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αIIbβ3 integrin : New allelic variants in Glanzmann Thrombasthenia, effects on mRNA splicing, expression and structure-function

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Short title :

New mutations in Glanzmann patients and carriers

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Abstract

Glanzmann thrombasthenia (GT) is an autosomal recessive inherited bleeding disorder characterized by an impaired platelet aggregation due to defects in integrin αIIbβ3 (ITGA2B, ITGB3), a fibrinogen receptor. Mutations from 24 GT patients and 2 carriers of various origins, Caucasian, North-African and Asian were characterized. Promoter and exon sequences of αIIb and β3 genes were amplified and directly sequenced. Among 29 identified mutations, 17 new allelic variants resulting from nonsense, missense and deletion / insertion mutations were described. RNA alterations were evaluated by using web-servers. The αIIb p.S926L, p.V903F, and β3 p.C38Y, p.M118R, p.G221D substitutions prevented complex expression at the surface of Cos-7 cells by altering the αIIb or the β3 subunit structure. As shown by free energy analyses applied on the resolved structure of αIIbβ3 and structural modeling of the mutant, the p.K253M substitution of β3 helped to define a key role of the K253 in the interaction of the αIIb β-propeller and the β3 β-I domains. Finally, the αIIb p.Q595H substitution allowed cell surface expression of the complex but its corresponding c.2800G>T mutation is predicted to alter normal RNA splicing. Characterization of GT mutations is of interest for prenatal diagnosis, genetic counseling and provides useful tools for structure-function relationship studies of αIIbβ3.

Keywords

αIIbβ3, ITGA2B, ITGB3, Glanzmann, protein structure modeling

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INTRODUCTION

Glanzmann thrombasthenia (GT) is an autosomal recessive bleeding disorder characterized by a prolonged bleeding time and an impaired platelet aggregation in response to physiological agonists such as ADP, thrombin, collagen and epinephrine [Caen, 1972]. GT results from quantitative and / or qualitative defects of the fibrinogen (Fg) platelet receptor, the glycoprotein αIIbβ3 complex (OMIM accession number #273800). Classically GT has been divided into 3 subtypes defined by the αIIbβ3 content of patient's platelets : Type I, no or only trace amounts of αIIbβ3; Type II and variant, respectively with low and near normal platelet surface content of functionally impaired αIIbβ3 [Nurden, 2005]. To date, more than 100 different mutations involved in GT phenotype have been described in the αIIb gene and 60 in the β3 gene (http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu). The nature of GT mutations is highly variable excepting some that are commonly identified in consanguineous populations such as the αIIb c.409-3_418del (IVS3-3del13) and the β3 c.2031_2041del (2031del11) respectively detected in Arab and Iraqi-Jewish GT populations [Newman et al., 1991], the αIIb c.1544+1G>A (IVS15+1/G>A) mutation in French Gypsies [Schlegel et al., 1995], or the β3 c.1053_1058del (1053CCCAGG) deletion in North-Africans [Morel-Kopp et al., 1997].

Characterization of GT mutations in patients is critical for prenatal diagnosis and genetic counseling. We report here the characterization of 29 mutations identified in 24 thrombasthenic patients and 2 asymptomatic carriers. Using site directed mutagenesis, cell expression models, *in silico* tools and the resolved structure of the αIIbβ3 complex, we have analyzed the pathogenic role of 17 newly described allelic variants. Structural modeling studies of an allelic variant of GT has revealed the importance of the Lys253 in the interaction of the αIIb β-propeller and the β3 β-I domains.

MATERIAL AND METHODS

Patients

Blood samples from patients were sent to our laboratory for confirmation of GT diagnosis and genetic analysis. Propositi and their detailed platelet phenotype are listed in Supp Table S1. Propositi 4 and 15 are GT carriers. Propositus 4 who had presented mild bleeding and healing difficulties during tympanoplasty was addressed to the laboratory for GT investigation after flow cytometry analysis revealed a 50 % reduction of αIIbβ3 platelet surface expression. Propositus 15 was unexpectedly found to be a GT type I carrier during neonatal alloimmune thrombocytopenia diagnosis following in utero fetal death. Her platelets were phenotyped HPA-3a negative but genetically HPA-3a/b as determined by PCR-Sequence Specific Primer testing (data not shown).

As shown in Supp Table SI, 19 propositi of 22 of confirmed platelet phenotype_ (86 %) were found to be of type I (< 5 % of αIIbβ3 and < 25 % of Fg normal platelet contents). Three propositi were found to be of type II as their platelets presented low but significant αIIbβ3 content (≥ 10 %) and a relatively high level of intra-platelet Fg (≥ 50 **%).** None of these patients had been previously studied for molecular gene defects. Patients described herein gave their informed consent for genetic analysis.

