Major mutations in calf-1 and calf-2 domains of glycoprotein IIb in patients with Glanzmann thrombasthenia enable GPIIb/IIIa complex formation, but impair its transport from the endoplasmic reticulum to the Golgi apparatus

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The crystal structure of integrin \( \alpha_v/\beta_3 \) comprises 3 regions of contact between \( \alpha_v \) and \( \beta_3 \). The main contact on \( \alpha_v \) is located in the \( \beta \)-propeller while calf-1 and calf-2 domains contribute minor interfaces. Whether or not contacts between calf-1 and calf-2 domains of glycoprotein (GP) IIb (\( \alpha \)-IIb) and GPIIa (\( \beta_3 \)) play a role in GPIIb/IIIa complex formation has not been established. In this study we analyzed the effects of 2 naturally occurring mutations in calf-1 and calf-2 domains on GPIIb/IIIa complex formation, its processing, and transport to the cell membrane. The mutations investigated were a deletion-insertion in exon 25 located in calf-2 and an in-frame skipping of exon 20 located in calf-1. Mutated GPIIb cDNAs were cotransfected in baby hamster kidney cells with normal GPIIia (\( \beta_3 \)) CDNA. Analysis by flow cytometry failed to demonstrate detectable amounts of GPIIIb or GPIIb/IIIa complex on the surface of cells transfected with each mutation, but immunohistochemical staining revealed their intracellular presence. GPIIIb was mainly demonstrable as pro-GPIIIb by immunoprecipitation of cell lysates expressing each mutation. Differential immunofluorescence staining of GPIIIb and cellular organelles suggested that most altered complexes were located in the endoplasmic reticulum. Homology modeling of normal GPIIib based on the \( \alpha_v/\beta_3 \) crystal structure revealed similar contacts between \( \alpha_v \) and \( \beta_3 \) and between \( \alpha \)-IIb and \( \beta_3 \). Introduction of the mutations into the model yielded partial disruption of the normal contacts in the corresponding domains. These data suggest that despite partial disruption of calf-1 or calf-2 domain, GPIIb/IIIa complex is formed but its transport from the endoplasmic reticulum is impaired. (Blood. 2003;101:4808-4815)

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Introduction

Platelet aggregation depends on fibrinogen binding to glycoprotein (GP) IIb/IIIa complex (\( \alpha \)-IIb\( \beta_3 \)), which is a calcium-dependent heterodimer expressed exclusively in megakaryocytes and platelets.\(^1\) GPIIb and GPIIIa are encoded by separate genes, and following their synthesis they are introduced into the endoplasmic reticulum (ER) where they undergo N-linked glycosylation, form disulfide bonds, and assemble in GPIIb/IIIa complexes. The complex is then transported to the Golgi apparatus for final oligosaccharide processing and cleavage of single-chain pro-GPIIb into mature GPIIb, comprising a heavy and light chain. From the Golgi apparatus the complex is transported to the open canalicular system and then to the \( \alpha \) granules or plasma membrane.\(^2,3\) The assembly of GPIIb and GPIIIa as a complex is a prerequisite for its surface expression.\(^2\)

