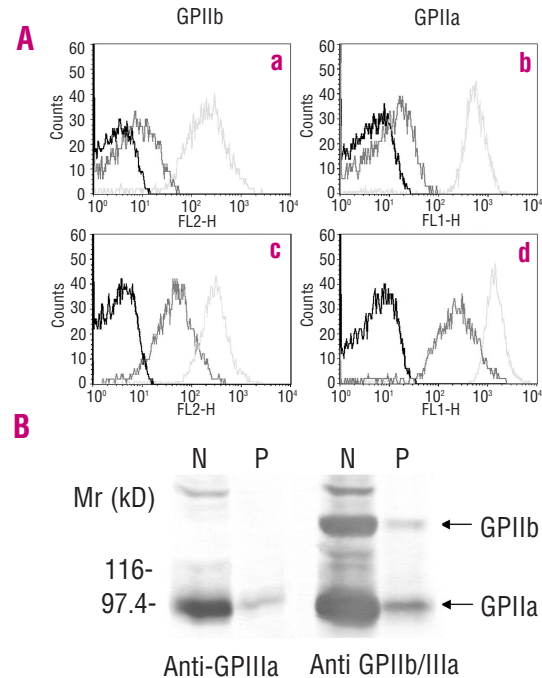


Figure 1. Measurements of GPIIb and GPIIIa in the patient's platelets. **A.** Detection of GPIIb (a,c) and GPIIIa (b,d) on the platelets' surface (a,b) or in permeabilized platelets (c,d) by flow cytometry using monoclonal anti-GPIIb and anti-GPIIIa antibodies; light gray: normal platelets, dark gray: patient's platelets, black: isotype control. **B.** Analysis of GPIIb and GPIIIa in the lysate of normal (N) and patient's (P) platelets by Western blotting. 30 µg platelet protein was loaded onto each lane. GPIIb and GPIIIa were immunodetected by a monoclonal anti-GPIIIa antibody or by a polyclonal antibody reacting with both GPIIb and GPIIIa.

(Figure 1A,c,d). 57,104±5,568 fibrinogen receptor copies were detected on control, and 2,556 copies (4.5%) on patient's platelets. The patient's two daughters expressed 31,157 and 35,030 receptor copies. In the patient's platelets the amount of GPIIb and GPIIIa was strongly reduced (Figure 1B). Only 6% and 16% of the amount present in control platelets were measured by scanning densitometry respectively.

Sequencing of patient's DNA revealed three novel heterozygous mutations in the GPIIb gene (www.ensembl.org, gene ID: ENSG00000005961): c.1771_1772insG causes frameshift and predicts a premature termination codon at amino acid 575 (p.Asp560GlyfsX16; X575GPIIb), c.339C>G and c.2437C>A resulting in p.Leu116Val (L116VGPIIb) and p.His782Asn (H782NGPIIb) amino acid exchange. One of the patient's daughters carried the c.2437C>A mutation. The other daughter carried the c.1771_1772insG along with c.339C>G mutation, indicating that these mutations are on the same allele. Neither point mutation could be detected in 50 unrelated individuals suggesting that they do not represent common polymorphisms. No mRNA representing the allele with c.1771_1772insG was detected in the patient's platelets. As the premature termination codon is 110 nucleotides 5' to the next exon-exon junction, in the nonsense-mediated