Monoclonal antibodies

Monoclonal antibodies (Moab) SZ1 (anti-GPIX), SZ22 (anti-αIIb), SZ21 (anti-β3), P2 (anti-αIIbβ3) and E3 (anti-Fg) were purchased from Beckmann Coulter-France (Villepinte, France). Moabs AP2 (anti-αIIbβ3) and AP3 (anti-β3) were obtained from Lucron Bioproducts

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(Lille, France). Moab Y2-51 (anti-β3) was purchased from DAKO (Trappes, France). Moab XIIF9 to β3 was a generous gift from G.Vezon (CRTS, Bordeaux, France). In some flow cytometry experiments, Moabs SZ22, SZ21 and P2 were directly conjugated to FITC.

Blood samples

Blood samples from patients were collected by venipuncture using 5mM ethylene-diamine-tetra-acetic acid as an anticoagulant. Platelet were separated by differential centrifugation and used or stored in 70% ethanol at –80°C until RNA was extracted. In some cases, 500 µl of whole blood were mixed with 1.3 ml of RNA Later (Applied Biosystem, Courtaboeuf, France) and were kept frozen at –80°C for subsequent RNA extraction.

Flow cytometry

For platelet glycoprotein and Fg content analysis, 50 µl of EDTA anticoagulated whole blood was diluted in 500 µl of phosphate saline buffer (PBS) pH 7.2 containing 1 % (wt/vol) BSA-fraction V (Euromedex, Souffelweyersheim, France) and 3 mM EDTA (PBE buffer). Cell were fixed by adding 1.5 ml of PBS - 3 mM EDTA, pH 7.2, containing 1.33 % (vol/vol) of a methanol-stabilized formaldehyde solution (Fisher Scientific, Illkirch, France) for 15 min at room temperature (RT). The reaction was stopped by adding 2 ml of PBE before cells were pelleted by centrifugation at 1500 g for 10 min. Pelleted cells were washed in 2 ml PBE before to be resupended in 1 ml PBE containing 0.1 % (wt/vol) sodium azide and store at 4°C until use. 40 µl of the fixed cells suspension were incubated with 3 µg/ml Moab in PBE (100 µl final volume), for 1 h at RT. After two washes in 2 ml PBE, the bound Moabs were detected by using a polyclonal goat anti-mouse IgG conjugated to FITC (Jackson Immunoresearch Europe, Newmarket, UK) diluted in PBE (1 µl for 10 ml) for 30 min at RT. Following one wash in 2 ml PBE, mean fluorescence intensity of cells was analyzed on an

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XL-MCL Flow cytometer (Beckman Coulter, Villepinte, France). To detect intraplatelet Fg, each incubation steps were done in presence of 1% (wt/vol) saponine (Fischer Scientific). Amounts of proteins are expressed as a percentage of the mean amounts obtained with normal platelets from 10 healthy donors.

To analyze αIIβ3 wild type or mutant complexes expressed at the surface of transfected Cos-7 cells, cells were collected by Trypsin-EDTA treatment and washed 2 times in PBS-1% (wt/vol) BSA. 5 x 10^5 cells were incubated in 100 µl PBS containing 3 µg/ml Moabs (SZ22, SZ21 or P2) conjugated to FITC for 1 h at RT. Cells were washed twice in 2 ml PBS-BSA before to be analyzed by flow cytometry. Dead cells were discarded from analysis by using the 7-AAD viability dye (Beckmann-Coulter). Results are expressed as a percentage of positive cells for the concerned antigen.

DNA/RNA extractions

Genomic DNA was isolated from peripheral blood according to a published procedure [Jallu et al., 2002] or by using the MagNA Pure Compact Instrument and the Nucleic Acid Isolation Kit I (Roche Diagnostics, Meylan, France). In some cases, DNA samples were obtained by using QIAamp DNA Blood Mini kit (Qiagen, Courtaboeuf, France). RNA extractions were done on platelets by using the Rneasy Protect MiniKit (Qiagen). DNAs and RNAs were extracted according to the manufacturers' instructions.

