

Three novel β -propeller mutations causing Glanzmann thrombasthenia result in production of normally stable pro- α_{IIb} , but variably impaired progression of pro- $\alpha_{IIb}\beta_3$ from endoplasmic reticulum to Golgi

E. J. R. NELSON,* J. LI,* W. B. MITCHELL,* M. CHANDY,† A. SRIVASTAVA† and B. S. COLLER*

*Laboratory of Blood and Vascular Biology, The Rockefeller University, New York, NY, USA; and †Department of Hematology, Christian Medical College, Vellore, Tamilnadu, India

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Summary. *Background:* Glanzmann thrombasthenia (GT) is an autosomal recessive bleeding disorder characterized by lack of platelet aggregation in response to most physiological agonists and caused by either a lack or dysfunction of the platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa). *Objectives:* To determine the molecular basis of GT and characterize the mutations by *in vitro* expression studies. *Patients:* We studied three unrelated patients from southern India whose diagnosis was consistent with GT. *Results:* Immunoprecipitation of the cell lysates and immunoblotting showed no detectable mature α_{IIb} in the G128S mutant, in contrast to 6% and 33% of the normal amount of mature α_{IIb} in the S287L and G357S mutants, respectively. Pulse-chase analysis demonstrated pro- α_{IIb} in the mutants comparable with the normal pro- α_{IIb} , but no conversion to mature α_{IIb} in the G128S mutant, and only trace conversion to mature α_{IIb} in the S287L and G357S mutants. The disappearance of pro- α_{IIb} in the three mutants was similar to that in cells expressing normal $\alpha_{IIb}\beta_3$ or α_{IIb} only. All three mutants demonstrated pro- $\alpha_{IIb}\beta_3$ complexes and co-localized with an ER marker by immunofluorescence. The G128S mutant showed no co-localization with a Golgi marker, and the other two mutants showed minimal and moderate co-localization with the Golgi marker. *Conclusions:* These three β -propeller mutations do not affect the production of pro- α_{IIb} , its ability to complex with β_3 , or its stability, but do cause variable defects in transport of pro- $\alpha_{IIb}\beta_3$ complexes from the endoplasmic reticulum to the Golgi.

Keywords: $\alpha_{IIb}\beta_3$ biogenesis, β -propeller mutations, Glanzmann thrombasthenia.

Introduction

Glanzmann thrombasthenia (GT) is a rare, autosomal recessive bleeding disorder characterized by a life-long mucocutaneous bleeding tendency and absent or severely reduced platelet aggregation in response to the physiological agonists ADP, epinephrine, and collagen, but relatively normal aggregation in response to ristocetin [1–3]. The disease is caused by either a lack or dysfunction of the platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb/IIIa), which serves as a receptor for fibrinogen, von Willebrand factor, and perhaps other adhesive glycoproteins [4]. Mutations in either the α_{IIb} or β_3 genes have been identified in more than 100 patients with GT, and include minor or major deletions, insertions, inversions, and point mutations [5–8]. α_{IIb} and β_3 are synthesized as two independent polypeptide chains and enter the endoplasmic reticulum (ER) where they form a complex, undergo N-linked glycosylation and form intrachain disulphide bonds [9]. The complexes are then transported to the Golgi apparatus for final oligosaccharide processing and cleavage of α_{IIb} into heavy and light chains, before being transported to the membranes of α granules and the plasma membrane [10]. The assembly of the $\alpha_{IIb}\beta_3$ complex appears to be a prerequisite for transport out of the ER and thus cell surface expression [11,12]. α_{IIb} that is not complexed to β_3 is likely retained in the ER and degraded, while the uncomplexed β_3 can either be degraded or combine with an αV subunit to form the vitronectin receptor, $\alpha V\beta_3$ [13].

Mutations that impair the synthesis of either α_{IIb} or β_3 prevent the export of sufficient numbers of $\alpha_{IIb}\beta_3$ complexes to the platelet surface resulting in GT [1]. However, α_{IIb} or β_3 mutations that have no apparent effect on the synthesis of

Correspondence: Barry S. Coller, Laboratory of Blood and Vascular Biology, The Rockefeller University, 1230, York Avenue, New York, NY 10021-6399, USA.

E-mail: collerb@rockefeller.edu

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either subunit can also give rise to thrombasthenia, either by perturbing the conformation of pro- $\alpha_{IIb}\beta_3$ complexes so they fail to be exported out of the ER [14–21] or by impairing $\alpha_{IIb}\beta_3$ function [22–28]. So far, 20 missense mutations causing GT have been reported within the β -propeller domain of α_{IIb} , the region that both complexes with β_3 and contributes to the ligand binding site. Thirteen of these mutations were associated with reduced surface expression, of which six mutations namely, G242D [15], V298F [20], E324K [19], R327H [16], I374T [20], and G418D [14] were directly demonstrated to disrupt biogenesis by preventing transport of pro- $\alpha_{IIb}\beta_3$ from the ER to the Golgi, leading to intracellular retention. In this paper, we describe three new missense mutations, G128S, S287L and G357S within α_{IIb} β -propeller blades 2, 5 and 6; all three are located on the upper face of propeller in the area involved in interaction with β_3 . We show that all three mutant pro- α_{IIb} were synthesized, stable and able to form a complex with β_3 . The G128S mutation completely prevents the transport of the pro- $\alpha_{IIb}\beta_3$ complex from the ER to the Golgi, whereas the S287L and G357S mutations variably impair transport from the ER to the Golgi and subsequent surface expression.

Patients

Patient 1, from Andhra Pradesh, was diagnosed as having GT at 4 years of age based on a history of easy bruising, epistaxis, prolonged bleeding time (more than 15 min), absent clot retraction, and absent *in vitro* platelet aggregation in response to ADP, epinephrine, and collagen. His parents were first-degree relatives (uncle-niece), but had no bleeding symptoms. Patient 2, also from Andhra Pradesh, was diagnosed at 5 years of age based on a history of epistaxis, gum bleeding, hematuria, reduced clot retraction, and absent *in vitro* platelet aggregation in response to ADP, epinephrine, and collagen, but a normal initial phase response to ristocetin. His parents were also first-degree relatives (uncle-niece) and had no bleeding symptoms. Patient 3, from Tamil Nadu, had more mild symptoms (gingival bleeding) and was diagnosed at 21 years of age based on prolonged bleeding time (more than 15 min), absent *in vitro* platelet aggregation in response to ADP, epinephrine, and collagen, but a normal initial phase response to ristocetin. His parents had no significant bleeding symptoms, but two of his paternal aunts died of bleeding complications before 10 years of age. There was no known consanguinity.

