

**THE EFFECT OF NOVEL GPIIB MUTATIONS CAUSING GLANZMANN
THROMBASTHENIA ON THE SYNTHESIS, INTRACELLULAR TRANSPORT,
SURFACE EXPRESSION AND LIGAND BINDING ABILITY OF THE
FIBRINOGEN RECEPTOR**

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Introduction

Glanzmann thrombasthenia is an autosomal recessive bleeding disorder characterized by prolonged bleeding time and PFA-100 closure times, absent platelet aggregation in response to all physiological agonists and by absent or diminished clot retraction. The abnormalities are related to quantitative or qualitative abnormalities of the platelet surface fibrinogen receptor complex (GPIIb/IIIa complex, $\alpha_{IIb}\beta_3$ integrin, CD41a). Clinical symptoms occur soon after birth and include episodic severe mucocutaneous bleeding frequently localizing to the gastrointestinal tract, unprovoked bruising, epistaxis and prolonged bleeding after trauma or surgical interventions. In women, copious menstrual hemorrhage accompanies the disorder. The fibrinogen receptor; is a complex formed by glycoproteins (GP) IIb and IIIa. It is expressed on the platelet surface and in the membrane of α -granules. In activated form it binds adhesive proteins (most importantly fibrinogen and von Willebrand factor) and it is essential to the mechanism of platelet aggregation. GPIIb is a platelet specific protein synthesized in megakaryocytes as a single chain protein (pro-GPIIb). In the endoplasmic reticulum pro-GPIIb undergoes N-glycosylation, internal disulfide bonds are formed, and it is assembled into a calcium dependent heterodimer with GPIIIa. Then, it becomes transported into the Golgi for final oligosaccharide processing and for proteolytic cleavage to yield an N-terminal heavy chain and a C-terminal light chain held together by an internal disulfide bond. The assembly of GPIIb and IIIa is a prerequisite for the expression on the platelet surface. GPIIIa also associates with α_v integrin to form the vitronectin receptor (integrin $\alpha_v\beta_3$; VnR) present in a number of cell types. A small number of VnR is also expressed on the platelet surface. Direct information on the three-dimensional structure of GPIIb and on the structural requirements of its interaction with GPIIIa is only available from a most recent study on crystal forms that include only the N-terminal β -propeller part of GPIIb (residues 1-452) and GPIIIa residues 1-440. Most of our knowledge on the interaction of the two integrins in the non-liganded closed (bent) conformation comes from close analogies with the known three-dimensional structure of the ectodomain of VnR. The crystal structure of integrin $\alpha_v\beta_3$ reveals 4 major domains, β -propeller, thigh, calf-1 and calf-2 domains, in α_v and 3 regions of contact between α_v and β_3 . The main contact on α_v is located in the β -propeller, while calf-1

and calf-2 domains contribute minor interfaces. Homology modeling of normal GPIIb based on the $\alpha_v\beta_3$ crystal structure revealed similar contacts in the GPIIb/IIIa complex.

The GT database (<http://sinaicentral.mssm.edu/intranet/research/glanzmann>) lists 62 records of mutations in the GPIIb gene and 41 records of mutations in the GPIIIa gene. GT can be caused by mutations in the GPIIb or GPIIIa genes and the two types of mutation can be phenotypically distinguished by the detection of the VnR or GPIIIa. GT can also be classified according to the number of GPIIb/IIIa receptors expressed on the platelet surface. In type I disease no or only very low number of fibrinogen receptors can be detected on the platelet surface, while in type II GT their number is strongly reduced (below 15%), but well measurable. In variant GT the number of surface expressed GPIIb/IIIa complexes is nearly normal or normal, but the fibrinogen receptor is functionally defective. The analysis of molecular defects and its consequences in GT patients has resulted in important information on the structure-function relationship and the biogenesis of GPIIb and GPIIIa molecules and on the causative nature of a particular mutation. In this work I report two patients with Glanzmann thrombasthenia. In the first case type I GT was caused by a novel homozygous deletion (1618delC) in exon 17 of the GPIIb gene causing premature termination of protein synthesis (STOP533). In the second patient with type II GT we detected three heterozygous mutations in the GPIIb gene: a single nucleotide insertion (1772insG) causing frameshift and premature stop-codon at position 575, and two point mutations (C339G and C2437A) resulting in L116V and H782N amino acid replacements, respectively. The structural-functional consequences of the mutations and their effect on the biogenesis of GPIIb were explored.

Patients

Patient 1

A six-year-old boy of Romany descent demonstrated umbilical bleeding and hematoma at the site of intramuscular injection after birth. At the age of six months he was admitted to the hospital due to severe epistaxis when the highly prolonged bleeding time (>20 min) and PFA-100 closure times (>300 sec with both collagen-ADP and collagen-epinephrine cartridges), the lack of platelet aggregation to ADP, arachidonic acid, epinephrine and collagen, and the absence of PRP clot retraction established the diagnosis of Glanzmann thrombasthenia. Since then he has had repeated episodes of severe mucocutaneous bleeding. There was no evidence of consanguinity and no family history of bleeding diathesis.

