Type II Glanzmann thrombasthenia in a compound heterozygote for the αIIb gene. A novel missense mutation in exon 27

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Background and Objectives. Glanzmann thrombasthenia is an autosomal recessive bleeding disorder characterized by a life-long hemorrhagic tendency and absent or severely reduced platelet aggregation in response to agonists, caused by quantitative or qualitative abnormalities in the platelet fibrinogen receptor, integrin αIIbβ3. The aim of this study was to identify the molecular genetic defect and determine its functional consequences in a patient with type II Glanzmann thrombasthenia.

Design and Methods. The expression of platelet αIIbβ3 was determined by flow cytometry and western blotting. Mutations were identified by sequencing both cDNA and genomic DNA. Functional characterization was assessed by exontrap and transient transfection analysis.

Results. Flow cytometry and western blot analysis revealed markedly reduced levels of platelet αIIbβ3, which may account for the residual fibrinogen binding detected upon platelet activation. Sequencing of genomic DNA revealed the presence of two mutations in the αIIb gene: a C1750T transition in the last codon of exon 17 changing Arg553 to STOP, and a C2829T transition in exon 27 that changes Pro912 to Leu. Sequence analysis of reversely transcribed αIIb mRNA did not detect cDNA from the C1750T mutant allele, and revealed a significant increase of the physiological splicing out of exon 28 in the cDNA carrying the C2829T mutation. Transient expression of [912Leu]αIIb in CHO-β3 cells showed a marked reduction in the rate of surface expression of αIIbβ3.

Interpretation and Conclusions. The results suggest that the thrombasthenic phenotype is the result of reduced availability of αIIb-mRNA, enhanced expression of exon 28-deleted transcripts, and defective processing of [912Leu]αIIb.

Key words: αIbβ3, Glanzmann thrombasthenia, splicing mutations, platelets.

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Glanzmann thrombasthenia is an autosomal recessive bleeding disorder characterized by a life-long hemorrhagic tendency and absent or severely reduced platelet aggregation in response to agonists.[1,2] The thrombasthenic phenotype is associated with quantitative or qualitative abnormalities in the platelet fibrinogen receptor, the αIIbβ3 integrin or glycoprotein (GP) IIb-IIIa, which can also serve as a platelet receptor for fibronectin, vitronectin and von Willebrand factor.[3-5] Heterozygous individuals, with 50-60% the normal amount of platelet αIIbβ3, have no abnormalities of platelet function and are clinically asymptomatic. The platelet content of αIIbβ3 is used to categorize the disease into three types:[1] type I, with total absence of platelet αIIbβ3; type II, with < 20% of normal platelet content; and variant Glanzmann thrombasthenia, with a near normal amount of αIIbβ3. A number of mutations, distributed worldwide, have been found to be associated with Glanzmann thrombasthenia[6,7] including minor or major deletions, insertions, inversions and mostly point mutations. The functional characterization of many of them has provided important information on the assembly, stability and intracellular transport of the complex.[2]

Most of the reported single nucleotide substitutions are located in the coding sequence, and they cause missense or nonsense substitutions at the amino acid level, producing either normal-sized non-functional or truncated proteins.[6-9] Mutations that alter mRNA splicing are frequently nonsense mutations[10,11] or mutations directly affecting the standard consensus splicing signals,[12] and typically lead to skipping of the neighboring exon. Less commonly, exonic splicing mutations are missense or even silent mutations that generate an ectopic site[13] that is used preferentially, or activate a cryptic splice site.[14] In nonsense-associated altered splicing, deletion of exons with premature termination codons appears to be mediated, at least in part, by a mechanism in which recognition of the translational reading-frame is involved.[15,16] Exon skipping is

also found in association with an increasing number of point mutations other than those of the nonsense type. Mechanisms involving alteration of pre-mRNA secondary structure and disruption or creation of cis-acting exonic signals, such as exonic splicing enhancers and silencers, have been proposed to explain exon skipping resulting from missense and silent mutations.[15] The production of different isoforms from the same transcription unit is very common in mammalian cells. Alternative splicing forms of the α3 and αIIb integrin subunits have been identified during screening of cDNA libraries and the cellular transcripts detected by reverse transcriptase polymerase chain reaction (RT-PCR).[17,18] The alternatively spliced form of αIIb results from an in-frame deletion of a 102 bp of exon 28.[18] As reported for other genes,[19] inherent weak splicing signals for exon 28 may account for its constitutional deletion. The low expression of the deleted transcript and the failure of αIIb^{-28} protein to reach the cell surface suggest that it does not have a significant biological function.