Materials and methods

Immunoblot analysis of platelet lysates

Immunoblot analysis of washed platelets was performed essentially as previously described [29] using murine mAbs specific for α_{IIb} (CD41, SZ22) and β_3 (CD61, SZ21) [Immunotech, Marseille, Cedex, France]. Samples for α_{IIb} were tested reduced to display both pro- α_{IIb} and the heavy chain of

mature (cleaved) α_{IIb} , and samples for β_3 were tested non-reduced.

Polymerase chain reaction, single strand conformation polymorphism analysis and DNA sequencing

A total of 38 polymerase chain reaction (PCR) reactions were performed on genomic DNA extracted from buffy coat cells using intragenic primers and conditions that were previously described [30,31] and additional primers and conditions that can be obtained by contacting one of us (B.S.C). Single strand conformation polymorphism (SSCP) analysis was performed by either an automated (PhastGel System; Pharmacia Biotech, Uppsala, Sweden) or manual method (Protean II Cell; Bio-Rad, Hercules, CA, USA). DNA sequencing was performed on amplicons that showed mobility shifts by SSCP analysis (Big Dye terminator kit; PE Biosystems, Foster City, CA, USA).

Site-directed mutagenesis

Mutant α_{IIb} cDNA constructs were generated by site-directed mutagenesis (QuikChange XL Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA). Wild-type α_{IIb} cDNA in the pEF1/V5-His mammalian expression vector was used as the template (kindly provided by Dr Junichi Takagi, Harvard University, Boston, MA, USA). After mutagenesis, the mutant cDNA was used to transform XL10-Gold ultracompetent cells as per the manufacturer's instructions (Stratagene).

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids (GIBCO, Carlsbad, CA, USA), and 1% each of penicillin and streptomycin. Cells were grown to 70–80% confluency and then transfected with vector alone (mock), or normal α_{IIb} cDNA alone (about 7.5 μ g), or normal or mutant α_{IIb} cDNA (about 7.5 μ g) co-transfected with an equal amount of normal β_3 cDNA in pcDNA 3.1/Myc-His vector (also provided by Dr Takagi) (Perfectin Transfection Reagent; Gene Therapy Systems, San Diego, CA, USA). Forty-eight hours after transfection, stable cell lines were generated by G418 selection (0.5 mg/mL, GIBCO) for 14 days. To ensure comparability among cell lines, stable cell lines were not selected for high surface $\alpha_{IIb}\beta_3$ expression.

Flow cytometry

Transfected cells were harvested [0.05% trypsin, 1 mM ethylenediaminetetraacetic acid (EDTA)], washed, and incubated with Alexa 488-labeled mouse mAbs 10E5 ($\alpha_{IIb}\beta_3$) or 7E3 ($\alpha_{IIb}\beta_3 + \alpha V\beta_3$) for 30 min 22 °C before dilution with phosphate-buffered saline (PBS) and analysis by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA, USA).

Immunoprecipitation and immunoblotting

Lysates of transfected cells [50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM N-ethyl maleimide, 0.2% protease inhibitor mixture (Protease Inhibitor Cocktail set III, Calbiochem, San Diego, CA, USA)] were precleared with protein G-Sepharose beads (Amersham Pharmacia Biotech), and then incubated with 2 μg of mouse anti-V5 tag (anti- α_{IIb}) mAb (Invitrogen Corporation, Carlsbad, CA, USA) at 4 °C overnight followed by protein G beads. Immunoprecipitates were eluted from the beads by incubating in sodium dodecyl sulfate (SDS) sample buffer, with (for α_{IIb}), or without (for β_3) 10% β -mercaptoethanol at 100 °C for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting was performed with mouse mAbs CA3 (anti- α_{IIb} ; Chemicon International, Temecula, CA, USA) or CD61 (anti- β_3 ; Dako Corporation, Carpinteria, CA, USA).

Pulse-chase analysis

Stably transfected cells were incubated in methionine- and cysteine-free DMEM for 30 min and then incubated with 480 μCi of ^{35}S protein labeling mix (Perkin Elmer Life Sciences, Boston, MA, USA) at 37 °C for 15 min. Cells were incubated with DMEM containing 1 mg mL⁻¹ each of cold methionine and cysteine for 0, 1, 2, 4, 8 and 24 h at 37 °C. Cells were then harvested and solubilized; after preclearing the lysates with protein G beads, 2 μg of anti-V5 tag antibody was added, followed by protein G beads. The beads were washed twice and immunoprecipitates were eluted in SDS sample buffer containing 10% β -mercaptoethanol. After SDS-PAGE, gels were fixed, washed, soaked in an autoradiographic image intensifying reagent (Autofluor; National Diagnostics, Atlanta, GA, USA), dried, and subjected to autoradiography.

Immunofluorescence microscopy

Transfected cells were grown overnight in DMEM on poly-L-lysine-coated glass cover slips, washed twice, and fixed in ice-cold acetone-methanol (1:1). The cells were washed, incubated in blocking solution [2.5% bovine serum albumin (BSA)/0.05% NP-40 in PBS] and reacted with either rabbit anti-calnexin polyclonal antibody (ER marker, 1:200 dilution; Stressgen Bioreagents, Victoria, BC, Canada) or rabbit anti-mannosidase II polyclonal antibody (Golgi marker, 1:100 dilution; US Biological, Swampscott, MA, USA), followed by labeling with TRITC-conjugated F(ab')₂ fragment of goat anti-rabbit IgG (H + L) (Jackson Immuno-research Laboratories, West Grove, PA, USA). The cells were washed thrice and then incubated with a 1:100 dilution of FITC-conjugated anti- α_{IIb} antibody (SZ22). The cover slips were then washed, air-dried, mounted onto a glass slide with antifade solution (Molecular Probes, Eugene, OR, USA), and analyzed using an inverted microscope (Olympus

IX70, Melville, NY, USA). Images were deconvolved using Deltavision software (Applied Precision, Issaquah, WA, USA).