Patient 2

A 52-year-old male patient was investigated, who has been suffering from recurrent gingival and occasional severe gastro-intestinal bleeding since early childhood. GT was diagnosed at the age of 30. No other family members showed any sign of bleeding diathesis and there was no evidence of consanguinity within the family. His bleeding time was >20 min and PFA-100 closure times were >300 sec with both collagen-epinephrine and collagen-ADP cartridges. The patient's platelets failed to aggregate in response to ADP, arachidonic acid, epinephrine and collagen, while ristocetin induced a reversible one-phase aggregation. Clot retraction of the patient was partially retained (45% versus 30% in normal controls).

Materials and methods

Preparation of plasma and platelet specimens

Venous blood samples from the patient and his two daughters were collected in a 1:10 volume of 0.105 mol L⁻¹ sodium citrate. Citrated blood samples were used for flow cytometry. Platelet poor plasma was separated by centrifugation at 2,000 g for 20 min. Washed platelet suspension was prepared¹⁹ and the final platelet pellet was solubilized in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Analysis of platelet lysate by SDS-PAGE and Western blotting

SDS-PAGE (7.5% gel) was carried out in reducing or non-reducing conditions. For the immunodetection of fibrinogen receptor components on Western blot, polyclonal sheep anti-GPIIb/IIIa antibody (Affinity Biologicals, Hamilton, Canada) that reacted with both GPs and monoclonal anti-GPIIIa antibody (Serotec, Kidlington, UK) were used as primary antibodies. When SDS-PAGE of platelet lysate was carried out in non-reducing conditions, the antibody stained GPIIb and GPIIIa equally well on the Western blot, while in reducing conditions it gave an intense reaction with GPIIb and only faintly stained GPIIIa. The reactions were developed by Vectastain Elite ABC sheep and mouse IgG kits (Vector, Burlingame, CA) according to the manufacturer's instructions. A GS-800 calibrated densitometer (Bio-Rad Hercules, CA) was used to determine the relative amount of GPIIb and GPIIIa in the patient's platelets.

Flow cytometric analysis of platelet glycoproteins

In direct labeling experiments platelets in 1:10 dilution of citrated blood were stained with combinations of fluorescently labeled antibodies against GPIb α (Dako, Glostrup, Denmark), GPIIb (Dako), GPIIIa (Sigma, St. Louis, MO) and the GPIIb/IIIa complex (Becton-Dickinson, Erenbodegen, Belgium). Samples were washed in phosphate buffered saline (PBS), fixed in 1% paraformaldehyde and analyzed in a FacsCalibur flow cytometer (Becton-Dickinson). Intracellular staining for GPIIb and GPIIIa was investigated following permeabilization of the platelets with 0.1% Triton-X 100. Fibrinogen receptor and VnR were quantitatively measured before and after activation with thrombin receptor agonist peptide (TRAP) using a highly sensitive indirect labeling technique. Calibration beads, unlabeled primary antibody for the GPIIb/IIIa complex and TRAP were components of the ADIAflo Platelet Gp kit (American Diagnostica, Greenwich, CT). The anti-VnR IgG was purchased from Serotec (Kidlington, UK). FITC-labeled goat anti-mouse IgG F(ab')₂ (Dako) was used as detecting antibody.

Polymerase chain reaction amplification and sequencing of *GPIIb* gene

Genomic DNA was isolated from buffy coats using QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). The exons of GPIIb gene were amplified using PCR primers

encompassing entire exons and short segments of flanking introns. Exons 2-3, 6-7, 8-9, 16-17, 19-20 and 23-24 were amplified in pairs together with the intervening introns. The following primers were used for amplification of exons 4, 16-17, 18 and 23-24:

4F 5' CAGGGCACAGGGAACAATCG 3';

4R 5' GGAGACAAGGAGGAGGGGTCAG 3';

16-17F 5'-CCTCAGACCAAGACACCCGTG-3',

16-17R 5'-CTCCCAGCCCTGCCAATC-3'.

18F 5' AGGAGAAGGTGGGATGTGTATGG 3',

18R 5' GACTGGCTGGATTTTGAGGTTTTTC 3',

23-24F 5' CCTCACCCCCAGAAAGTCAC 3'

23-24R 5' CAGGATGTAGAGCAGGTCGG 3'.

To exclude the possibility that the C339G and C2437A mutations represent common polymorphisms in the Hungarian population, DNA from 50 randomly selected healthy individuals (100 alleles) were investigated. In these cases only exons 4 and 24 of the GPIIb gene were amplified and sequenced.

Sequence analysis of platelet mRNA

Total RNA was isolated from platelets of both patients with Nucleospin RNA II kit (Macherey-Nagel GmbH, Düren, Germany). Reverse transcription was performed using First Strand cDNA Synthesis Kit for RT-PCR (AMV), (Roche Diagnostics GmbH, Mannheim, Germany). The following primers were used to amplify overlapping segments of patient's GPIIb mRNA derived cDNA encompassing exons 12 to 27:

1F 5'GCAGCCGCGAGGCCCCACG'3, 1R5'GGGCTCAGCTTGTCCCGGAA'3;

2F5'CCACTGGGCACAACATTCCT'3, 2R5'TCATTCTCCTTCTTCTGATT'3;

3F5'AGATTGTGCTGCTGGACGTG'3, 3R5'AAGGCCAGCACCGTGACCAT'3.