We characterize here the genetic defect in a case of type II Glanzmann thrombasthenia caused by compound heterozygosis of the αIIb gene due to nonsense C1750T and missense C2829T mutations.

Design and Methods

Antibodies

Murine monoclonal antibodies specific for β3 (H1AG11) and αIIb (2BC1) were raised in our laboratory using as antigen αIIbβ3 heterodimer isolated from human platelets.[20] The anti-αvβ3 monoclonal antibody LM609 was from Chemicon (Temecula, CA, USA). The AK2 monoclonal antibody (anti-GPIbα) was from Serotec (Oxford, UK), and SZ1 (anti-GPIX) was from Beckman Coulter (Fullerton, CA, USA). Fluorescein isothiocyanate-conjugated (FITC) F(ab')$_2$ fragment of rabbit anti-mouse immunoglobulin (Ig) was purchased from Dako A/S (Denmark). The horseradish peroxidase-conjugated goat anti-mouse antibody was from Bio-Rad Laboratories (Hercules, CA, USA).

Case report

The patient is a 2-year-old female from China with a clinical diagnosis of Glanzmann thrombasthenia. She has frequent epistaxes and mucocutaneous hemorrhages that started immediately after birth. The patient's bleeding time was prolonged and she had platelets of normal size and number. Platelet aggregation in response to ADP, collagen, adrenaline and arachidonic acid was less than 5% that of control platelets and no clot retraction was observed. Ristocetin-induced platelet agglutination and plasma fibrinogen content were within the normal range. No information about family antecedents was available since the child was adopted.

Flow cytometry analysis of platelets surface receptors

Platelet-rich plasma was obtained from venous whole blood by centrifugation at 150 g for 20 min at room temperature. Platelets were sedimented at 1,000 g for 10 min, washed with 0.5 mM EDTA in phosphate-buffered saline, and incubated, at a concentration of 10^6 cells/100 μL, with the specific monoclonal antibody for 30 min. After washing, the platelets were incubated in a 1:25 dilution of FITC-conjugated F(ab')$_2$ fragment of rabbit anti-mouse Ig for 20 min. Samples were analyzed in a Coulter flow cytometer, model EPICS XL.

Binding of fibrinogen to activated platelets

FITC-labeled human fibrinogen was prepared as previously described.[13] The platelet-rich plasma was diluted with Hepes/citrate buffer, pH 7.4 (5 mM Hepes, 2 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 3 mM KCl, 134 mM NaCl, 12 mM $NaHCO_3$, 12.9 mM Na citrate, 0.1% glucose, 0.1% bovine serum albumin and 1 mM $CaCl_2$) to a final concentration of 5×10^7 platelets/mL. Platelets (60 μL) were activated for 5 min at room temperature with one or more of the following activating agents: 1 mM dithiothreitol (BRL, Life Technologies Co.), 50 μM adenosine 5'-diphosphate (Sigma), 1 mM epinephrine (Sigma). Then, 7.5 μg of FITC-fibrinogen were added and, after 15 min at room temperature, platelets were washed and resuspended in Hepes/citrate buffer for flow cytometry analysis.

Total platelet content of platelet αIIb, β3 and αvβ3

Platelet proteins were solubilized in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.05% Tween 20) supplemented with 1 mM phenylmethyl sulfonyl fluoride and 5 μL/100 μL protease inhibitor cocktail (Sigma). After 20 min on ice, lysates were cleared by centrifugation and resolved on sodium dodecyl sulfate (SDS)-7.5% polyacrylamide gels under non-reduced conditions, transferred to PVDF membranes and then incubated with mixed monoclonal antibodies against αIIb and β3. The specific bound antibodies were detected with a peroxidase-conjugated goat anti-mouse IgG and visualized using the ECL chemiluminescent system. Western blots were quantified by densitometry scanning of the autoradiographs. To determine the platelet content of αvβ3, 300 μg of solubilized platelet protein were pre-cleared and immunoprecipitated for 4 hours at 4°C with 1.5 μg of anti-αvβ3 monoclonal antibody. The immunoprecititates were treated for 2 hours with protein A-sepharose CL-4B (Amershan Biosciences), washed and eluted by boiling for 10 min in 25 μL of non-reduced loading buffer, and analyzed by western blotting with the anti-β3 monoclonal antibody as described above.