A diminished amount or dysfunction of the GPIIb/IIIa complex causes Glanzmann thrombasthenia (GT). GT is transmitted in an autosomal recessive manner and is caused by mutations in either GPIIb or GPIIIa genes.\(^4\) So far more than 60 mutations causing GT have been described and listed in a database.\(^5\) Expression of some of these mutations in heterologous cell cultures has shed light on the functional importance of particular domains within GPIIb and GPIIIa in the assembly of GPIIb/IIIa complex. Similar information has also been derived from immunohistochemical analyses of soluble GPIIb/IIIa complex and from the crystal structure of soluble \( \alpha_v/\beta_3 \), which is closely related to \( \alpha \)-IIb\( \beta_3 \).\(^6,7\) By immunohistochemical examinations it was demonstrated that the C-terminal region of GPIIib, including parts of calf-1 and calf-2 domains, is involved in the contact between GPIIb and GPIIIa.\(^6\) The crystal structure of the extracellular part of integrin \( \alpha_v/\beta_3 \) revealed that while the main contact between \( \alpha_v \) and \( \beta_3 \) lies in the \( \beta \)-propeller of \( \alpha_v \) and \( \beta_A \) domain of \( \beta_3 \), additional contacts between calf-1 and calf-2 domains of \( \alpha_v \) and epidermal growth factor (EGF)–3, EGF-4, and \( \beta \) tail domain (\( \beta_TD \)) domains of \( \beta_3 \) were also depicted. The latter contacts were small and discontinuous and therefore believed not to occur in the membrane-bound receptor.\(^7\) This apparent weakness of the contacts could also be inferred from an electron microscopy study that demonstrated that GPIIb/IIIa complexes had a globular head with 2 flexible tails that were sometimes joined together and sometimes spread apart.\(^8\) However, a recent electron microscopy study that included physicochemical measurements of normal and mutated \( \alpha_v/\beta_3 \) and \( \alpha \)-IIb\( \beta_3 \) demonstrated that in the low-affinity state, in solution, and on cell surface, the tails of the \( \alpha \) and \( \beta \) subunits have substantial interactions with one another.\(^9\) This study also showed that activation of \( \alpha_v/\beta_3 \) resulted in a dramatic
rare rearrangement to an extended conformation in which no association between the tails of the α and β subunits was displayed.9

We have previously described 2 major mutations in the calf-1 and calf-2 domains of the GPIIb gene in patients with GT. One mutation in calf-2, a 6-bp deletion and 31-bp insertion in exon 25, leads to cryptic splicing of exon 25 and results in substitution of Leu786–Asn795 (LHNLPGPTVNY) by 8 alternative amino acids (GLHLIHQQ).10 A second mutation in calf-1, an A→G transition in the acceptor splice site of intron 19–exon 20 junction, results in 4 alternatively spliced mRNAs, all lacking exon 20 with 3 of them remaining in-frame.11 In an attempt to clarify the importance of the contact between calf-1 and calf-2 of GPIIb and EGF-3, EGF-4, and βTD of GPIIIda, we analyzed the effects of these major mutations in calf-1 and calf-2 on GPIIb/IIa complex formation, its processing, and transport to the cell membrane.

Materials and methods

Antibodies

A rabbit polyclonal antibody recognizing both reduced GPIIb and nonreduced GPIIIda was previously produced in our laboratory.12 Marine monoclonal antibodies, AP3 specific for GPIIIda and AP2 specific for GPIIb/IIa complex, were generously provided by Dr Thomas Kunicki from Scripps Research Institute (La Jolla, CA), and monoclonal antibody 10E5 specific for GPIIb/IIIa complex was a gift from Dr Barry Coller from Rockefeller University, New York, NY. Murine monoclonal antibodies SZ22 against the heavy chain of GPIIb and P2 recognizing GPIIb only when complexed with GPIIIa were purchased from Immunotech (Marseilles, France). Monoclonal antibody CA3 against human GPIIb and polyclonal rabbit antihuman-β3 antibody were purchased from Chemicon International (Temecula, CA). Rabbit antirat manniosidase II antiserum was purchased from Dr Kelley W. Moremen, University of Georgia, Athens, and rabbit anticanine calcinein carboxyl terminus was purchased from Strengene Biotechnologies (Victoria, BC, Canada). Goat antimouse immunoglobulin G (IgG) fluorescein isothiocyanate (FITC) conjugate, goat antirabbit IgG agarse conjugate, goat antirabbit IgG peroxidase conjugate, goat antirabbit IgG agarose conjugate, and goat antirabbit IgG peroxidase were purchased from Sigma (St Louis, MO). Goat antirabbit IgG Texas red (TR) conjugate was purchased from Jackson ImmunoResearch Laboratories, West Grove, PA.

Preparation of GPIIb mutant cDNA constructs

Platelet GPIIb mRNA of an Iranian-Jewish patient with GT bearing a deletion-insertion mutation in exon 25 was cloned into normal pGEM7-GPIIb (generously provided by Dr Peter Newman, Blood Center of Southeastern Milwaukee, WI) to create GPIIb-alt.23 as described previously.10 Platelet GPIIb mRNA was also prepared from an Iraqi-Jewish patient for GPIIb/IIa complex formation, its processing, and transport to the cell membrane.