Quantitation of platelet GpIIb mRNA

Total RNA was isolated from platelets of 3 unrelated controls and the first patient as described above. After reverse transcription a 177 bp segment of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA and a 523 bp segment of GpIIb cDNA were amplified with primers F: 5'-AGGGCTGCTTTTAACTCTG-3', R: 5'-CTGGAAGATGGTGATGGG-3' and F: 5'-AGATTGTGCTGCTGGACGTG-3', R:5'-

AAGGCCAGCACCGTGACCAT-3', respectively. All PCR reactions were performed in Light Cycler 2.0 equipment (Roche Diagnostics GmbH, Mannheim, Germany) in triplicates using SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, UK). Activation of the Taq polymerase for 10 min was followed by 40 cycles of denaturation (95 °C, 30 sec), annealing (55 °C, 30 sec), and elongation (72 °C, 1 min). Crossing point of the reactions was defined as the point where amplification curve turned into the log-linear phase, and the fluorescence first reached a level significantly higher than the background. It was automatically calculated by the Light Cycler Software 4.0. Relative quantification of GPIIb mRNA in the patient's platelets was performed according to the following mathematical formula: $GpIIb \text{ mRNA Ratio (patient/control)} = E_{(GPIIb)}^{\Delta C_p \text{ GPIIb (control-sample)}} / E_{(GAPDH)}^{\Delta C_p \text{ GAPDH (control-sample)}}$. E represents the efficacy of the PCR reaction, which was calculated using the following equation: $E = 10^{(-1/\text{slope})}$, where slope stays for the slope of the curve obtained by plotting the crossing points of the 1-512 fold diluted cDNA of both GAPDH and GPIIb against the logarithmic scale of dilution.

Site directed mutagenesis, transfection of BHK cells

The pGEM7 plasmid containing wild type GPIIb cDNA was a generous gift from Dr. Peter Newman (Blood Center of Southeastern Milwaukee, WI). Preparation of pcDNA3 plasmid (Invitrogen, San Diego, CA) containing wild type GpIIb cDNA and the geneticin resistance gene was performed as described previously.¹³ The C440G, 1773insG, C2438A mutations were introduced separately using the Quickchange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). PCEP4 plasmid (Invitrogen) containing wild type GPIIIa cDNA and the hygromycin resistance gene was prepared from pCDNA3-GPIIIa plasmid (a generous gift of Dr. D.L. French from Mount Sinai School of Medicine, New York, NY). Baby Hamster Kidney (BHK) cells were co-transfected with pcDNA3 plasmid containing either wild type or mutated GPIIb cDNA and PCEP4 plasmid carrying wild type GPIIIa cDNA using LipofectAMINE reagent (Gibco, Pailey, United Kingdom). Cells transfected only with the respective plasmids were considered as mock cells. BHK cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mg mL⁻¹ glutamine and 5% fetal calf serum (FCS) (Biological Industries, Beit-Haemek, Israel). The transfected cells were selected with medium containing 0.5 mg mL⁻¹ geneticin (Gibco) and 0.5 mg mL⁻¹ hygromycin (Roche). GPIIIa-mock cells transfected only with PCEP4-GPIIIa were selected with 0.5 mg

mL⁻¹ hygromycin (Roche). Mock cells transfected with plasmids lacking GPIIb and GPIIIa cDNA were selected with both antibiotics.

Assesment of surface expressed GPIIb on transfected BHK cells

The surface appearance of GPIIb in transfected BHK cells was investigated by confocal laser scanning microscopy (CSLM). For fluorescence confocal microscopy live cells grown on glass coverslips were stained at 4°C degrees with PE conjugated anti-GpIIb (Dako, Golstrup, Denmark) or FITC-coupled anti-CD41a (Becton Dickenson) monoclonal antibodies for 10 minutes. Cells were then washed and fixed with 1% PFA for 10 minutes, and mounted in Mowiol. A Zeiss LSM510 was used to image immunoreactivity through a 63x Plan Aplanachromat NA=1.4 oil immersion objective. For FITC labels, 488 nm excitation, corresponding dichroic mirror and 505-550 nm bandpass emission filter was used. PE was detected using excitation and 543 nm, and emission over 560 nm.

Immunoprecipitation of GPIIb and GPIIIa from transfected BHK cells

BHK cells co-transfected with normal GPIIIa and normal or mutant forms of GPIIb were lysed in lysis buffer (20 mmol L⁻¹ Tris HCl, pH 7.5, 150 mmol L⁻¹ NaCl, 1% Nonidet P-40, 1% deoxycholic acid, 5 mmol L⁻¹ CaCl₂ and Complete protease inhibitor mix (Roche)). Proteins were immunoprecipitated from the cell lysates by a rabbit polyclonal antibody recognizing both GPIIb and GPIIIa (Affinity Biologicals) or a monoclonal antibody against GPIIIa (Becton-Dickinson). Immunoprecipitates were subjected to SDS-PAGE and Western blotting as described above.