Results

Platelet content of $\alpha_{IIb}\beta_3$

Immunoblot analysis of platelet lysates showed no detectable pro- α_{IIb} or mature α_{IIb} in any of the patient samples; trace amounts of β_3 were detected only in samples from patients 2 and 3, but no β_3 was detectable in the sample from patient 1 (data not shown).

Mutation detection

The SSCP analysis of the genomic DNA of patient 1 revealed a mobility shift in the PCR product of exon 4 of α_{IIb} and DNA sequencing confirmed a G → A nucleotide substitution at position 475 of the pro- α_{IIb} sequence. This predicts a substitution of glycine to serine at amino acid position 128 within the β -propeller domain at the end of the S3-S4 loop of blade 2, just before the third β strand (W2S3) [32,33]. In patient 2, a mobility shift was observed in the PCR product of exon 11 of α_{IIb} , and this was found to be a result of C → T nucleotide substitution at position 953 of the pro- α_{IIb} sequence. This predicts a substitution of serine to leucine at amino acid position 287, which is located in the β -propeller domain just before the beginning of the first β strand in blade 5 (W5S1). In patient 3, a mobility shift was observed in the PCR product of exon 12 of α_{IIb} and a G → A nucleotide substitution at position 1162 of the pro- α_{IIb} sequence was subsequently identified. This predicts a substitution of glycine to serine at amino acid position 357, which is also located in the β -propeller domain, in the highly conserved region just before the first β strand in blade 6 (W6S1).

Expression of mutant $\alpha_{IIb}\beta_3$ receptors in HEK 293 cells

Fifty-two percent of cells transfected with normal α_{IIb} and β_3 bound 10E5 (MFI-43), whereas mock transfected cells (vector alone) showed minimal 10E5 binding (5%). In sharp contrast, only 0, 2, and 8% of the G128S, S287L and G357S mutant cells bound 10E5 above background, and among the positive cells the extent of binding was lower (MFIs 14, 14, and 35). Immunoprecipitation of the cell lysates with an antibody to α_{IIb} , followed by immunoblotting with an antibody to α_{IIb} showed the presence of pro- α_{IIb} in the normal and all three mutant cells (Fig. 1, top panel). While mature α_{IIb} was seen in the normal $\alpha_{IIb}\beta_3$ cells, there was no detectable mature α_{IIb} in the G128S mutant, 6% of the normal value of mature α_{IIb} in the S287L mutant, and 33% of the normal value in the G357S mutant. Immunoprecipitation with an antibody to α_{IIb} also pulled down β_3 as part of the $\alpha_{IIb}\beta_3$ complex (Fig. 1, bottom panel). β_3 was not

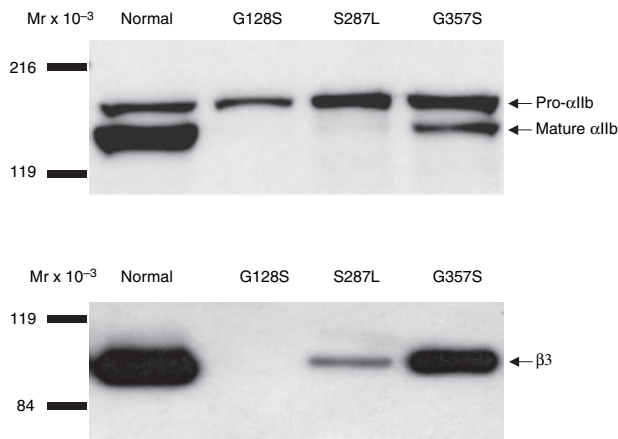


Fig. 1. Immunoprecipitation and immunoblotting of the transfected HEK 293 cells. Cell lines were solubilized in lysis buffer and immunoprecipitated with an antibody to the V5 epitope tag of the α_{IIb} subunit. Immunocomplexes were analyzed by immunoblotting under reducing (α_{IIb}) or non-reducing (β_3) conditions using an antibody to α_{IIb} (CA3, top) or β_3 (CD61, bottom). Pro- α_{IIb} , mature α_{IIb} and β_3 are indicated by arrows.

detected in cells expressing the G128S mutant, while 6% and 35% of the normal amount of β_3 was pulled down in complex with the S287L and G357S α_{IIb} mutants, respectively.

Pro- α_{IIb} subunits of the G128S, S287L and G357S mutants are synthesized and degraded at rates similar to normal pro- α_{IIb} , but show little or no progression to mature α_{IIb}

Cells expressing normal $\alpha_{IIb}\beta_3$ demonstrated maximum synthesis of pro- α_{IIb} within 1–2 h, progressive maturation of a portion of the pro- α_{IIb} to mature, cleaved α_{IIb} over approximately 8 h, and disappearance of pro- α_{IIb} between 8 and 24 h (Fig. 2A). Cells transfected with normal α_{IIb} alone gave a pattern of pro- α_{IIb} synthesis and disappearance very similar to the cells co-transfected with both normal α_{IIb} and β_3 , with no obvious evidence of more rapid degradation. Pro- α_{IIb} molecules were also synthesized at the normal rate by the three mutant $\alpha_{IIb}\beta_3$ cells, but there was no progression to mature α_{IIb} in the G128S mutant, only trace progression to mature α_{IIb} in the S287L mutant, and slightly more progression to mature α_{IIb} in the G357S mutant (Fig. 2A). Of note, the small amounts of mature α_{IIb} observed in the S287L and G357S mutants appeared very early in the time course and did not increase thereafter in density. The rates of disappearance of pro- α_{IIb} in the three mutant cells were very similar to the rates in the cells expressing α_{IIb} alone and normal $\alpha_{IIb}\beta_3$, with a half-disappearance time of approximately 4 h (Fig. 2B). In contrast to cells transfected with normal $\alpha_{IIb}\beta_3$, however, all three mutant cells showed some increase in relative pro- α_{IIb} values over the first 2 h. Thus, despite their defects in progression to mature α_{IIb} , the G128S, S287L and G357S mutations did not affect either the production of pro- α_{IIb} or its rate of degradation.