Cell culture and cotransfection of GPIIb and GPIIIda cDNAs in baboon hamster kidney (BHK) cells

BHK cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 2 mg/mL t-glutamine and 5% fetal calf serum (Biological Industries, Beit-Haemek, Israel). The cells were transfected with 1 μg pCDNA3-GPIIIda (generously provided by Dr D. L. French from Mount Sinai School of Medicine, New York, NY) using LipofectAMINE reagent (Gibco, Paisley, United Kingdom). The transfected cells were selected with medium containing 0.7 mg/mL G418 (Gibco). BHK cells resistant to G418 and stably expressing GPIIIda were transfected with 1 μg of either normal or 1 of the 2 mutated forms of pCEP4-GPIIb using LipofectAMINE reagent. The transfected cells were selected with medium containing 0.7 mg/mL G418 and 0.5 mg/mL hygromycin (Boehringer Mannheim, Germany). Mock were cells expressing GPIIIda and transfected with 1 μg pCEP4 (Invitrogen) and selected for resistance to both hygromycin and G418.

Assessment of surface expression of GPIIb/IIa complex by flow cytometry

Transfected cells were harvested with phosphate-buffered saline (PBS) and 5 mM EDTA [ethylenediaminetetraacetic acid], centrifuged, and incubated in growth medium for 30 minutes at room temperature. The cells were resuspended in PBS (5 × 10⁵ cells per 100 μL) and incubated with monoclonal antibodies against either GPIIIa (SZ22, 15 μg/mL), GPIIIda (AP3, 15 μg/mL), or GPIIb/IIa complex (10E5, 30 μg/mL) for 30 minutes at room temperature. Cells were then washed with PBS, resuspended in 100 μL PBS, and incubated for 30 minutes at room temperature with fluorescein isothiocyanate (FITC) conjugate goat antimaion IgG diluted 1:50. Finally, the cells were diluted to 5 × 10⁵/mL, and surface fluorescence was analyzed in a Coulter flow cytometry (EPICS XL, Louton, United Kingdom).

Immunoprecipitation of GPIIb and GPIIIda

BHK cells transfected with normal GPIIIda and either normal or mutant forms of GPIIb (5 × 10⁶ cells) were lysed in lysis buffer (20 mM Tris [tris(hydroxymethyl)aminomethane] HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholic acid, and complete protease inhibitor mix [Boehringer Mannheim]). Cell lysates were incubated in ice for 30 minutes and centrifuged for 20 minutes at 16 000g to remove debris. The supernatant was incubated with 20 μg rabbit polyclonal antibodies recognizing both GPIIb and GPIIIda or with 1 μg CA3 monoclonal antibody against GPIIb overnight at 4°C followed by incubation with goat antirabbit IgG agarose or goat antimaion IgG agarose, respectively, for 5 hours at 4°C and then washed 5 times with TBST (20 mM Tris HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100) and eluted at 100°C with reducing loading buffer (40 mM Tris [pH 6.8], 1.5% SDS, 8% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol). Immunoblot analyses were performed by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transfer to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA), and subjection to SZ22 monoclonal antibody against GPIIb followed by goat antimaion IgG peroxidase conjugate (Sigma). Immunoreactive bands on the membrane were detected using enhanced chemiluminescence (ECL) kit (Amersham, Buckinghamshire, United Kingdom) and x-ray film exposure. Prestained high molecular weight proteins (Bio-Rad Laboratories, Richmond, CA) were used to estimate the molecular weights of the precipitated proteins. In some experiments, immunoprecipitated proteins were treated with endoglycosidase H (New England Biolabs, Beverly, MA) as recommended by the manufacturer before the samples were analyzed on SDS-PAGE as described.