Sequencing analysis

Sequencing was performed as previously described [Quintanar et al., 1998] or by GenoScreen (Lille, France). Primers defined by Kato and coworkers [Kato et al., 1992] and Jin and co-workers [Jin et al., 1993] were respectively used to amplify coding sequences of

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αIIb and β3 genes from genomic DNA. Primers to amplify promoter sequences of αIIb and β3 have been respectively defined according to the genes' reference sequences NG_008331.1 and NG_008332. αIIb promoter sequences were studied by using the following primer pairs ("a" forward, "b" reverse) :

IIbP1a CGCCAGAATTCTCAGTCACGAAGCTTGGCTCAAGACGGAG

IIbP1b CCAGCCAGATATCAGCTATGTACTACCACCGTGCTAGTCC

IIbP2a CGCCAGAATTCTCAGTCACGGGTAAAGATTCAAGAGACAT

IIbP2b CCAGCCAGATATCAGCTATGTCAGATTCCTCCACAGGAAG

IIbP3a CGCCAGAATTCTCAGTCACGGAAGGGAAGGAGGAGGAGCT

IIbP3b CCAGCCAGATATCAGCTATGCTTCCCTTACGGCTCACCTC

Underlined sequences are non genomic sequence for sequencing PCR products. Promoter and exon 1 sequences of β3 gene were amplified with the primer pair

IIIaE1a ATGTGGTCTTGCCCTCAACAGGTAG

IIIaE1b CACGCTCTCACCCAGGAAGTTACAG by using the Advantage GC genomic PCR kit (Clontech, Saint-Germain-en-Laye, France) and then sequencing was done with the forward primer 3aseq-P1a CCGGAAAACCAAACTAAGGC and the reverse primer 3aseq-P1b GACCAGTCCTCCGCGTTTGC.

All identified mutations were confirmed by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis on a second PCR product. Restriction enzymes are listed in tables 1 and 2 with the corresponding mutations. The experimental details of these RFLP assays can be obtained upon request. When the mutation did not introduce restriction modification, sequencing was done on a second PCR-product. For nucleotide numbering the A nucleotide of the ATG start was designated +1 (cDNA ITGA2B and ITGB3 GenBank accession numbers NM_000419.3 and NM_000212.2 respectively). For amino acid numbering used in the text, the first amino acids of the mature αIIb and β3 proteins were

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designated +1. Residue numbering according to the Human Genome Variation Society (HGVS) nomenclature that designed as +1 the translation initiating methionine (http://www.hgvs.org/mutnomen) is also shown in tables 1 and 2. Both numberings differ by 31 and 26 residues that correspond to the signaling peptides of αIIb and β3 respectively. All identified allelic variations have been deposited on the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) and their corresponding accession numbers are indicated in tables 1 and 2.

Site directed mutagenesis and in-vitro expression

Site directed mutagenesis and *in vitro* expression in Cos-7 cells were done as previously described [Jallu et al., 2002] or by using the QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Massy, France) according to the manufacturer's instructions. In some mutagenesis experiments, plasmid pcDNA3.1-Zeo containing the αIIb cDNA was used instead of pcDNA3.1-Neo (Invitrogen, Cergy Pontoise, France).

Minigene study

To study a mutation predicted to affect mRNA splicing, an αIIb gene fragment covering exons 17 to 20 and containing the mutation was synthesized and introduced in the plasmid Exontrap (Mobitech, Goettingen, Germany). The wild type form of the sequence was obtained by site directed mutagenesis. Synthesis, cloning and site directed mutagenesis were done by the Epoch Biolabs, Inc. Company (Sugar Land, TX, USA). Following transient transfection in Cos cells, total RNA was extracted and mRNA amplified for the exon 17 to 20 and directly sequenced.

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Western-blot analysis

Trypsin-EDTA collected Cos-7 cells were washed 2 times in PBS and 1 time in 50 mM Tris-HCL pH 7.6, 150 mM NaCl. Cells were resuspended (1.5 x 10^7 cells) in 100 µl of the same Tris buffer containing 1% (wt/vol) Triton-X 100 and 2 mM para-Methyl Sulfonyl Fluoride (PMSF). They were lysed for 30 min at 4°C and the lysate centrifuged at 16.000 g for 30 min at 4°C. The supernatant was diluted with 4 volumes of a 150 mM NaCl solution containing 2.5 % (wt/vol) Sulfate Dodecyl Sodium (SDS), 31.25 mM n-ethylmaleimide and 2.5 mM PMSF. The SDS lysate was heated to 100°C for 5 min before 35 µl were used for western-blot analysis as previously described [Jallu et al., 1994] with a minor modification : bound antibodies were detected by using anti-mouse IgG conjugated to peroxidase associated with a chemiluminescence detection technique.

Computational analyses

The effect of mutations on intron splicing has been predicted *in silico* by using the following tools : Genscan (http://genes.mit.edu/GENSCAN.html [Burge and Karlin, 1998]) and NNSPLICE 0.9 (http://www.fruitfly.org/seq_tools/splice.html [Reese et al., 1997]). Modifications of the splicing factors binding sites pattern resulting from the mutations were evaluated by using the ESEFinder tool [Cartegni et al., 2003; Smith et al., 2006].