Detection of pro- $\alpha_{IIb}\beta_3$ complexes in normal and mutant receptors

To assess whether the mutant pro- α_{IIb} subunits formed complexes with β_3 , immunoprecipitations of pulse-labeled cells 1 h after the chase were performed with antibodies to β_3 . Cells transfected with normal $\alpha_{IIb}\beta_3$ demonstrated pro- α_{IIb} immunoprecipitating with antibody to β_3 (Fig. 2C) whereas cells transfected with α_{IIb} alone did not (data not shown). All three mutant cells demonstrated pro- α_{IIb} immunoprecipitating with antibodies to β_3 (Fig. 2C). The pro- $\alpha_{IIb}\beta_3$ complexes of all three mutants were also detected up to 8 h postchase in a separate experiment (data not shown).

Progression of $\alpha_{IIb}\beta_3$ from the ER to Golgi to surface membrane in normal and mutant cells

Fluorescent labeling of α_{IIb} in cells transfected with normal $\alpha_{IIb}\beta_3$ demonstrated strong staining at the periphery of the cell consistent with $\alpha_{IIb}\beta_3$ surface expression, and diffuse staining throughout the remainder of the cell (Fig. 3A, left top and bottom panels). The ER (calnexin) stain was distributed diffusely throughout the cell in clusters that were more accentuated near the periphery (Fig. 3A, top center panel), while the Golgi (mannosidase II) stain was present in more discrete clusters, primarily in the periphery (Fig. 3A, bottom center panel). The merged image of α_{IIb} and calnexin demonstrated extensive co-localization, seen as yellow, indicating the presence of α_{IIb} in the ER (Fig. 3A, top right panel). The cell surface $\alpha_{IIb}\beta_3$, however, appeared green, indicating that it did not co-localize with the calnexin stain, and thus served as a control. In the normal $\alpha_{IIb}\beta_3$ cells, α_{IIb} stain also co-localized with the mannosidase-II stain (Fig. 3A, bottom right panel), indicating the presence of α_{IIb} in the Golgi. The insert figures in Fig. 3A are from mock transfected cells demonstrating the absence of α_{IIb} , but the presence of calnexin- and mannosidase-II positive organelles. The G128S mutant showed enhanced staining for α_{IIb} in a ring-like configuration within the cell, but no surface α_{IIb} staining (Fig. 3B, left top and bottom panels). Co-localization with calnexin (Fig. 3B, top center and right panels) indicated that the vast majority of the α_{IIb} was in the ER. In the α_{IIb} and mannosidase-II merged image, the mannosidase-II-positive organelles stained red rather than yellow, indicating little or no presence of G128S $\alpha_{IIb}\beta_3$ in the Golgi (Fig. 3B, bottom center and right panels). The S287L mutant cells demonstrated barely detectable surface labeling for α_{IIb} , strong ring-like staining for α_{IIb} inside the cell that co-localized with calnexin, indicating an ER localization, and some co-localization with mannosidase-II (Fig. 3C). In contrast, a small amount of surface α_{IIb} was detectable in the G357S mutant cells and there was stronger co-localization with mannosidase-II, indicating that more G357S $\alpha_{IIb}\beta_3$ was transported to the Golgi. The strong ring-like staining for α_{IIb} , which co-localized with calnexin was also observed in these cells, indicating the presence of G357S α_{IIb} in the ER (Fig. 3D).