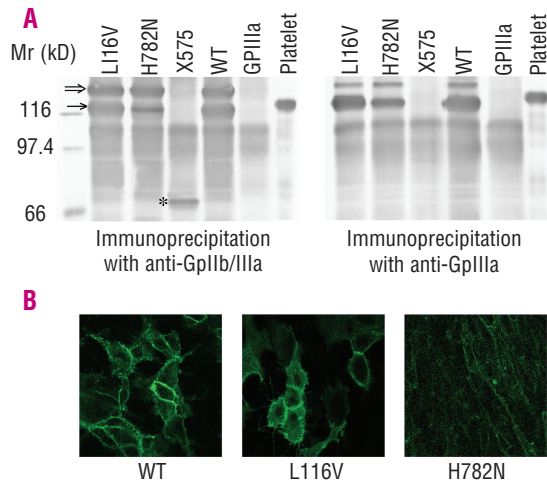


Figure 2. Co-expression of wild type or mutant GPIIb with wild type GPIIIa in BHK cells. The following GPIIb mutants were expressed: L116V, H782N, X575. **A.** Immunoprecipitation and Western blot analysis of GPIIb from transfected BHK cells. A polyclonal antibody reacting with both GPIIb and GPIIIa or a monoclonal anti-GPIIIa antibody was used for immunoprecipitation. SDS PAGE was carried out in reducing condition. The polyclonal antibody against GPIIb and GPIIIa was used for immunodetection. In reducing condition, GPIIIa shows poor reaction with the antibody. For comparison, platelet lysate was also subjected to SDS PAGE and immunodetection. The first lane represents Mr standard proteins. =>: pro-GPIIb; ->: mature GPIIb; *: truncated GPIIb. **B.** Surface expression of GPIIb/IIIa complex on transfected BHK cells was investigated by confocal laser scanning microscopy. FITC-labelled monoclonal antibody against GPIIb/IIIa complex was used for immunolabelling. As no GPIIb/IIIa was detected on the surface of cells co-expressing X575 GPIIb and wild type GPIIIa, this experiment is not shown in the Figure.

decay-competent region of the transcript, it is very likely that the mutant GPIIb-mRNA is eliminated by nonsense-mediated decay mechanism.⁵ A similar effect of GP mutations causing early stop codon has been reported in some cases.^{3,6-9}

The polyclonal antibody against both integrin subunits immunoprecipitated pro-GPIIb and mature GPIIb from BHK cell expressing WT or L116V mutant to the same extent and anti-GPIIIa antibody co-immunoprecipitated the mature proteins, indicating their normal complex forming ability (Figure 2A). In fact, considerable surface labelling of WTGPIIb/IIIa and L116VGPIIb/IIIa was observed (Figure 2B). The extent of fibrinogen binding to ac<M>ivated GPIIb/IIIa in cells expressing WTGPIIb and L116VGPIIb was also identical (*not shown*). The amount of mature H782NGPIIb was diminished while that of its pro-form was similar to the WT (Figure 2A) suggesting hindered maturation. The surface expression of H782NGPIIb/IIIa was strongly reduced (Figure 2B). The X575GPIIb was expressed in a limited amount as a truncated protein of 70 kDa that was not co-immunoprecipitated by anti-GPIIIa (Figure 2A). This finding and the lack of its surface expression suggest that it was unable to form complex with GPIIIa. This mutation is located in the C-terminal part of the thigh domain and results in the loss of both calf domains and the distortion of thigh domain. Two other non-sense mutations in the thigh domain also prevented complex formation with GPIIIa.^{3,9} There is an extensive, ~7 nm² contact between the thigh domain and