For immunoprecipitation of the GPIIb/IIa complex, cells were lysed in the same lysis buffer but containing 5 mM CaCl₂. The complex was precipitated either by 3 μg P2 or 5 μL rabbit antiserum against GPIIIda followed by adding IgG agarose conjugate antimaion or antirabbit antibody, respectively, and elution in sample buffer with or without 5% β-mercaptoethanol. Western blot analyses were performed using SZ22 and AP3 monoclonal antibodies followed by goat antimaion IgG peroxidase conjugate.
Immunohistochemistry

Transfected BHK cells were grown on glass slides to subconfluence, washed in Tris-buffered saline (pH 7.6) with 5 mM CaCl₂ (TBS-Ca), dried for 24 hours at room temperature, and fixed in acetone for 15 minutes at 4°C. Cells were then incubated in TBS-Ca containing S222, AP2, or 10E5 monoclonal antibodies (10 to 20 μg/ml) using alkaline phosphatase–anti-alkaline phosphatase kit (APAAP, Dako, Glostrup, Denmark). Antibody binding to the cells was visualized as red staining using light microscopy and the Duet system (BioView, Rehovot, Israel).

Immunofluorescence microscopy

Transfected cells were grown on 12-mm cover slips, washed in PBS, and fixed in acetone methanol (1:1) as previously described. Cells were incubated for 1 hour at room temperature with 1:1000 dilution of S222 monoclonal antibody against GPIIb, and either rabbit antirat mannansidase II or rabbit anticanine calnexin carboxyl terminus, followed by 1 hour of incubation with 1:70 FITC conjugate goat antimouse IgG or 1:40 TR conjugate goat antirabbit IgG, respectively. The cover slips were coated with antifade solution (Oncor, Gaithersburg, MD) and analyzed using the Duet system.

Pulse-chase analysis

Transfected cells were metabolically labeled with [35S]methionine and chased for 1 to 24 hours as previously described. Total GPIIb was immunoprecipitated from cell lysates with 3 μg S222 monoclonal antibody, and the GPIIb fraction complexed with GPIIIa was precipitated with AP3 or 10E5 antibodies. The immunoprecipitations were carried out at 4°C for 16 hours, which was followed by incubation with agarose-conjugated goat antimouse IgG (50 μl) for 5 hours at 4°C. Recombinant proteins without reducing agent were electrophoresed by SDS-PAGE, and the gel was fixed in isopropanol–acetic acid–water (25:10:65), vacuum dried, and exposed to Biomax MS film using Biomax Transcreen-LE (Kodak, Rochester, NY) at −70°C. Relative amounts of the separated proteins were estimated by densitometry (Clinscan2, Helena Laboratories, Beaumont, TX). In some experiments, immunoprecipitated proteins were treated with peptide N-glycosidase F (PNGaseF) (New England Biolabs) before they were subjected to SDS-PAGE.

Molecular modeling of GPIIb

Because αIIb and α₃ are homologous proteins with 36% identity in amino acid sequence and because αIIbβ3 (GPIIb/IIIa complex) and α₂β₃ share the same β₃ subunit, we used the recently described crystal structure of human α₂β₃ (PDB:1JV2) as a template for modeling β-propeller, calf-1, and calf-2 domains of GPIIb. Homology modeling was done separately for calf-1 and calf-2 domains in wild-type and in GPIIb-del.20 and GPIIb-alt.25 using Modeler of InsightII software (Accelrys, San Diego, CA). The Modeler performs automated homology modeling by simultaneously combining information from the sequence alignment and structures of multiple reference proteins. The sequences of αIIb (XP_045985), α₃ (XP_002379), and α₂ (XP_039011) were aligned by Pileup algorithm (GCG, Accelrys). Normal and mutant GPIIb models and the α₂ template were superimposed by structure using the appropriate InsightII option. The amino acids of calf-1 and calf-2 domains of αIIb located in the interface with β₃ subunit at 0.5 nm (5 Å) distance were marked and the nature of the contact analyzed using contacts of structural units (CSU) software.