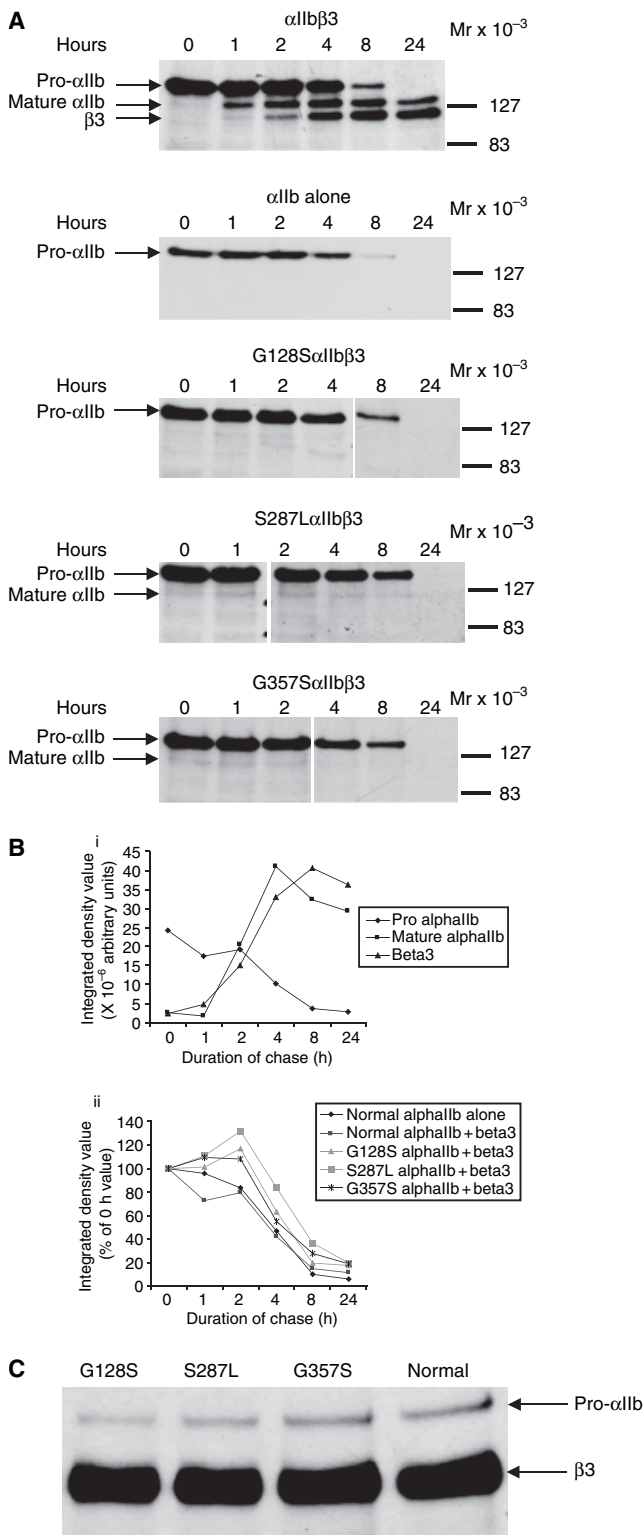


Fig. 2. Pulse-chase analysis of transfected HEK 293 cells expressing normal or mutant $\alpha_{IIb}\beta_3$ receptors. (A) Stable cell lines expressing normal or mutant $\alpha_{IIb}\beta_3$ were pulsed with ³⁵S-methionine- and cysteine-containing medium for 15 min and chased in medium containing unlabeled methionine and cysteine for 0, 1, 2, 4, 8, and 24 h. The cell lysates were immunoprecipitated with an antibody to the V5-epitope tag on α_{IIb} . Samples were electrophoresed under reduced conditions. Bands representing pro- α_{IIb} , mature α_{IIb} , and β_3 are indicated by arrows. (B) (i) Kinetics of pro- α_{IIb} , mature α_{IIb} , and β_3 in cell lines transfected with normal α_{IIb} and β_3 subunits as judged by band densities. There was a gradual decrease in pro- α_{IIb} levels and this was mirrored by both an increase in mature α_{IIb} over 24 h and an increase in β_3 associated with α_{IIb} . (ii) Kinetics of pro- α_{IIb} in cell lines transfected with α_{IIb} only or normal or mutant $\alpha_{IIb}\beta_3$ receptors. All three mutant α_{IIb} subunits demonstrated similar rates of degradation, which were similar to the rates in the normal $\alpha_{IIb}\beta_3$ and α_{IIb} only cell lines. (C) Immunoprecipitation with an antibody to β_3 (7H2) 1 h after pulse-chase. Bands representing pro- α_{IIb} and β_3 are indicated by arrows.

[33] using the MOLEcule analysis and MOLEcule display (MOLMOL) software. The residues glycine 128 and serine 287 are at the top of the propeller and lie within the interface with the β_A domain of β_3 , while the glycine 357 is buried deeper within the propeller structure (Fig. 4A). All three mutations are located among the residues contributing to the highly conserved ‘cage’ motif, which consists of two concentric rings of predominantly aromatic residues within the β -propeller structure [32]. This motif has been shown to contain a consensus sequence, $(X_{17-33}\{\phi\phi G\phi X_{13-20}PX_{2-15}GX_{5-8}\})_7$, where X is any residue and ϕ is an aromatic residue (Fig. 4B) [32]. It is defined by a mostly aromatic 4-residue ‘cup’ ($\phi\phi G\phi$) that precedes the first β strand in each propeller blade (A), a proline immediately following the second β strand (B), referred to as **Pro-B**, and an invariant glycine at the beginning of the third β strand (C), referred to as **Gly-C** [32]. Glycine 128 is located at the position of the invariant glycine in blade 2. The conservation of glycine at this or an adjacent relative location in all seven β -propeller blades of α_{IIb} , α_V , α_1 - α_{11} , α_M , α_L , and α_X subunits in humans indicates the importance of having the small size and/or flexibility of glycine in this region. The serine 287 is located immediately adjacent to the $\phi\phi G\phi$ ‘cup’ in blade 5, and is conserved in α_1 , α_2 , α_4 - α_{11} , α_L , and α_X subunits in humans. The glycine 357 is located directly within the ‘cup’ of blade 6 and is conserved in α_V , α_1 - α_{11} , α_L , α_M , and α_X subunits in humans. Of especial note, a previously described GT mutation (G418D) is in the same position as glycine 357 in the ‘cup’ of α_{IIb} blade 7 [14]. The three mutated α_{IIb} residues are also conserved across species, including α_{IIb} of mouse, rat, rabbit, horse, pig, and dog.

Discussion

We have characterized three separate α_{IIb} mutations in the β -propeller domain, in blades 2 (G128S), 5 (S287L), and 6 (G357S), in three unrelated patients with GT from southern India. The pro- α_{IIb} of the G128S mutant was synthesized in HEK 293 cells and formed a complex with β_3 but we could not

Locations of the three mutations in the crystal structure of $\alpha_{IIb}\beta_3$ and their potential structural implications

The locations of the α_{IIb} β -propeller mutations, G128S, S287L, and G357S were identified on the crystal structure of $\alpha_{IIb}\beta_3$