Sequence analysis of reversed transcribed mRNA and genomic DNA fragments of αIIb and β3

Total platelet RNA was obtained using the guanidinium isothiocyanate procedure. Screening for mutations was carried out by direct sequencing of PCR amplified overlapping fragments of reversed transcribed αIIb and β3 mRNA. The oligonucleotide pair used for amplification and sequencing of the 3' fragment of αIIb-cDNA have been previously reported.[14]

Genomic DNA was isolated from peripheral blood cells. DNA was amplified with Taq polymerase using oligonucleotides from intronic flanking regions. A 890-bp DNA fragment encompassing exons 27 to 29 was amplified using the oligonucleotide pair: sense-intron 26: 5'-atgatggggtgatgggccgga-3', and antisense-intron 29: 5'-aacctgggtgtgtgcaactct-3'. The 1468-bp DNA fragment containing exons 13 to 18 was amplified using the oligonucleotide pair: sense-intron 12: 5'-aataacaatcagccacttcct-3', and antisense-intron 18: 5'-acttggcactaaccctaatcc-3'. Direct DNA sequencing was performed in an automatic Applied Biosystems sequencer.

Exontrap analysis

The exontrap vector system (Mo Bi Tec GmbH, Göttingen, Germany) was used to analyze whether the C2829T mutation in exon 27 alters the splicing pattern of the mutant transcript. Genomic DNA from a control and the patient was used for PCR-amplification of a 890-bp DNA fragment encompassing exons 27-29 and the adjacent regions of introns 26 and 29, as described above. The PCR products were subcloned and cloned into an Xho I-Bam HI digested exontrap vector. Sequence analysis was performed to verify the correct insertion and absence of errors potentially caused by the Taq polymerase. The constructs with the normal or mutant αIIb sequence were transiently transfected into CHO cells using Lipofectamine reagent (Invitrogen) and, 48 hours after transfection, total RNA was extracted and RT-PCR was performed as previously described.[10]

Transient expression of wild and mutant αIIb

Expression plasmid containing mutant αIIb-cDNA was prepared as followed. Total RNA from the patient was used for RT-PCR amplification of a 1114 bp fragment of αIIb-cDNA, using the sense (1995-2015) and antisense (3129-3109) oligonucleotides, as described above. The PCR product was digested and ligated into the Apa I- and HinD III- digested pcDNA3.1(-)/Myc-His B vector (Invitrogen, San Diego, CA, USA) containing the wild-type αIIb-cDNA, which was previously treated to disrupt the Apa I site in polylinker. To generate wild and mutant expression plasmids containing the *b* allelic form of the HPA-3 (Ile843Ser) polymorphism, a BamH I cDNA fragment from the patient was exchanged for the corresponding segment in wild and mutant vectors. Nucleotide sequencing was performed to verify the correct orientation of the cloned fragments. CHO cells stably expressing β3 (CHO-β3) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Cells were transiently transfected by the diethyl aminoethyl-dextran method with 15 μg of either normal or mutated αIIb constructs. Forty-eight hours after transfection the cells were harvested and the surface expression of αIIbβ3 complexes was determined by flow cytometry analysis.

Results

The patient is a 2-year old female from China whose history of bleeding episodes started immediately after birth. The clinical diagnosis of Glanzmann thrombasthenia was based on a prolonged bleeding time, normal platelet count, and absence of spontaneous or agonist-induced platelet aggregation.

The platelet content of αIIbβ3 was estimated by flow cytometry and western blot analysis. As shown in Figure 1A, platelets from the patient incubated with antibodies against αIIb and β3 subunits showed detectable positive fluorescence relative to the signal obtained by incubating cells only with the second fluorescent anti-mouse IgG, but the mean fluorescence intensities accounted for less than 10% of surface expression of αIIbβ3 relative to control platelets. The analysis also revealed a normal surface content of the platelet GPIb-IX complex. Quantification of platelet αIIbβ3 by western blot analysis (Figure 2) showed results consistent with those of flow cytometry. The platelet content of αvβ3 was estimated by immunoprecipitation analysis using the monoclonal antibody LM609 that recognizes a site on the αvβ3 vitronectin receptor present when αv and β3 are complexed together. As shown in Figure 2, similar amounts of the complex were detected in the patient and the control when the immunoprecipitated proteins were blotted with an anti-β3 monoclonal antibody.