Results

GPIIb is formed but not surface expressed in cells transfected with GPIIb mutants

Fluorescence-activated cell sorter (FACS) analyses with monoclonal antibodies S222 and 10E5 recognizing GPIIb and GPIIb/IIIa, respectively, demonstrated lack of surface expression of GPIIb and GPIIb/IIIa in cells transfected with alt.25 or del.20 mutations (Figure 1). Using AP3 monoclonal antibody against GPIIIa detected GPIIIa on the surface of cells containing normal as well as mutant GPIIIa. This resulted from complex formation between human GPIIIa and hamster α₂, as previously described. Immunohistochemistry experiments using S222 antibody showed that expression of GPIIb occurred in cells transfected with normal GPIIb and GPIIb-alt.25 (Figure 2). In cells containing the GPIIb-del.20 mutation, only weak staining with S222 was observed. These results indicate that the mutated forms of GPIIb were absent from the cell membrane but present intracellularly.

To further analyze the nature of the mutant forms of GPIIb, cell lysates were first immunoprecipitated with polyclonal antibody against GPIIIa and GPIIIIa and then subjected to SDS-PAGE with β-mercaptoethanol followed by Western blotting using S222 monoclonal antibody. Cells transfected with normal GPIIb displayed 2 bands corresponding to mature GPIIb and pro-GPIIb (Figure 3A). Cells transfected with GPIIb-alt.20 exhibited only one band, which migrated as pro-GPIIb, and cells transfected with GPIIb-del.20 exhibited a fast-migrating pro-GPIIb corresponding to the predicted deletion of 50 amino acids of exon 20 (Figure 3A). In these experiments the amount of both mutated GPIIb proteins, particularly GPIIb-del.20, was lower than the amount of wild-type (WT) GPIIb. When immunoprecipitation was carried out with a monoclonal antibody CA3 against GPIIIa, and immunoblotting was performed with S222 monoclonal antibody, an additional smaller GPIIb-related band was observed in both GPIIb-alt.25 and GPIIb-del.20 (Figure 3B). These smaller bands probably represent in vivo–generated degraded GPIIb because cell lysates were prepared in the presence of protease inhibitor cocktail contained in the commercial tablets.

GPIIb/IIIa complex is formed in cells expressing the GPIIb mutants

GPIIb/IIIa complex formation in cells expressing the GPIIb mutations was examined by immunostaining with 2 distinct monoclonal antibodies against the complex, 10E5 and AP2 (Figure 2). Compared with WT cells transfected with normal GPIIb, the cells transfected with GPIIb-alt.25 or GPIIb-del.20 reacted only faintly with 10E5 monoclonal antibody. Use of monoclonal antibody AP2 clearly showed the presence of the
complex in cells transfected with GPIIb-alt.25 and less conspicuously in cells transfected with GPIIb-del.20.

To further analyze whether or not the mutants of GPIIb formed GPIIb/IIIa complexes, we examined the coprecipitation of mutants and normal GPIIb with GPIIIa using an antibody against GPIIIa. Cell lysates expressing each mutant of GPIIb (and normal GPIIIa) were first immunoprecipitated with polyclonal antibody against GPIIIa and then immunoblotted with a mixture of monoclonal antibodies SZ22 against GPIIb and AP3 against GPIIIa. Figure 4A-B shows that both GPIIIa and GPIIb were clearly demonstrable in reduced and nonreduced gels containing WT and the mutants of GPIIb. Notably, in nonreduced conditions, the SZ22 antibody similarly recognized WT GPIIb and the mutants of GPIIb (Figure 4B). Immunoprecipitation of cell lysates with monoclonal antibody P2 recognizing GPIIb only when complexed with GPIIIa also revealed pro- and mature normal GPIIb, pro–GPIIb-alt.25, and a band corresponding to pro–GPIIb-del.20 (Figure 3C). Degraded GPIIb (Figure 3B) was absent in these gels. When GPIIb complexed with GPIIIa was precipitated by AP3 and analyzed with SZ22 in immunoblotting and long exposure, small amounts of mature GPIIb-alt.25 and a minute amount of mature GPIIb-del.20 were demonstrable (Figure 4D). Taken together, these experiments indicate that GPIIb/IIIa complexes are formed in cells harboring GPIIb-alt.25 and GPIIb-del.20.
normal GPIIb/IIIa increased during the antibody as described. Figure 6B demonstrates that the amount of GPIIb-alt.25 decayed slightly faster and GPIIb-del.20 decayed slightly slower than WT GPIIb. The generalization and stability of the normal GPIIb/IIIa complex was examined by immunoprecipitation of the complex by 10E5 monoclonal antibody as described. Figure 6A shows that while WT mature GPIIb was resistant to EndoH digestion, which removes only non-N-glycosylation, GPIIb-alt.25 and GPIIb-del.20 mutants of GPIIb were EndoH-sensitive similarly to WT pro-GPIIb, indicating normal N-glycosylation. GPIIb were precipitated by polyclonal antibodies against GPIIb and treated with EndoH. Shown is Western blot analysis using anti-GPIIb antibody (SZ22) of SDS-PAGE in the presence of β-mercaptoethanol of cell lysates. Notable are resistance to EndoH for mature GPIIb in WT cells and sensitivity to EndoH for pro-GPIIb in cells containing GPIIb mutants as well as WT pro-GPIIb.