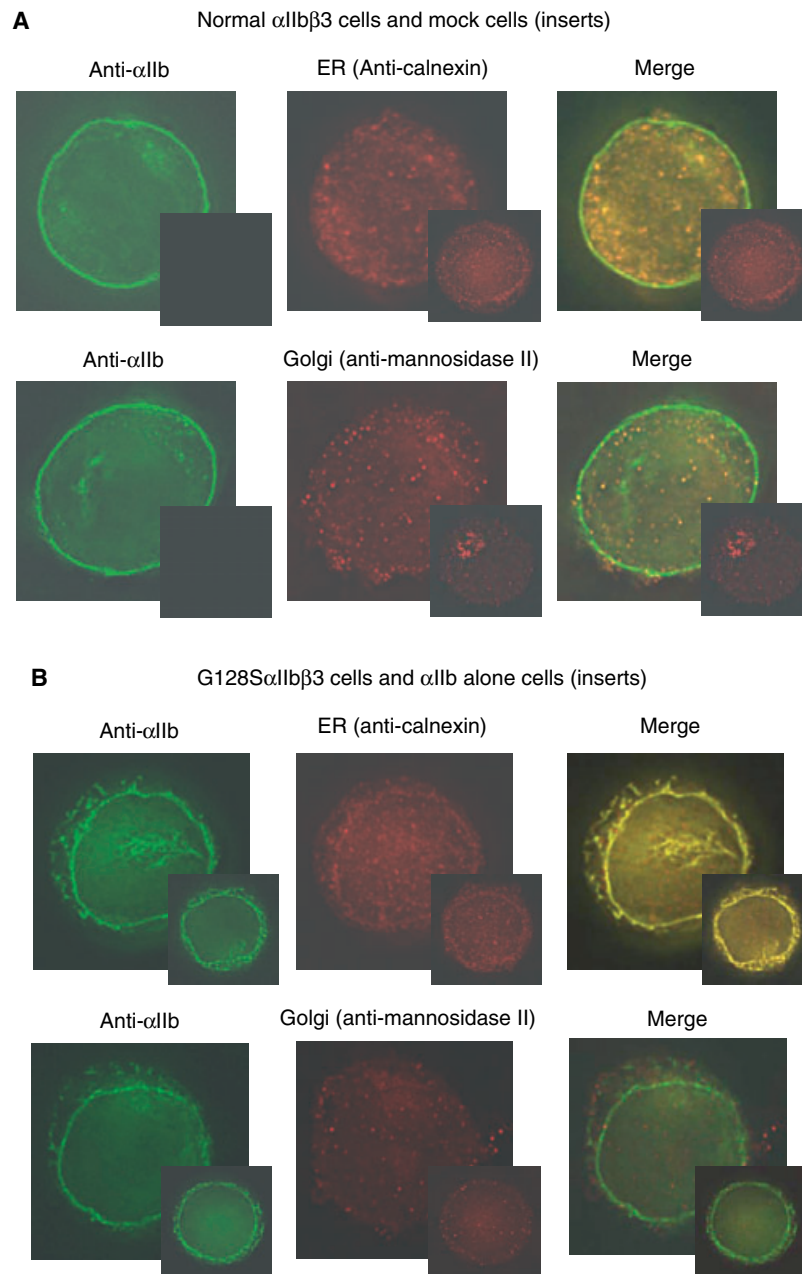


Fig. 3. Co-localization of α IIb in the ER and Golgi apparatus in the transfected HEK 293 cells. Transfected cells were labeled with an anti- α IIb antibody (green), and either an antibody to an ER component (calnexin) or a Golgi component (mannosidase II) (red). (A) Cells transfected with normal α IIb β 3 showed strong labeling of the cell surface, evident as a green outline, indicating normal α IIb β 3 expression. In addition, there is α IIb staining throughout the cell, some of which co-localizes with the ER marker and some with the Golgi marker, as indicated by the yellow color of the merged images. In contrast, mock transfected cells, shown here as inserts adjacent to the corresponding images of the normal cells, showed no α IIb staining either outside or inside the cell, but did show staining of the ER and Golgi. (B) Surface staining of α IIb was not observed in cells transfected with the G128S α IIb β 3 mutant. α IIb staining of the G128S α IIb β 3 mutant co-localized with the ER marker, indicated by the yellow color of the merged image, but not with the Golgi marker. The intensity of ER staining by anti-calnexin antibody in the G128S α IIb β 3 mutant cells was greater than in the normal α IIb β 3 cells. These results are comparable to cells transfected with α IIb alone (inserts). (C) S287L α IIb β 3 mutant cells demonstrated minimal amounts of α IIb on the surface. This mutant α IIb co-localized with the ER marker, but showed only faint co-localization with the Golgi marker, implying egress of only a small amount of S287L α IIb β 3 from the ER to Golgi. (D) In contrast, the G357S α IIb β 3 mutant cells demonstrated faint, but detectable surface labeling, and showed α IIb staining co-localizing with both the ER and the Golgi markers.

identify any mature α IIb β 3 complex either inside or on the surface of the cells. Moreover, although the G128S α IIb β 3 colocalized with the ER marker, it did not colocalize with the

Golgi marker. As a result we conclude that the α IIb G128S mutation profoundly interferes with transport of the pro- α IIb β 3 complex to the Golgi apparatus and subsequent processing to

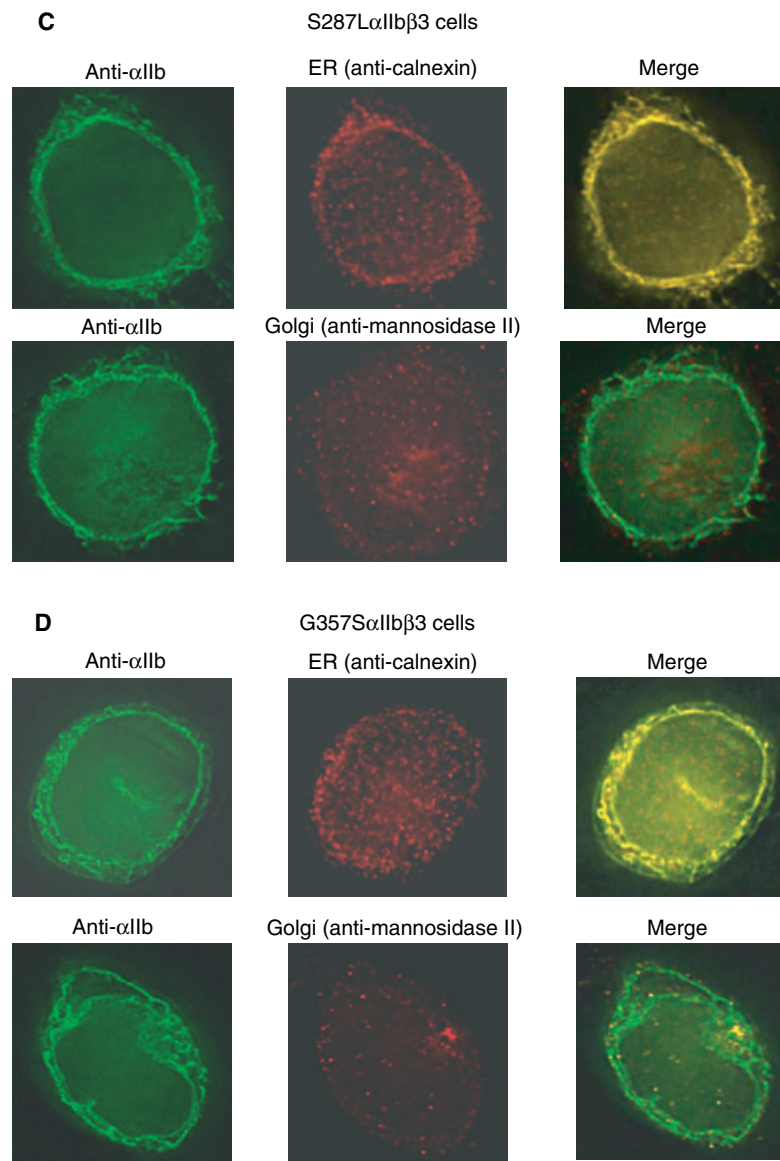


Fig. 3. Continued.

mature, cleaved α IIb. Although HEK 293 cells differ from megakaryocytes, our data indicating the need for pro- α IIb β 3 transport to the Golgi as a prerequisite for processing of pro- α IIb to mature α IIb are similar to those previously reported in megakaryocytes [10].