Analysis of fibrinogen binding of surface expressed GPIIb(L116V)/GPIIIa by flow cytometry

For fibrinogen binding, 10⁷ cells mL⁻¹ BHK cells co-transfected with L116V mutant or wild type GPIIb and wild type GPIIIa were resuspended in Tris buffered saline (TBS) containing 0.1% bovine serum albumin (BSA) (Sigma) and 0.25 mM MgCl₂ and incubated for 1 hour at room temperature with 0.1 mg mL⁻¹ human fibrinogen (Sigma) in the presence or absence of 10 µg mL⁻¹ PT25-2 monoclonal antibody (Takara, Shiga, Japan) known to induce steric change of GPIIb/IIIa. 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 cytometry. Background fluorescence was obtained by using the above procedure with mock transfected BHK cells.

Pulse-chase analysis

Transfected cells were kept on methionine-free media for 1 hour, and then pulsed with $500 \mu\text{Ci mL}^{-1}$ [^{35}S] methionine (Merck, Darmstadt, Germany) for 30 min. After washing out the isotope the cells returned to normal culturing condition and were chased for 0, 1, 3, 6 and 24 hours. The cells were lysed and GPIIb/IIIa was immunoprecipitated. The immunoprecipitate was subjected to SDS-PAGE (7.5% gel), in reducing condition, the gel was fixed in isopropanol-acetic acid-water (25:10:65) and soaked in Amplify solution (Amersham Biosciences, Little Chalfont, UK). Finally, the gels were vacuum dried and exposed to Hyperfilm High Performance Autoradiography Film (Amersham) at -70°C . Relative amounts of the separated proteins on the fluorogram were estimated by a GS-800 calibrated densitometer (Bio-Rad).

Intracellular localization of GPIIb in transfected cells

Transfected BHK cells were grown in DMEM (10% FCS) onto 12 mm round coverslips under selection pressure (0.5 mg mL^{-1} geneticin and/or hygromycin) for 2 days. Cells were washed in PBS, then fixed and permeabilized for 2 min in methanol chilled to -20°C . Coverslips were washed in PBS containing 0.05% Triton X-100 and 0.5% BSA (TX-BSA-PBS), and then incubated for 40 min at room temperature with a mixture of fluorescent labeled antibodies dissolved in TX-BSA-PBS. The mixture contained the following antibodies: monoclonal mouse antibody against protein disulfide isomerase (PDI) (ascites, 1:200 dilution) (Affinity Bioreagents, Golden, CO), $1 \mu\text{g mL}^{-1}$ anti-Golgin-97 mouse monoclonal antibody (Invitrogen) and $4 \mu\text{g mL}^{-1}$ phycoerythrin (PE) labeled 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 (GAMIG), respectively, using the Zenon labeling and blocking kit (Invitrogen). Cells were then washed 3 times in TX-BSA-PBS, mounted in $5 \mu\text{L}$ Mowiol (0.1 M Tris-HCl, pH 8.5, 25 % glycerol (w/v), and 10% Mowiol 4-88, Hoechst, Frankfurt, Germany) on pre-cleaned microscopic slides and investigated by CLSM. AlexaFluor 488 was excited with a 488 nm Ar

ion laser and detected through a 505-550 nm band-pass filter. PE was excited with a 543 nm HeNe laser and detected through a 560-625 nm band-pass filter. AlexaFluor 647 was excited with a 633 nm HeNe laser and detected through a 650 long-pass filter. Pinholes were set to obtain 1 μm optical slices. 512 \times 512 pixel images were taken with pixel times of 6.4 μs , and 2 \times line-averaging. All images were obtained in multi-track mode. With the given settings, there was no channel cross-talk as determined from using single-labeled samples.

Laser scanning cytometry and image analysis

Laser scanning cytometry was performed using an iCys instrument (CompuCyte, Cambridge, MA) based on an Olympus IX-71 inverted microscope with a 40x plan semiapochromatic objective. In this series of experiments nuclei were also stained by adding 1 $\mu\text{g mL}^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) to the incubation buffer for the last 5 minutes of incubation. DAPI was excited with a 405 nm diode laser, AlexaFluor 488 and PE were excited with a 488 nm Ar ion laser, and AlexaFluor 647 with a 633 nm HeNe laser. Emission was detected through specific filters as described for CLSM. From each sample 112 confluent image tiles, each 1000 \times 768 pixels in size were scanned to allow evaluation of ~ 1500 cells.