**Oligosaccharide processing of the mutated GPIIb**

Because both mutants of GPIIb were produced in transfected cells and assembled as GPIIb/IIIa complex, we investigated the glycosylation of the GPIIb mutants. The initial stage of glycosylation, which takes place in the endoplasmic reticulum (ER), was examined by treatment of SZ22-immunoprecipitated cell lysates with peptide N-glycosidase F (PNGaseF), which cleaves the bonds between the innermost oligosaccharides and the asparagine in N-linked glycoproteins. Figure 5A shows that PNGaseF treatment resulted in the same changes in the migration of nonreduced GPIIb in WT, del.20, and alt.25 cells, indicating that both normal GPIIb and mutants of GPIIb underwent similar N-glycosylation. Complex N-glycosylation in the Golgi was assessed by resistance to endoglycosidase H (EndoH) digestion, which removes only noncomplex N-mannose oligosaccharide chains in the ER. Figure 5B shows that while WT mature GPIIb was resistant to EndoH digestion (migration was similar), both mutants del.20 and alt.25 GPIIb were EndoH-sensitive similarly to WT pro-GPIIb as depicted by a change in their migration in reduced gel.

**Stability of the GPIIb mutants**

To examine intracellular stability of the GPIIb mutants, cells harboring them were pulse labeled with [35S]methionine for 30 minutes and then lysed or chased with unlabeled methionine for 1 to 24 hours and then lysed. Total GPIIb was immunoprecipitated from cell lysates using SZ22. Figure 6A demonstrates that GPIIb levels in cells containing WT GPIIb decayed gradually and almost disappeared after 7 hours. GPIIb-alt.25 decayed slightly faster and GPIIb-del.20 decayed slightly slower than WT GPIIb. The generation and stability of the normal GPIIb/IIIa complex was examined by immunoprecipitation of the complex by 10E5 monoclonal antibody as described. Figure 6B demonstrates that the amount of normal GPIIb/IIIa increased during the first 2 hours and then declined. Because similar experiments with the mutants did not yield analyzable gels, probably due to diminished affinity of 10E5 to the mutated complexes, we precipitated the mutated complexes by AP3. The results showed that the GPIIb mutants complexed with GPIIIa and degraded in a pattern similar to WT complexes (Figure 6B). Collectively, these experiments suggest that mutant GPIIb/IIIa complexes formed and degraded at a rate that is similar to WT GPIIb/IIIa complex.