The S287L and G357S mutations in patients 2 and 3 respectively, resulted in abnormalities similar to those in patient 1, but the defects in α IIb β 3 complex formation, transport to the Golgi, and surface expression were less severe. Of note, the pro- α IIb in all three mutations was synthesized and disappeared with kinetics that were similar to normal pro- α IIb when expressed either alone or in combination with normal β 3. Similar observations have been made with other mutations affecting the α IIb β -propeller domain

[14,15]. Moreover, as the overall disappearance of pro- α IIb from cells lacking β 3 is not more rapid than in cells containing normal β 3, these data suggest that the rate of pro- α IIb degradation is not primarily determined by the formation of the pro- α IIb β 3 complex. Immunoprecipitation with anti- β 3 antibody detected only a small amount of pro- α IIb in complex with β 3 in cells expressing normal and mutant α IIb β 3 receptors.

The availability of the crystal structures of α V β 3 and α IIb β 3 permits us to correlate the molecular abnormalities in the α IIb β -propeller domain in GT patients with their functional consequences [32,33]. The various mutations identified in the β -propeller domain thus far, including the ones reported in this paper, are depicted in the ribbon diagram model of the

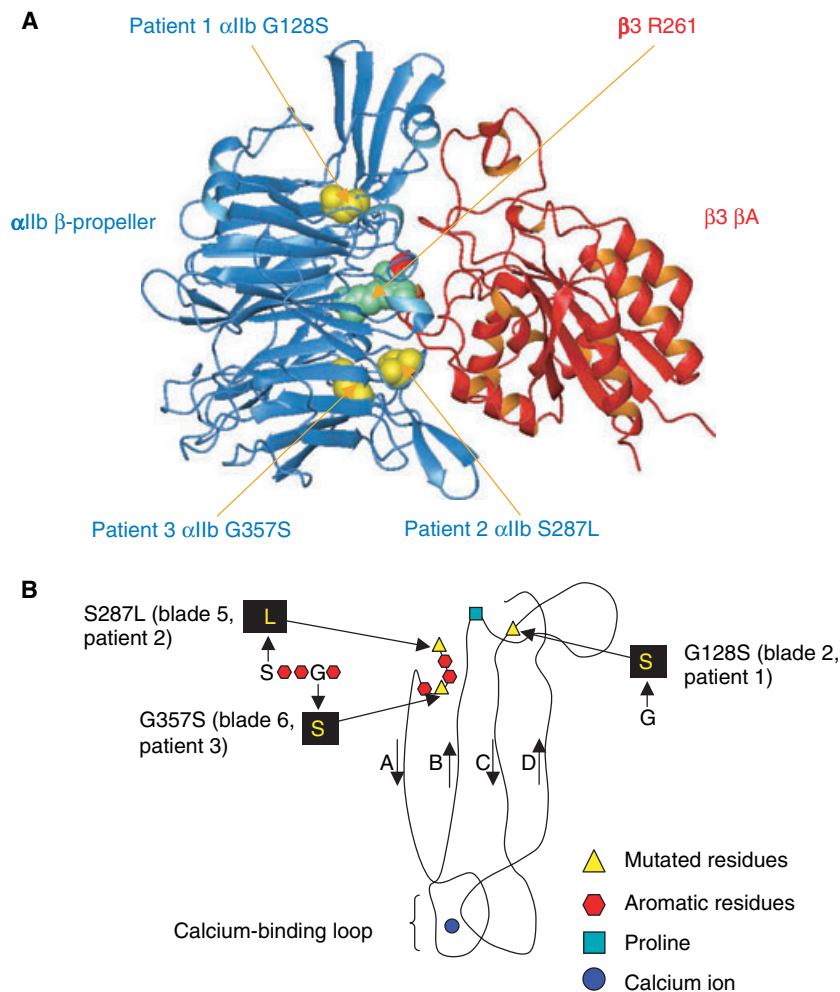


Fig. 4. Location of the three mutations in the $\alpha_{IIb}\beta_3$ crystal structure. (A) The crystal structure of $\alpha_{IIb}\beta_3$ [33] is shown, highlighting the interface between the β -propeller domain of α_{IIb} (blue) and the βA domain of β_3 (red). The mutated residues of α_{IIb} (yellow), and the R261 of β_3 (green) are represented by space-filling models, and the remainder of the protein is shown as a ribbon diagram. (B) Schematic diagram of one blade of the α_{IIb} β -propeller (viewed from the side), derived from the crystal structure, showing residues of the cage motif and the relative positions of the three mutations reported in this study, which lie in blades 2, 5 and 6, respectively.