The capacity of Glanzmann thrombasthenia platelets to bind soluble fibrinogen was assessed by incubation in the absence or presence of different activators, and the binding of FITC-fibrinogen was estimated by flow cytometry analysis. As shown in Figure 1B, in response to the stimulation with agonists some of the platelets from the patient exhibited a detectable increase in surface fluorescence. However, the values of fluorescence, calculated as a product of the percent of gated positive cells and the values of mean channel of fluorescence, in the activated platelet population from the patient was approximately 10-fold lower than those in the control. Dithiothreitol stimulated the binding of control platelets to fibrinogen, but no effect was observed on platelets from the patient.

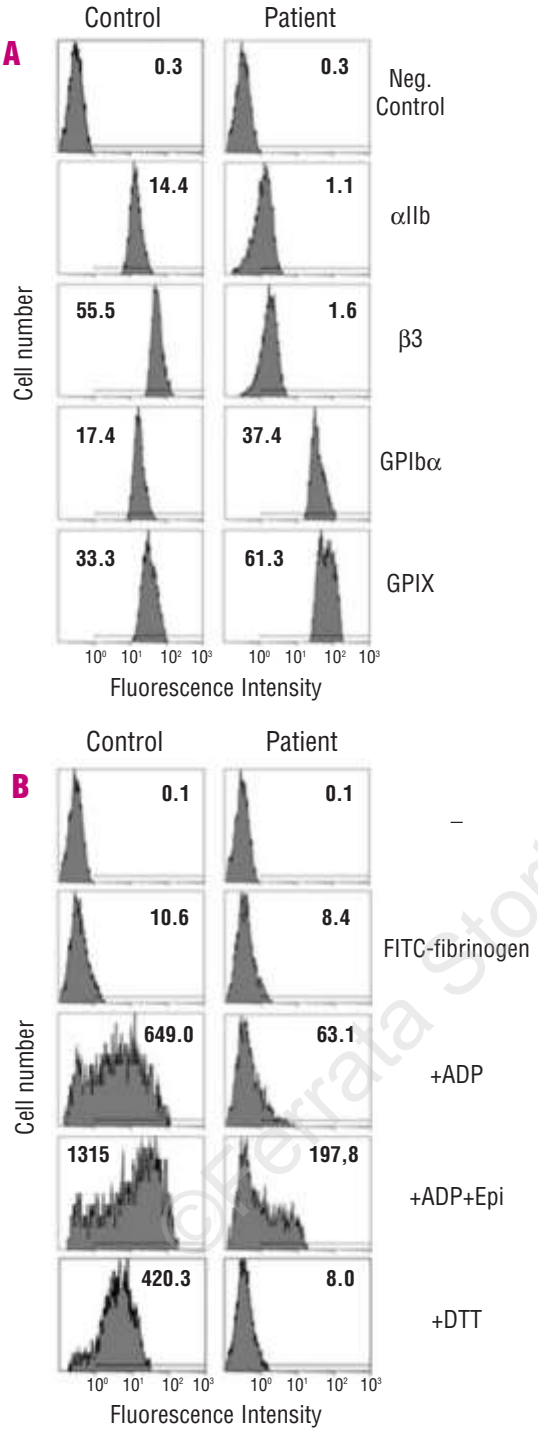

Figure 1. Flow cytometry analysis of platelet surface αbβ3 and fibrinogen binding to platelets. (A) Surface expression of αIIbβ3 and GPIbIX. Washed platelets were incubated with specific monoclonal antibodies against either αIIb (2BC1), β3 (H1AG11), GPIbα (AK2), or GPIX (SZ1), as described in the *Methods*. The negative control represents the fluorescence of platelets incubated only with the second FITC-labeled antibody. The mean channel of fluorescence is indicated in each panel. (B) Binding of soluble fibrinogen to platelets from the thrombasthenic patient and a control. Platelets were processed as described in the *Methods* and preincubated for 5 min with the indicated activator before addition of FITC-fibrinogen. Numbers correspond to the values of fluorescence calculated as a product of the percent of gated positive cells and the value of mean channel of fluorescence.