**Cellular localization of alt.25 and del.20 GPIIb mutants**

To determine the cellular localization of GPIIb-alt.25 and GPIIb-del.20 mutants, immunofluorescence staining was performed using concomitantly SZ22 monoclonal antibody against GPIIb and rabbit polyclonal antibodies against calnexin (an ER marker) or SZ22 monoclonal antibody against GPIIb together with rabbit polyclonal antibodies against mannose-2 II (a Golgi marker). Secondary antibodies used were antimouse FITC and antirabbit Texas red conjugates, which depict GPIIb in green, the ER or Golgi in red, and localization of GPIIb and the organelle markers in yellow. ER-calnexin distributed mainly around the nuclei (Figure 7A in red) while Golgi–mannose-2 II was aggregated in clumps (Figure 7B in red). In WT transfected cells GPIIb was distributed evenly all over the cell, including the plasma membrane (Figure 7A in green), and a fraction colocalized with both calnexin and mannose-2 II (yellow, Figure 7A and 7B, respectively). This suggested that some fraction of WT GPIIb could be found in the ER or the Golgi apparatus. In cells expressing the mutants GPIIb-del.20 and alt.25, GPIIb (green) was distributed mainly around the nuclei as calnexin (Figure 7A, red and yellow) while the mannose-2 II was aggregated in red clumps (Figure 7B). Only in 1 of 20 cells expressing GPIIb-alt.25 and in 1 of 25 cells expressing GPIIb-del.20 was a fraction of GPIIb colocalized with mannose-2 II—that is, was present in the Golgi. These results fit with the finding of minute amounts of mature GPIIb in immunoprecipitation experiments (Figure 4D). Taken together, these data suggest that the mutants GPIIb-del.20 and alt.25 were localized mostly in the ER and only trace amounts reached the Golgi apparatus.
The effects of the GPIIb mutants on the contact between GPIIb and GPIIIa

The models of β-propeller, calf-1, and calf-2 domains of GPIIb were structurally superimposed with β-propeller, calf-1, and calf-2 domains of αIIb, and the contacts of the respective domains with β3 subunit seemed to be similar (Figure 8A). The crystal structure of αβ3 was originally resolved at a resolution of 0.31 nm (3.1 Å), which does not enable accurate determination of interatomic distances. Nevertheless, approximations of important interactions, which stabilize the structure of the complex and the size of the interface between α and β3, were calculated by CSU software. The size of the αβ-propeller interface with β3 was the largest (24.28 nm²), whereas the sizes of the αβ-calf-1 and calf-2 interfaces with the β3 subunit were 2.74 nm² (9 amino acids) and 8.54 nm² (14 amino acids), respectively. Amino acid alignment of α and αIIb at the contact areas demonstrates that 3 of 9 amino acids were conserved in calf-1 and 5 of 14 amino acids were conserved in calf-2. None of the changes in the remaining amino acids was predicted to significantly hamper the contacts between calf-1 and calf-2 domains with the corresponding domains of β3.

In the calf-1 domain of GPIIb-del.20, 50 amino acids are missing (Figure 8B, black), among which 3 take part in the contact...
with β3 (1.64 nm²). Modeling of calf-1 of GPIIb-del.20 yielded a model with a score of 13.0, which is below the minimum score (17.8) for a correct protein of this size. Consequently, this model was not further analyzed. In the calf-2 domain of GPIIb-alt.25 the amino acid backbone is predicted to be shifted due to insertion of 8 amino acids replacing 10 amino acids of wild-type GPIIb. As a result of this shift, His788 was no longer in a position that allows contact with Tyr594 of β3. Also, the distance between Glu785 and Tyr594 was increased in comparison with the normal interactions (Figure 8C). Hence, GPIIIb-alt.25 is predicted to disrupt the contact of calf-2 domain with EGF-4 of β3 (1.47 nm²) but does not affect the other contacts with βTD.

**Discussion**

The presented data show that 2 major mutants, GPIIb-del.20 and GPIIb-alt.25 in the calf-1 and calf-2 domains, respectively, enable GPIIb/IIIa complex formation but impair its transport from the ER to the Golgi apparatus, leading to absent surface expression of this complex. The mutants of GPIIb were detectable mostly as pro-GPIIb (Figures 3-5) and were localized mainly in the ER (Figure 7), findings that are consistent with conversion of pro-GPIIb to mature GPIIb in the Golgi apparatus. Immunoprecipitation and pulse-chase experiments revealed that mutant GPIIb/IIIa complexes did form, although in apparently decreased amount, despite the markedly deranged GPIIb protein mutants. The stability of the mutant proteins when complexed with normal GPIIIa appeared to be unimpaired (Figure 6). However, because of the limitation of pulse-chase experiments it cannot be ruled out that some degradation of GPIIb mutants occurred within the 30 minutes of the pulse (Figure 6). The presence of a GPIIb degradation product derived from the mutant proteins but not from normal GPIIb (Figure 3B) is consistent with such an assumption. Decreased amounts of mutant GPIIb proteins were also observed in immunostaining experiments (Figure 2) and immunoblotting analyses performed under reduced conditions (Figures 3, 4A,C,D, 5B) but not in immunofluorescence experiments (Figure 7) and immunoblotting analyses performed under nonreduced conditions (Figures 4B, 5A). Conceivably, variable recognition of the mutants of GPIIb by the antibodies under the different experimental conditions was responsible for the discrepant results. Together the data suggest that GPIIb-del.20 has a substantially greater negative effect on overall GPIIb synthesis than GPIIb-alt.25.