Quantitative digital image processing tasks were performed using the C programming environment of SCIL-Image (TNO, Delft, The Netherlands). For determining the ratio of average GPIIb immunofluorescence in the Golgi versus the ER, individual cells were identified based on nuclear DAPI fluorescence, and their boundaries obtained from the merged fluorescence signal from the nucleus, the Golgi, the ER and the expressed GPIIb. Binary masks on cells were created by thresholding the overlay images; occasional holes in cell masks were filled with dilation-erosion cycles using a 3 \times 3 mask and a connectivity of 8 pixels. The background-corrected fluorescence of immuno-labeled GPIIb was used as input channel. The histogram mean of cells from images of unlabeled samples taken with identical instrument parameters were taken as background values. Additional binary masks for separating the Golgi and the ER were generated by thresholding anti-Golgin-97 and anti-PDI immunofluorescence images. Average GPIIb labeling intensities were determined for both the ER and Golgi regions and the ratio of the two was calculated in each cell. 1766, 1443 and 1859 cells transfected with the wild type, H782N and L116V proteins, respectively, were

used for the calculations. The mean \pm S.D of these ratios was used to characterize the relative abundance of GPIIb in the Golgi with respect to that in the ER.

Results

Patient 1

Flow cytometric analysis of platelet GpIIb, GpIIIa, GPIIb/IIIa complex and VnR

Individual components of the fibrinogen receptor and the GpIIb/IIIa complex itself were investigated by direct labeling flow cytometry, quantitative flow cytometry and by Western blotting. The absence of GpIIb on the platelet surface and intracellularly after permeabilization with Triton X100 was unequivocal, while the presence of a small amount of GpIIIa could not be excluded by direct labeling technique. The lack of fibrinogen receptor complex on the patient's platelets (receptor count <50 /cell), as demonstrated by quantitative flow cytometry, indicated type I Glanzmann disease. Activation of platelets with thrombin receptor agonist peptide increased the surface expression of GpIIb/IIIa complex in control platelets but failed to induce any increase in patient's platelets. The proband's parents expressed approximately 39 % of GPIIb/IIIa complex in comparison with control platelets (mother: 22.415 receptor/platelet, father: 22.075 receptors/platelet, control: 57.104 receptors/platelet). The number of VnR on thrombasthenic platelets was found to be higher (377/platelet) than in normal platelets (97 ± 36 /platelet, $n=14$). It is interesting that the activation of thrombasthenic platelets by TRAP induced a significant increase in the surface exposure of VnR (634/platelet). In control platelets no increase in surface VnR was observed.

SDS-PAGE and Western blotting of platelet lysates

Using a polyclonal antibody that reacted with both components of the fibrinogen receptor a faint, but well detectable band corresponding to GPIIIa was demonstrated in the patient's sample by Western blotting. GPIIb showed up as a heavy band in the control sample, however no traces of it could be found in the patient's platelets. The presence of GPIIIa in the patient's platelets was confirmed by using a specific anti-GPIIIa monoclonal antibody for immunodetection.

Identification of the thrombastenic genotype

The above results indicated that the genetic defect lies in the GPIIb gene. Indeed, direct DNA sequencing detected a novel deletion of a cytosine in exon 17 (at position 1618) in homozygous form. Parents were heterozygous for the same deletion. The single base deletion caused a frameshift beginning at amino acid 509 of the mature protein that resulted in a stop codon at amino acid position 533. Direct sequencing of mRNA-derived GPIIb cDNA extending from exon 6 to 24 revealed no alternative mRNA processing in patient's samples.

1618delC causes nonsense-mediated decay of GPIIb mRNA

In order to reveal the effect of the nonsense mutation on mRNA stability we have performed relative quantitation of GPIIb mRNA in control and patient's platelets and normalized results to the glyceraldehyde phosphate dehydrogenase (GAPDH) housekeeping gene as a reference. To calculate the efficacy of PCR reactions we diluted cDNA samples 1-512 fold in an exponential manner and plotted their Cp values (mean, n=3) versus the dilution rate in a logarithmic scale. In both cases the linearity was high (Pearson correlation coefficient $r > 0,99$), and slope of the curve was determined. Efficacy was calculated according to the formula $E = 10^{1/\text{slope}}$. Sample to control GPIIb mRNA ratio was calculated using the equation: $R = E_{(\text{GPIIb})}^{\Delta\text{Cp GPIIb (control-sample)}} / E_{(\text{GAPDH})}^{\Delta\text{Cp GAPDH (control-sample)}}$. E value derived from mean Cp values for GAPDH and GPIIb were 2.47 and 3.26, respectively. Patient's and controls' samples were run in triplicates with a parallel non-RT control. R value calculated for all the three different controls was 0,06 % in average, which shows that patient's platelets express less than 0,1% GpIIb mRNA compared to control platelets.

Analysis of transfected BHK cells

Surface expression of neither GPIIb nor GPIIb/IIIa complex could be detected on BHK cells by CLSM: The anti-glycoprotein IIb/IIIa antibody immunoprecipitated significant amount of pro-GPIIb and mature GPIIb from the lysate of BHK cells expressing wild type GPIIb and IIIa molecules. No GPIIb was immunoprecipitated from cells expressing only GPIIIa or from mock transfected cells. From cells carrying the stop533 mutation a faint band of a 60 kD protein was detected by the anti GPIIb/IIIa antibody. This protein was not

recovered when the immunoprecipitation was carried out with an antibody against GPIIIa while the same antibody co-immunoprecipitated the wild type mature GPIIb. These findings suggest that the truncated protein corresponds to the truncated GPIIb molecule and does not form complex with GPIIIa integrin.