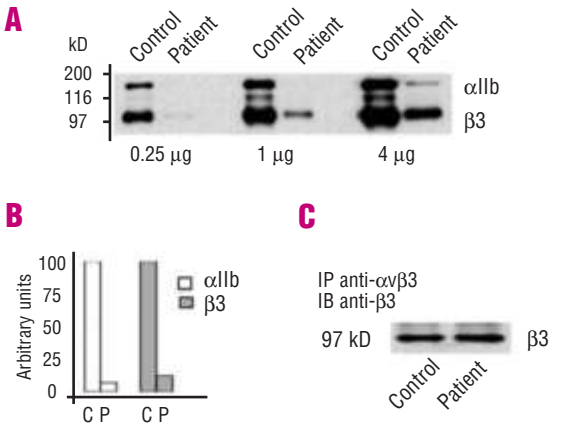

Figure 2. Analysis of platelet αIIb, β3 and αvβ3 content. (A) Immunoblot analysis of αIIb and β3. Platelet lysates were resolved on SDS-7.5% polyacrylamide gels under non-reduced conditions and transferred to PVDF membranes. Immunoblotting with 1 μg/mL of the mixed monoclonal antibodies was performed as described in the *Methods*. (B) The analysis was repeated three times using different protein concentrations and data were quantified by densitometry. (C) Analysis of platelet αvβ3 content. Immunoprecipitation of αvβ3 and western blotting analysis of the immunoprecipitates were performed as described in the *Methods*.

Identification of mutations in the αIIb gene

The normal amount of platelet αvβ3 in the patient suggested that the reduced levels of αIIbβ3 complex were not due to a limited availability of β3. Thus, we first searched for mutations in the αIIb gene by sequencing PCR-amplified overlapping fragments of reversed transcribed αIIb-mRNA. This analysis revealed a homozygous single C2829T substitution in exon 27 that would result in the change of Pro to Leu at position 912 in the mature αIIb peptide (Figure 3). The analysis also showed homozygosis for the HPA-3 polymorphism that results from a T to G mutation in exon 26 changing Ile843 to Ser.

Since the presence of the mutation could not be evaluated in the biological parents we considered it necessary to verify the homozygous status by analyzing the genomic DNA. Sequence analysis of a DNA fragment encompassing exons 27-29 demonstrated that the patient was heterozygous for the C2829T transition (Figure 3), suggesting the presence of another αIIb mutation that would result in absent or very limited amounts of mRNA since no transcripts were detected in the RT-PCR analysis. Thus, we analyzed the coding and partial intronic sequences of αIIb by sequencing PCR products encompassing one or more exons. This analysis demonstrated the presence of a heterozygous C1750T transition in the last codon of exon 17 that changes residue Arg553 to STOP codon. The resulting aberrant transcript is predicted to encode a severely truncated αIIb subunit consisting of only 552 amino acids, 456 residues shorter than the normal protein, lacking the transmembrane and cytoplasmic domains. These mutations were not found in a significant number of DNA samples

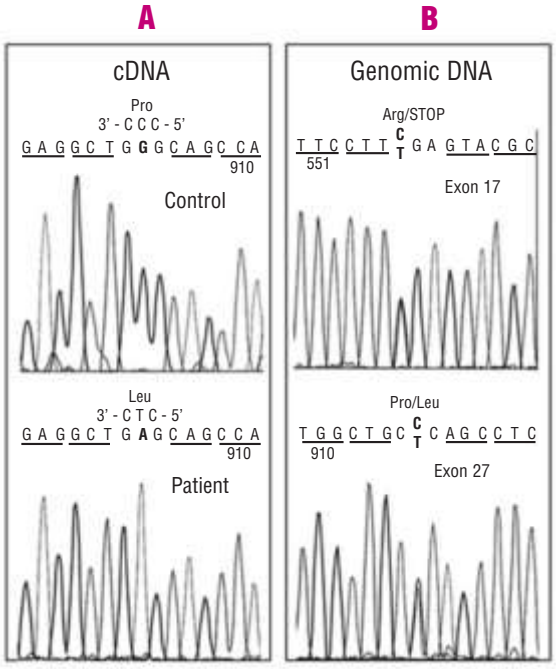

Figure 3. Identification of mutations in the αIIb gene. (A) Direct sequencing of the antisense strand of the 3' overlapping RT-PCR product showing a homozygous G to A substitution that changes Pro to Leu in the Glanzmann thrombasthenia patient. (B) Sequence analysis of genomic DNA fragments revealed the heterozygous status of the missense mutation found in the cDNA (lower panel), and the presence of a nonsense Arg/STOP mutation as result of a C/T transition in the last codon of exon 17 (upper panel).