Other, albeit point mutations in the calf-2 domain, for example, Gln747Pro,18 or in the calf-1 domain, for example, Cys674Arg,19 were also associated with preserved intracellular complex formation yet with profoundly reduced surface expression. Moreover, major mutations in the calf-2 domain of GPIIb, for example, skipping of exon 28,20,21 Ser870Stop,22 and deletion of the whole GPIIb light chain,23 were also shown to enable GPIIb/IIIa complex formation but impaired carbohydrate processing and surface expression of the complex. Finally, even a mutation associated with a deletion of both calf-1 and calf-2 domains (Arg597Stop) was recently found to enable formation of an unstable and rapidly degradable GPIIb/IIIa complex.24 Taken together, these observations suggest that significant changes in calf-1 or calf-2 domains do not prevent GPIIb/IIIa complex formation but do impair its transport to the cell surface, probably by its retention and degradation in the ER.

Our analysis of the α,β3 crystal structure and the predicted structure of αIIbβ3 model showed that the main intersubunit contact between αIIb and β3 subunit is the interface of the β-propeller of GPIIb and the βA domain of GPIIIa similar to what was described for the interface of α, and β3 in the α,β3 crystal. Disruptions of essential components in the β-propeller have consequently been shown to be associated with rapid degradation of the uncomplexed GPIIb mutants (del Ala106-Gln111, del Ser129-Ser161, Glu324Lys)25. In contrast, missense mutations in the calcium-binding domain of the β-propeller, located away from the interface with β3, Gly242Asp, Arg327His, Gly418Asp25 were shown to enable GPIIb/IIIa complex formation but resulted in its retention in the ER. These observations indicate that the calcium-binding domain is also essential for the transport of the GPIIb/IIIa complex from the ER to the Golgi.25,26

Our analyses of the GPIIb/IIIa model that was based on the crystal structure of α,β3 showed that significant contacts exist between calf-1 of α, or αIIb and EGF-3 of β3 and between calf-2 of α, or αIIb and EGF-4 and βTD of β3. Similar contacts between GPIIb and GPIIIa have also been shown by immunochromic methods6 and by a recent electron microscopy study that involved physicochemical measurements.9 In our analyses of the model, which are purely theoretical and unsupported by experimental data, the respective contacts were partially disrupted in mutant GPIIb-alt.25 and del.20. In GPIIb-alt.25 there was abolishment of the contact between amino group of Arg783 in α3, or of His877 in αIIb and the aromatic ring of Tyr594 in β3, which is a known stabilizing structure,27,28 and in GPIIb-del.20 there was a deletion of 3 amino acids that are in the contact area with β3 (Figure 8). Notwithstanding these significant disruptions, intracellular GPIIb/IIIa complex formation was formed and was stable. This supports the notion that the contacts between GPIIb and GPIIIa in the region of calf-1 and calf-2 domains are not essential for GPIIb/IIIa complex formation but apparently stabilize its bent conformation.9 We speculate that the partial disruption of these contacts in del.20 and alt.25 mutants gives rise to conformational changes that are responsible for retention of the aberrant GPIIb/IIIa complex in the ER.

**Acknowledgments**

We thank Dr Malka Reichart from Bioview and Maya Sokolovsky for their technical help with the visual computerized microscopy. We are grateful to the Bioinformatics Unit of the George S. Wise Faculty of Life Sciences at Tel Aviv University for providing technical assistance and computation facilities and to Barak Rotblat for his help with the protein modeling.

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