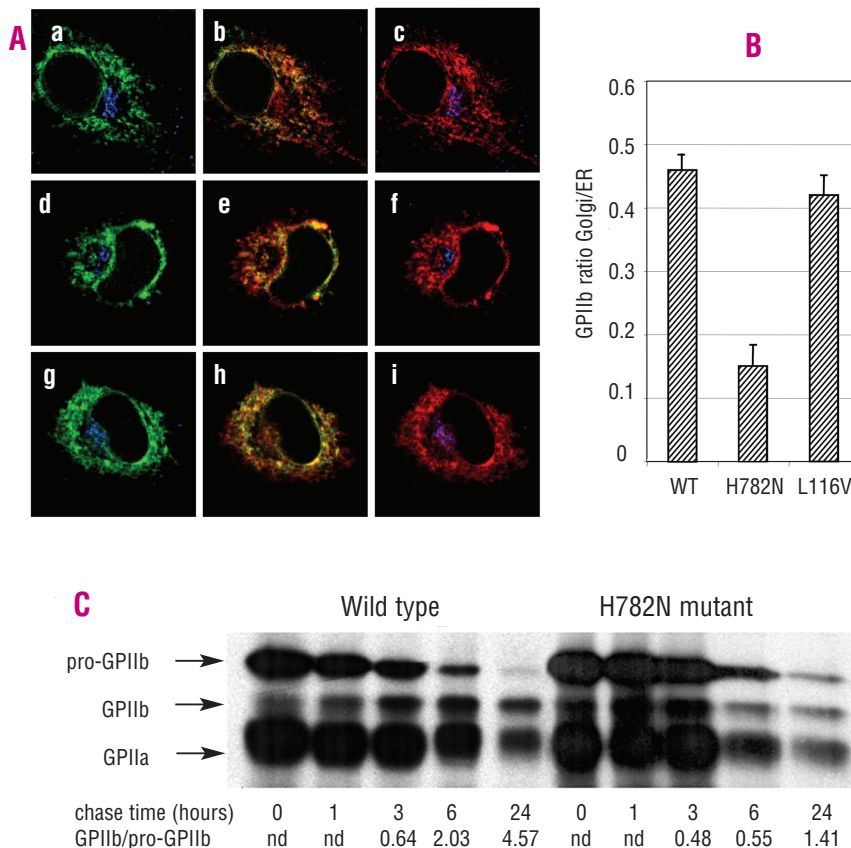


Figure 3. The fate of wild type and mutant GPIIb molecules in transfected BHK cells. **A.** Intracellular localization of wild type and mutant GPIIb by immunofluorescent staining. Cells co-transfected with wild type GPIIIa and wild type (a-c), H782N mutant (d-f) or L116V mutant (g-i) GPIIb were stained for an ER marker (protein disulfide isomerase), a Golgi marker (Golgin-97) and GPIIb. ER, Golgi and GPIIb appear in green, blue and red colors, respectively. a,d,g: double labelling for ER and Golgi; b,e,h: double labelling for GPIIb and ER; c,f,i: double labelling for GPIIb and Golgi. Overlap of labelling ER and GPIIb appears in yellow, while overlap of staining for Golgi and GPIIb results in purple color. **B.** Determination of the relative abundance of wild type and mutant GPIIb molecules in the ER and in the Golgi by laser scanning cytometry. Labelling intensity of GPIIb in the Golgi relative to that in the ER was measured for the three GPIIb variants. Means±S.D are shown on the graph. 1766, 1443 and 1859 cells transfected with the WT, H782N and L116V proteins respectively were used for the calculations. **C.** Pulse chase analysis of BHK cells co-transfected with wild type or H782N mutant GPIIb and WT GPIIIa. The cells were pulsed for 30 mins with ³⁵S-methionine and chased for various time, as indicated in the figure. The ratio of mature GPIIb and pro-GPIIb was determined by quantitative densitometry. nd: not determined (due to the heavy bands it was not possible to correctly separate individual bands after the 0 and 1 hour chase periods).

the β -propeller¹⁰ that might be important for the correct orientation of β -propeller for interaction with GPIIIa. The absence and/or distortion of part of the thigh domain could alter the orientation of β -propeller and block the complex formation with GPIIIa.

CLSM of permeabilized BHK cells showed co-localization of WTGPIIb and L116VGPIIb with both ER and Golgi markers (Figure 3A), indicating normal synthesis and transport from ER to Golgi. H782NGPIIb co-localized with the ER, while co-staining for GPIIb and the Golgi seemed less remarkable. As demonstrated by LSC, staining of WTGPIIb and L116VGPIIb in the ER and in the Golgi were of the same proportion, while the relative amount of the H782NGPIIb in the Golgi was significantly lower (Figure 3B).

Pulse-chase experiments showed that the degradation of WT and H782NGPIIb forms (pro-GPIIb plus mature GPIIb) followed the same kinetic (Figure 3C). By contrast, the conversion of H782Npro-GPIIb into mature GPIIb was significantly slower than that of WTpro-GPIIb. These findings indicate normal degradation, but delayed maturation of H782N mutant. Maturation of L116VGPIIb occurred with the same kinetics as the WT protein (*not shown*). Normal synthesis, maturation, intracellular transport, surface expression and fibrinogen binding ability indicated that L116V was not a causative mutation. The H782N mutation is localized to the calf-2 domain and contact between the calf-2 domain of GPIIb and GPIIIa has been demonstrated.^{11,12} The partial disruption of this

contact due to mutation does not influence the complex formation with GPIIIa, but could be responsible for the impaired transport of the aberrant complex from the ER.^{4,13,14} A similar correlation between retention in the ER and impairment of GPIIb maturation has been reported.⁴ A further disturbance in the transport of the H782NGPIIb/IIIa complex beyond the cis-Golgi that could contribute to the poor expression of GPIIb/IIIa on the surface of platelets and co-transfected BHK cells cannot be excluded.

Authors' Contributions

GL performed the molecular genetic analysis, the immunoprecipitation experiments, essential parts of the transfection and pulse chase experiments, participated in designing the study, in the immunofluorescent studies and in writing the manuscript; NR co-ordinated and performed the transfection experiments, the characterization of the *p.Leu116Val* mutant and participated in writing the manuscript; ZBo provided the study subject and established diagnosis; GV co-ordinated and performed the immunofluorescent studies; JK co-ordinated and performed the flow cytometry experiments; ZBe carried out the hemostasis diagnostic characterization; LM contributed to study design, co-ordinated the study, interpreted the data and was mainly responsible for preparing the manuscript.

All authors approved the version submitted for publication.

Conflict of Interest

The authors reported no potential conflicts of interest.

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