Patient 2

Flow cytometric analysis of platelet GpIIb, GpIIIa and GPIIb/IIIa complex

Flow cytometric analysis showed well detectable but strongly reduced amount of surface expressed GPIIb and GPIIIa on patient's platelets compared to controls. Permeabilization of platelets with 0.1% Triton X-100 significantly increased the labeling of GPIIb and GPIIIa. However, even after permeabilization the intensity for staining GPIIb in the patient's platelets was much lower than in control platelets. Quantitative flow cytometry using a monoclonal antibody against GPIIb/IIIa complex detected $57,104 \pm 5,568$ copies of fibrinogen receptor on control platelets, while patient's platelets expressed only 2,556 copies (4.5%) of GPIIb/IIIa receptor complex. Activation of the patient's platelets with TRAP increased the receptor count to 4,148/platelet. Platelets from the patient's two daughters expressed 31,157 and 35,030 receptors, after activation with TRAP the number of receptors increased to 45,061 and 44,788.

SDS-PAGE and Western blotting of platelet lysates

The lysates of the patient's and control platelets were analyzed by Western blotting under non-reducing condition using either a monoclonal antibody against GPIIIa or a polyclonal antibody recognizing both GPIIb and GPIIIa. Both components of fibrinogen receptor were unequivocally present in patient's platelets but in a strongly reduced amount, as compared to controls. By scanning densitometry analysis of the immunoblot, the relative amount of GPIIb and GPIIIa in patient's platelets was found to be 6 and 16% of the amount measured in controls, respectively.

Analysis of genomic DNA

Exon-to-exon direct sequencing of the patient's DNA revealed three novel heterozygous mutations in the GPIIb gene: 1772insG in exon 18 causing a frameshift

predicting a premature stop codon at amino acid position 575, C2437A in exon 24 resulting in the H782N amino acid replacement, and C339G in exon 4 responsible for the L116V amino acid exchange. One of the patient's daughters carried the 1772insG along with C339G mutation, while the other daughter carried only the C2437A mutation. These data imply that 1772insG and C339G mutations are situated on the same allele, while C2437A is positioned on the other allele. Neither C339G nor C2437A mutation could be detected in 100 alleles from randomly selected individuals, which suggests that neither of them represents a common polymorphism.

Sequence analysis of platelet mRNA

Platelet total RNA from the patient and his daughters was reverse transcribed, and cDNA was subjected to PCR amplification encompassing exons 12 to 27 of the GPIIb cDNA. Direct sequencing of the PCR products showed that patient's platelets expressed only the allele with the C2437A mutation, while the allele containing the 1772insG mutation could not be detected. Platelets of the daughter with the C2437A allele expressed both wild type and mutant mRNA's, while in the platelets of the other daughter only wild type mRNA could be demonstrated.

Expression and complex formation of mutant GPIIb proteins in transfected BHK cells

Using a PE-labeled monoclonal antibody against GPIIb and the GPIIb/IIIa complex CSLM demonstrated considerable surface labeling of BHK cells expressing wild type GPIIIa and wild type or L116V mutant GPIIb. As it was shown by flow cytometry, the cells expressing wild type GPIIb/IIIa or GPIIb(L116V)/GPIIIa complex showed considerable fibrinogen binding following the activation of GPIIb by PT25-2 antibody. The extent of fibrinogen binding to the two types of transfected cells was identical. The surface expression of H782N mutant GPIIb was significantly reduced, but detectable, while the STOP606 mutant GPIIb was not expressed on the cell surface, at all.

In order to assess the relative amount of pro-GPIIb compared to mature GPIIb and to estimate complex forming ability of mutant GPIIb molecules, lysates of transfected BHK cells were subjected to immunoprecipitation using either a polyclonal antibody recognizing both GPIIb and GPIIIa or a monoclonal antibody recognizing only GPIIIa. SDS PAGE of the immunoprecipitates was performed in reducing condition to distinguish between mature

GPIIb and pro-GPIIb and the Western blots were stained with the polyclonal antibody recognizing both GPIIb and GPIIIa. Immunoprecipitation with the antibody against both integrin subunits resulted in equal amount of wild type and L116V mutant pro-GPIIb and mature GPIIb suggesting that an equal amount of GPIIb was expressed in both cases. In the case of H782N GPIIb mutant the amount of mature GPIIb was diminished while that of pro-GPIIb did not seem to change. It is to be noted that mature GPIIb expressed in BHK cells migrated somewhat faster than the platelet protein what might be due to a somewhat different state of its glycosylation in the two cell types. In the BHK cells expressing STOP606 mutant GPIIb no staining for GPIIb of the normal Mr was detected, however a band of approximately 70 kDa, most likely corresponding to the truncated form of GPIIb was clearly demonstrated. When immunoprecipitation was carried out with the anti-GPIIIa antibody, wild type GPIIb and the L116V mutant co-immunoprecipitated with GPIIIa to the same extent. The same antibody co-immunoprecipitated significantly less H782N GPIIb, while the amount of co-immunoprecipitated pro-GPIIb remained un-changed. When anti-GPIIIa antibody was used for immunoprecipitation the truncated GPIIb could not be detected on the Western blot. This finding suggests that it was unable to form complex with GPIIIa.