$\alpha_{IIb}\beta_3$ in Fig. 5A,B. The vast majority of the missense mutations that have been characterized result in decreased or absent $\alpha_{IIb}\beta_3$ surface expression. The two missense mutations that affect ligand binding (Y143H [27] and P145A [26]) are located adjacent to the α_{IIb} -specific cap domain that has been implicated in ligand binding [33]. Many of the other mutations are at the interface between the α_{IIb} β -propeller domain and the β_3 βA -domain, where one might have expected them to interfere with complex formation between pro- α_{IIb} and β_3 . However, in our studies [20] and in studies conducted by others [14–17,19,24,28], immunoprecipitation experiments demonstrated the presence of pro- $\alpha_{IIb}\beta_3$ complexes. These observations are, in fact, consistent with the studies of Wilcox *et al.* [16], Basani *et al.* [17], and McKay *et al.* [34], who demonstrated that recombinant forms of α_{IIb} containing only the first three blades of the propeller could

form a complex with β_3 . Even among mutations affecting the non-cap region of the first three blades of α_{IIb} (Y143H [27], P145A [26], P145L [26], F171C [21], L183P [24], and the G128S that we now report), only the F171C mutation resulted in failure to form the pro- $\alpha_{IIb}\beta_3$ complex, and even in this case it is uncertain whether this resulted from it selectively altering the interface between pro- α_{IIb} and β_3 or from it causing more general disruption of pro- α_{IIb} folding as a result of the introduction of a new unpaired cysteine residue. Several α_{IIb} β -propeller mutations affect the regions in blades 4–7 that are near to or in the β -hairpin calcium binding loops [14–17,19]. These mutations also do not affect pro- $\alpha_{IIb}\beta_3$ complex formation, but dramatically affect receptor maturation and surface expression.

The major limiting step in $\alpha_{IIb}\beta_3$ biogenesis in the mutations we describe and most of the previously described mutations is

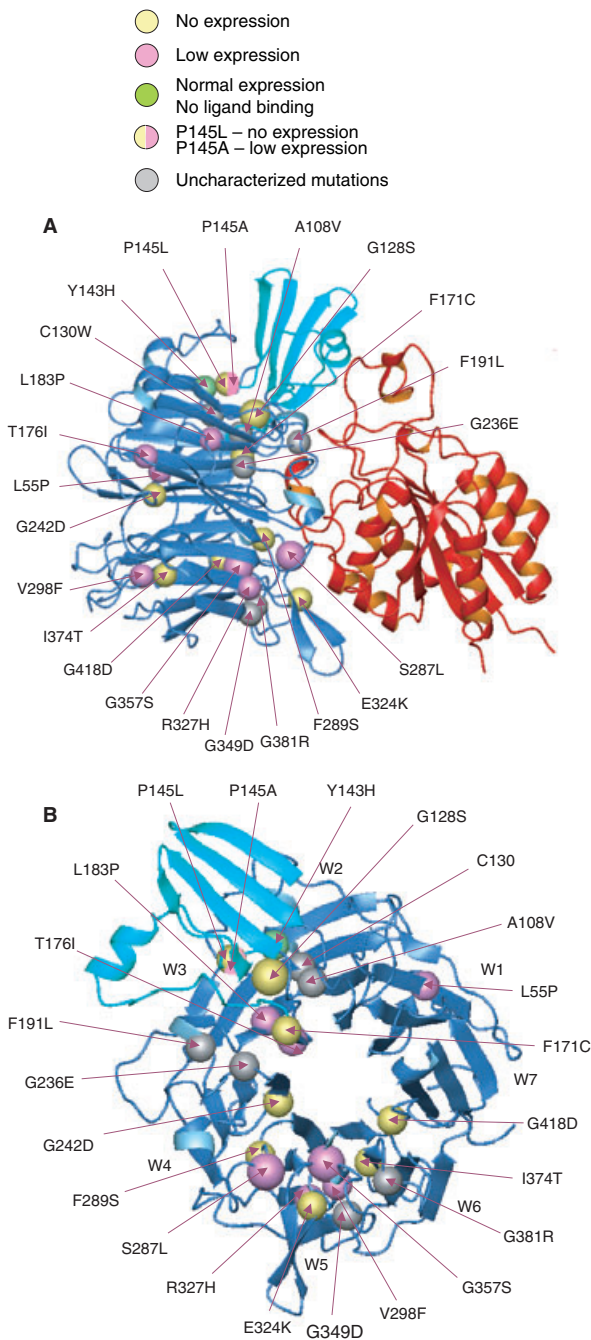


Fig. 5. Localization of the β -propeller mutations identified thus far in the α IIb β 3 crystal structure. (A) Crystal structure of the interface between the β propeller domain of α IIb (blue) and β A domain of β 3 (red) showing the location of the various β -propeller mutations reported thus far (including the ones reported in this paper). Mutations that result in no or low surface expression of α IIb β 3 are depicted in yellow and magenta, respectively. The Y143H mutation which affected ligand binding, but had little effect on surface expression, is shown in green, and lies adjacent to the cap region (cyan), implicated in ligand binding. Note that the mutations P145A, T176I, and L183P affected ligand binding as well as surface expression. The substitution of leucine or alanine for proline at position 145, respectively, resulted in no or low expression and this is depicted by a sphere that is half yellow and half magenta. Published mutations on which no data are available on either platelet surface expression or *in vitro* expression are depicted in gray. (B) Crystal structure of the β -propeller domain of α IIb, as seen from the surface facing the β 3 β A domain showing the various mutations published in the literature (including the ones in this paper). The central core formed by two concentric rings of aromatic residues is key to interacting with the β A domain of β 3, which is not shown here.

mutant proteins [14–16]. It is unlikely that the quality control mechanism involves a ligand-like molecule as some mutations that profoundly affect ligand binding do not result in markedly reduced receptor expression, as for example, β 3 D119Y [22,35] and R214Q [36,37]. Alterations in binding to a chaperone-like protein(s) seem likely.

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Conflict of interest disclosure

Dr Coller is an inventor of abciximab and in accord with Federal Law and the policies of the Research Foundation of the State University of New York, shares in royalties paid to the Foundation for sales of abciximab. No other authors have conflicts of interest.

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not, therefore, complex formation, but rather transport from the ER to the Golgi for carbohydrate modification, pro- α IIb cleavage, and transport to the cell surface. It is likely, therefore, that a molecular mechanism exists to assess the quality of the folding of pro- α IIb and/or the conformation of the pro- α IIb β 3 complex as a prelude to transport out of the ER. Direct support for alterations in the conformation of the pro- α IIb β 3 complex as a result of a number of the reported mutations (V298F, I374T, G242D, and G418D) derives from studies demonstrating lack of binding of monoclonal antibodies that are α IIb β 3 complex-specific or conformation-specific to the

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