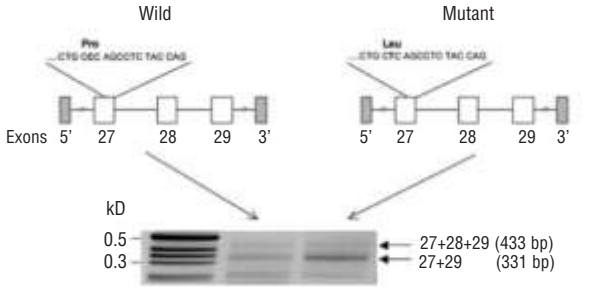

Figure 4. Exontrap analysis of αIIb genomic fragments. Fragments of normal and mutant genomic DNA of αIIb were cloned into the expression vector pET01, in which the intron containing the multicloning site is framed by the 5' donor and 3' acceptor splice sites. CHO cells were transiently transfected with the normal or mutated αIIb construct and the splicing patterns deduced by agarose gel and sequence analysis of the RT-PCR products, as described in the *Methods*.

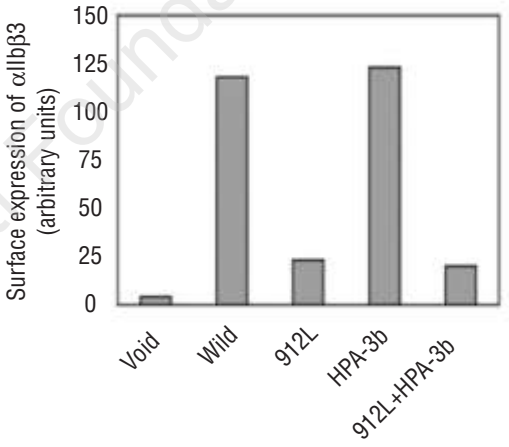

Figure 5. Surface expression of αIIbβ3 in transiently transfected CHO-β3 cells. CHO cells stably expressing β3 subunit were transfected with pcDNA3 plasmid containing either normal or mutated αIIb cDNAs. Forty-eight hours after transfection the surface expression of αIIbβ3 complexes was determined by flow cytometry using the 2BC1 monoclonal antibody. The fluorescence values were calculated as a product of the percent of gated positive cells and the value of the mean channel of fluorescence. Data are representative of three independent transfection experiments.

from normal healthy donors. Moreover, no other mutations were identified in the patient after complete analysis of β3 and αIIb coding sequences.

Exontrap analysis of normal and mutant αIIb alleles

αIIb mRNA with deleted exon 28 (αIIb-28) is a rare alternatively spliced product found in normal platelets. However, a significantly increased number of deleted forms of the transcript was observed in the patient when the DNA sequence of individual clones was analyzed (*data no shown*). To determine whether the C2829T transition induces skipping of exon 28 we analyzed the splicing of this portion of the αIIb gene transfected into CHO cells, using the exontrap vector system. Figure 4A schematically displays the minigene constructs used in these experiments. We examined the αIIb transcripts generated from the normal and mutant alleles by RT-PCR. The construct containing the wild sequence yielded products of similar intensity of approximately 400 and 300 bp. However, a near 100% relative increase of the 300 bp product, as quantified by densitometry scanning, was observed in the RT-PCR analysis of cells transfected with the mutant C2829T construct (Figure 4). Sequence analysis revealed that the amplified products correspond to normal and exon 28 deleted forms of the αIIb transcript.