Pulse-chase analysis of transfected BHK cells

At the end of the pulse period BHK cells containing wild type GPIIb/IIIa complex expressed predominantly pro-GPIIb and GPIIIa and only a faint band represented mature GPIIb. During the chase period the total intensity of the ³⁵S-methionone label and the intensity of the bands corresponding to pro-GPIIb and GPIIIa gradually decreased on the fluorogram. In parallel, the amount of mature GPIIb increased up-to 6 hours at which time its amount exceeded that of pro-GPIIb. After 24 hours pro-GPIIb appeared only as a faint band, while radiolabeled mature GPIIb was still present in a considerable amount. Quantitative densitometric analysis measured 2.03 and 4.56 mature GPIIb:pro-GPIIb ratios at 6 and 24 hours, respectively. Evidently, pro-GPIIb was gradually transformed into its mature form by proteolytic cleavage. L116V pro-GPIIb was converted into mature GPIIb with the very same kinetics as its wild type counterpart (not shown). In the case of H782N GPIIb transfected cells the general rate of elimination of the radiolabeled GPIIb forms and GPIIIa did not differ significantly from that observed in the cells transfected with wild type GPIIb. At 6 hours and 24 hours the total amount of wild type pro-GPIIb and mature GPIIb was 36% and 21% of the

0 time value, while at the same intervals the sum of H782N pro-GPIIb and mature GPIIb was 36% and 18%. However, the process of pro-GPIIb maturation slowed down, the GPIIb:pro-GPIIb ratios at 6 and 24 hours were only 0.55 and 1.41, respectively.

Intracellular localization of wild type and mutant GPIIb in transfected BHK cells

To determine the intracellular localization of wild type and mutant GPIIb, triple immunofluorescent labeling for GPIIb, for PDI (an ER marker) and for Golgin-97 (a Golgi marker) was performed in BHK cells co-transfected with any of the different GPIIb variants and with wild type GPIIIa. Immunofluorescent staining was analyzed by CLSM. In cells transfected with wild type GPIIb, staining for GPIIb co-localized with both ER and with Golgi markers. Cells transfected with L116V GPIIb mutant showed similar co-localizations. This finding indicates normal synthesis and transport of the L116V GPIIb molecule from the ER to the Golgi. H782N GPIIb co-localized with the ER, while co-staining for GPIIb and the Golgi seemed less remarkable. To obtain quantitative data on the relative abundance of GPIIb in the ER and in the Golgi triple stained cells were analyzed by laser scanning cytometry. The results confirm that in the ER and in the Golgi staining of wild type and L116V GPIIb are of the same proportion, while the relative abundance of the H782N variant in the Golgi is significantly lower than that of the other two forms (0.15 vs 0.46 and 0.42).

Discussion

We have investigated two patients suffering from recurrent haemorrhagic episodes from early childhood. The lack of platelet aggregation in response to all physiological agonists, the total absence of GPIIb/IIIa on the surface of platelets and the absence of clot retraction indicated type I Glanzmann thrombasthenia in the first case. The findings that the second patient's platelets failed to aggregate in the presence of the usual agonists while retained their ability to support clot retraction, together with the highly impaired, but detectable expression of surface expressed fibrinogen receptors and the strongly reduced amount of both GPIIb and GPIIIa in the platelets' lysate unequivocally indicated the diagnosis of type II Glanzmann thrombasthenia.

Molecular genetic analysis of the first patient revealed a novel homozygous deletion (1618delC) causing frame shift and predicting a premature termination codon after 24 altered

amino acids at position 533 (STOP533). Molecular genetic analysis of the second patient detected three heterozygous novel mutations, C339G, 1772insG and C2437A, in the GPIIb gene. The C339G and C2437A point mutations resulted in L116V and H782N amino acid replacements localized in the β -propeller and in the calf-2 domains, respectively. The single base insertion caused a frameshift predicting a novel stop codon at position 575 following 16 altered amino acids. Molecular genetic analysis of family members and sequence analysis of platelet mRNA revealed that the C339G and 1772insG mutations localized to the same allele, while the C2437A mutation was situated on the other allele.

The absence of truncated GPIIb molecules (STOP533 in patient 1 and STOP 575 in patient 2) in the patients' platelets posed the question if this was due to the lack of GPIIb-mRNA or to the failure of truncated GPIIb to associate with GPIIIa. We demonstrated that the 1618delC mutation leading to a frameshift and to a premature stop codon at position 533 resulted in the practical absence of specific mRNA in the patient's platelets. In the second patient, the failure to detect the mutant mRNA that codes for the truncated protein suggests the instability of the transcribed product. As in both cases the premature termination codons lie in the nonsense-mediated decay-competent region of the transcript, it is very likely that GPIIb-mRNA with the premature termination codons are eliminated by nonsense-mediated decay mechanism in both patients. A few studies have reported the effect of nonsense mutations on the amount of GPIIb-mRNA. A 288delC mutation and a C→A transversion at position +2 of the exon 5-intron 5 boundary, both leading to frameshift and premature termination of polypeptide synthesis, caused strong reduction of GPIIb transcript. A similar effect was observed with stop codons at position 584 and at position 597.

Data on the binding of truncated GPIIb to GPIIIa in cells co-expressing mutated GPIIb and wild type GPIIIa could provide information on the fine details of the structural requirements of their interaction. Truncated forms of GPIIb caused by nonsense mutations in the calf-2 domain and mutant GPIIb with partial disruption of calf-1 or calf-2 domain retain their ability to form complex with GPIIIa. R597→Stop mutation at the C-terminal end of the thigh domain considerably impaired, but did not completely eliminate complex formation between the truncated protein and GPIIIa. The GPIIb mutations leading to premature stop codon (STOP533, STOP575) are located at the C-terminal end of the thigh domain and results in the distortion of the C-terminal part of this domain and the linker connecting the thigh and the calf-1 domains. Both calf domains are lost. There is an extensive, $\sim 7 \text{ nm}^2$

contact between the thigh domain and the β -propeller and this contact might be important for the proper orientation of β -propeller required for interaction with GPIIIa. The data suggest that the absence and/or distortion of part of the thigh domain, and perhaps also the N-terminal part of the calf-1 domain, alters the orientation of β -propeller and through this mechanism it blocks the complex formation with GPIIIa.

Although the L116V mutant, being on the allele with the STOP606 mutation, was not expressed in the second patient's platelets, it showed normal complex formation with GPIIIa and normal surface expression in co-transfected BHK cells. As L116 is located in the cap sub-domain that is considered to be the fibrinogen binding domain it seemed interesting to test if the L116V substitution influenced the binding of fibrinogen to its receptor. The exchange of a hydrophobic amino acid (Leu) by another hydrophobic residue (Val) was without any effect on fibrinogen binding following the activation of GPIIb/IIIa receptor by PT25-2 antibody. These results indicate that L116V is a non-causative mutation, which, if expressed in platelets, would not cause thrombasthenia.

H782N mutant GPIIb showed diminished surface expression on platelets and on BHK cells co-expressing the mutant protein and wild-type GPIIIa. The amount of H782N pro-GPIIb was similar to that of its wild-type counterpart, while the amount of mature H782N GPIIb was reduced in platelet lysates. The H782N GPIIb was able to form complex with GPIIIa. Pulse-chase analysis revealed that the conversion of H782N pro-GPIIb into mature GPIIb was delayed, however the degradation of the mature two-chain protein was not accelerated. These data are consistent with confocal fluorescent microscopic and laser scanning cytometric findings showing that the transport of H782N pro-GPIIb/IIIa complex from the ER to the Golgi is impaired. A number of mutations in the GPIIb gene hamper the biogenesis of mature GPIIb/IIIa complex probably by preventing its transport from ER to Golgi, since in some cases a correlation between retention in the ER and prevention of GPIIb maturation had been shown, like in our case. Three of them, V951M, Q747P and a mutant with the insertion of 8 alternative amino acids beginning at position 786, were localized to the calf-2 domain. A contact between the calf-2 domain of GPIIb and GPIIIa has been demonstrated. The partial disruption of this contact by mutations does not influence the complex formation with GPIIIa, but could be responsible for the impaired transport of the aberrant complex from the ER. The H782N mutation is also localized to the calf-2 domain and such a mechanism could operate in this case, too. The poor expression of GPIIb/IIIa on

the surface of platelets and on the surface of co-transfected BHK cells might not be fully explained by the relatively moderate impairment in the transport and maturation of mutant pro-GPIIb. Disturbance(s) in the transport of the H782N GPIIb/IIIa complex at stage(s) beyond the cis-Golgi cannot be excluded.

The thesis was based on the following publications:

1. Losonczy G, Rosenberg N, Kiss C, Kappelmayer J, Vereb G, Kerényi A, Balogh I, Muszbek L. A novel homozygous mutation (1619delC) in GPIIb gene associated with Glanzmann thrombasthenia, the decay of GPIIb-mRNA and the synthesis of a truncated GPIIb unable to form complex with GPIIIa. *Thromb Haemost.* 2005;93:904-9. (IF: 3,056)
2. Losonczy G, Rosenberg N, Boda Z, Vereb G, Kappelmayer J, Hauschner H, Bereczky Z, Muszbek L. Three novel mutations in the glycoprotein IIb gene in a patient with type II Glanzmann thrombasthenia. *Haematologica* 2007 in press. (IF: 4,575)

Other publications:

1. Balogh I, Poka R, Losonczy G, Muszbek L. High frequency of factor V Leiden mutation and prothrombin 20210A variant in Romanies of Eastern Hungary. *Thromb Haemost.* 1999;82:1555-6. (IF: 4,983)
2. Tsorbatzoglou A, Módis L, Losonczy G, Biró Z, Berta A. 40%ige Glukose-Augentropfen gegen Hornhautödeme. Eine seltene Nebenwirkung. *Der Ophthalmologe* 2007 in press. DOI: 10.1007/s00347-006-1436-2. (IF: 1,559)