Transient expression of mutant forms of αIIb in CHO-β3 cells

Figure 5 depicts the results obtained by expressing either normal [912P]αIIb or mutant [912L]αIIb into CHO cells that stably express β3 on the cell surface associated with endogenous α subunits (CHO-β3 cells). In view of the simultaneous presence and close proximity of the mutated residue and the HPA-3b polymorphism (843Ser), we also analyzed the effect of transfecting αIIb-cDNA containing both substitutions. The rate of surface exposure of αIIbβ3 complexes was similar in cells transfected

with either normal or polymorphic αIIb-cDNA. However, the P912L substitution markedly decreased the expression of αIIbβ3 complexes at the cell surface in the presence of both forms of the HPA-3 polymorphism.

Discussion

The present work was aimed at investigating the molecular basis of the bleeding disorder presented by a patient with a clinical phenotype of Glanzmann thrombasthenia. The platelet content of αIIbβ3, assessed by flow cytometry and western analysis, indicated that the patient had type II Glanzmann thrombasthenia. The normal expression of platelet αvβ3 suggested that a limited availability of αIIb, but not of β3, subunit was involved in the etiopathogenesis of the disease.

Sequence analysis of αIIb-cDNA from the patient revealed the presence of a C to T homozygous transition at position 2829 in exon 27. The predicted effect of this mutation is the P912L substitution in αIIb. The simultaneous homozygosis of the HPA-3 polymorphism, together with the impossibility of performing genetic analysis in the parents, led us to confirm the presence of a unique mutation at the DNA level. Sequencing of the corresponding genomic DNA fragment revealed that the mutation was present in only one allele and that the patient was carrying another point substitution: a C1750T transition in the last codon of exon 17 that creates a premature termination stop codon in the 553Arg position and does not produce mRNA detectable in our experimental conditions of PCR amplification. This nonsense mutation had been previously found in a heterozygous state in six unrelated patients: three from China, two from Japan and one from France.[8] Since no polymorphic changes were detected at this position in 107 normal individuals from a Chinese province[9] the site might represent a mutational hotspot in the αIIb gene. In principle, the failure to detect the mutant C1750T transcript is consistent with the abnormal transcript being removed by nonsense-mediated decay.[16] The predicted mutant transcript would encode a truncated αIIb protein, 456 residues shorter than the normal protein, lacking the transmembrane and cytoplasmic domains, and a portion of the extracellular carboxyterminal fragment. However, cDNA sequence analysis in one of the reported cases, in which the trace amounts of the aberrant mRNA could be amplified due to the complete inactivation of the other αIIb allele,[21] demonstrated a markedly reduced amount of a transcript containing a 75-bp deletion in the 3' boundary of exon 17 that would code for a protein carrying an in-frame deletion of 25 residues.

Since the C1750T mutation generates trace amounts of mRNA, the total αIIb protein detected in the patient would, in practice, come from the C2829T mutant allele. The C2829T substitution is the first reported mutation located in exon 27 of αIIb, which codes for the aminoterminal region of the light αIIb chain. Transient transfection experiments demonstrated that [912L]αIIb resulting from the C2829T transition can be expressed at the cell surface as an αIIbβ3 complex. However, the 5-fold reduction in the rate of expression indicates some restriction in one or more of the steps involved in the maturation and traffic of the complex.

Unexpectedly, analysis of this mutation at the mRNA level revealed a significant increase in the number of transcripts in which exon 28 was deleted. This association was confirmed by exontrapping analysis of genomic DNA encompassing either the normal or mutated exon 27, which demonstrated that the identified mutation was forcing skipping of exon 28 in αIIb-mRNA. αIIb with deleted exon 28 (αIIb^{-28}) is a rare, alternatively spliced product of the αIIb transcript that may represent approximately 3% of the normally spliced form in control platelets.[22] Skipping of exon 28 has been previously reported to increase as a result of two different mutations in this exon. In one case the mutation created a stop in the last codon of exon 28 and, at the same time, changed the consensus 5' splice site of intron 28 forcing the exon deletion in virtually all the transcripts.[22] In contrast, the 5-bp duplication reported by Peretz *et al.*[23] abolished the normal stop codon without creating a new one and, as in the case reported here, a significant amount of non-deleted transcript was detected. In both cases, as observed in our study, the transcriptional rate and intracellular stability of the αIIb^{-28} mRNA seemed not to be decreased with respect to the normal transcript. However, it was not expressed as a stable αIIbβ3 heterodimer at the surface of either platelets or transfected cells.[22,24] According to these observations, the platelet αIIb in the present patient with Glanzmann thrombasthenia would come from the normal size transcript. The defective mRNA splicing promoted by the mutation may explain the discrepancy between the relative percents of expression of the mutant protein observed in platelets (less than 10% of the control) and transfected cells (20% of the control). Skipping of the mutant exon is a common mechanism of gene inactivation induced by single point mutations; nevertheless, it is less frequent that, as in this case of Glanzmann thrombasthenia, the mutation affected splicing of a contiguous or remote exon.[19,25,26] The presence of weak splicing signals in the deleted exons seems to be a common requisite in all cases. However, in contrast to other reports,[19,25,26] the αIIb mutation does not introduce a premature stop codon into the transcript.

The effects of nonsense mutations on the stability and splicing of mRNA have been extensively studied leading to the proposal that the reading frame undergoes surveillance before splicing.[27] However, less is known about the mechanism involved in altered splicing caused by misssense mutations. Since exon skipping can also be associated with apparently silent mutations, it has been suggested that both missense and silent mutations must

act altering *cis*-sequences that are important for correct splicing.[15] Binding of serine/arginine-rich (SR) proteins to such exonic splicing enhancer (ESE) or silencer (ESS) sequences can promote exon definition by modulating recruitment of the splicing machinery. Disruption of these exonic splicing sequences has been proposed as the underlying mechanism in a number of mutations.[28-30] Potential ESE that would facilitate recognition of splice sites and prevent exon skipping can be identified computationally but, in many instances, it is difficult to predict whether a mutation will alter splicing based on sequence alone. The scores for individual SR protein putative recognition sites, estimated by using the available ESEfinder program,[31] were not decreased in the mutant C2829T sequence. Paradoxically, the mutation was predicted to increase the score of a site for the SR protein SC35 from 2.418530 to 3.062970 (threshold value for the SC35 motif: 2.383). Although exon skipping has been coupled to disruption of SR sites,[15] it has also been shown to be promoted by overexpression of the SC35 splicing factor.[32,33]

The proportion of mutations identified at the DNA level which are predicted to affect mRNA splicing has often been underestimated.[28,34] Many recent reports show an increasing number of human genetic diseases due to exonic mutations that alter pre-mRNA processing.[15] In some instances, the identification of alternative transcripts may be the only way to explain the mechanism by which a mutation is causing a disease. The mutations identified in the present study are examples of genetic defects which need to be characterized at both the genomic and RNA levels in order to obtain a correct interpretation of their pathogenetic mechanism. The functional consequences of the C2829T substitution suggest that, as reported for another human diseases, disruption of exonic splicing elements may represent the underlying, so far underestimated, mechanism of gene inactivation displayed by a number of Glanzmann thrombasthenia mutations. Finally, in the present patient with Glanzmann thrombasthenia the HPA-3a (843Ile) allele is silent at the protein level. HPA-3 (Ile843Ser) is a common polymorphism of platelet αIIb that gives rise to post-transfusion purpura and neonatal alloimmune thrombocytopenic purpura.[35] Thus, this case is an example in which the genotype analysis without complementary serological typing could have led in some circumstances to misinterpretation of clinical symptoms. As reported for the HPA-1 polymorphism of β3, HPA-3 seems not to be associated with any measurable increase of the risk for cardiovascular disease.[36] However, in a post-stroke mortality study, patients with the *a* allele had a worse survival than those homozygous for the *b* allele.[37]

In summary, in the present work we have analyzed the pathogenic mechanism in a thrombasthenic patient carrying two mutations in exons 17 and 27 of the αIIb subunit. The reduced αIIbβ3 in the platelet membrane seems to be the result of unstable αIIb mutant transcripts, enhanced expression of aberrant αIIb^{-28} transcripts, and inefficient processing of [912Leu]αIIb protein. The detected binding of labeled fibrinogen suggests that the expressed mutant protein might be functional but not sufficient to support platelet aggregation.

AJ performed the mRNA and DNA sequence analysis and prepared the construct plasmids for transfections. DP carried out the transfection experiments; PL performed the cytometry analysis of platelets and participated in manuscript preparation; VJ conducted the clinical study of the patient and platelet aggregation analysis; CG-M coordinated the study and drafted the paper.

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