To compare wild type and a mutant headpiece of αIIbβ3, modeling has been done using the structure (PBD code 2VDL) published by Springer et co-workers [Springer et al., 2008]. Residues 1-452 of αIIb and 109-352 of β3 were taken into account. The interface between the protein chains has a correct resolution (2.75 Å) and the residues implicated at the interface have all good normalized B-factor values. A structural model of the mutant has been built. This model was checked using ProCheck software to analyze the quality of the modification [Laskowski et al., 1993] and the side-chain position was analyzed with SCWRL [Canutescu et

al., 2003]. Finally, the FastContact web server (http://structure.pitt.edu/servers/fastcontact/) was used to estimate the direct electrostatic and desolvation interaction free energy between two proteins [Camacho and Zhang, 2005; Champ and Camacho, 2007]. The computational estimation of the binding free energy is based on a statistically determined desolvation contact potential and Coulomb electrostatics with a distance-dependent dielectric constant [Camacho and Zhang, 2005]. This free energy provides a reasonably good estimate of experimental binding affinities from complex crystal structures.

RESULTS

Clinical GT diagnosis was confirmed in our laboratory by immunological or biochemical studies (see Supp Table S1). Genomic DNA samples from patients were studied for molecular defects in promoter and exon sequences of genes coding for αIIb and β3 by direct sequencing of the corresponding PCR products. Allelic variations identified in these genes are reported in tables 1 and 2. To avoid erroneous identification due to amplification errors, all sequence alterations have been confirmed on a second PCR product either by a PCR-RFLP technique when a restriction site modification was observed or by new sequencing analysis in other cases. Restriction enzymes that have been used are listed in tables 1 and 2 with their corresponding allelic variant. When available, DNAs from patient's relatives were also analyzed and used to confirm the allelic variations (data not shown).

Sequence alterations detected in the αIIb gene

For 18 propositi, a total of 20 mutations was identified in the gene coding for αIIb (see table 1). Five lead to nonsense codons : patients 1, 6, 7, 8 and 17. It should be noted that patient 17 was phenotype GT type II because the second mutation that had not been identified allowed residual expression of αIIbβ3 (see Supp Table S1). Three mutations directly affect a splice site : patients 9 and 12 with a single point mutation and 3 unrelated propositi (GT

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patients 3 and 5, and the carrier 4) who present the same 13 base pair deletion c.1442-13_1442-1del. However for these latter propositi, an unknown common ancestry cannot be excluded as all are European Caucasians who live in the north-east of France. Two heterozygous single base insertions were identified in patient 2. These mutations are predicted to result in frame-shift anomalies creating a downstream stop codon and premature termination. A 10 base pair deletion identified in patient 14 also leads to a frame shift anomaly. Other gene anomalies identified affecting αIIb, were missense mutations. Three of them (patients 6, 10, 15 and 16) have not been described so far and 6 (patients 3, 11, 12, 13 and 18) have already been reported (see references in Table 1). Among the 9 heterozygous compounds, 4 second obligatory mutations (patients 5, 7, 17 and 18) remain to be identified (NI abbreviation (not identified) in tables).

Sequence alterations detected in the β3 gene

Ten mutations were identified in the gene coding for the β3 glycoprotein for 8 propositi (see Table 2). One single point mutation (patient 20) is responsible for a nonsense codon and a second (patient 19) disrupts an acceptor splice site. An homozygous 4 base pair deletion located downstream of the donor splice site (c.1125+3_1125+6del) of intron 8 has been identified as the unique mutation in patient 25. In patient 26, two silent homozygous mutations, c.1143C>A and c.1260G>A (20507C>A and 20624G>A with genomic numbering : accession number J05427) located in exon 9 are observed. These mutations have been reported to be responsible for type I GT [Jin et al., 1996]. Though silent, their association induces exon 9 skipping and the introduction of five intronic bases in the final transcript [Jin et al., 1996]. Analysis of the mRNA from patient 26 has confirmed this deletion / insertion mechanism (data not shown). Finally five single point missense mutations were

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identified (patients 21, 22, 23 and 24). In two heterozygous patients the second obligatory GT mutation remains to be determined.

Flow cytometry analysis of mutant complexes expressed in COS-7 cells

Seven missense mutations not previously reported in the literature have been further studied by expressing the mutant complexes in COS-7 cells to assess their role in the GT phenotype. Cell surface expression of the mutant complexes was assessed by flow cytometry using Moabs SZ22 to αIIb, SZ 21 to β3 or P2 to the αIIbβ3 complex (see Figure 1). αIIb p.S926L (patient 16) and p.V903F (patients 6 and 15) and β3 p.C38Y (patient 21), p.M118R (patient 22), p.G221D and p.K253M (patient 24) substitutions prevented normal complex expression, mimicking the type I GT phenotype observed for the patients. For the αIIb p.S926L (patient 16) and β3 p.G221D and p.K253M (patient 24) mutants the percentage of positive cells expressing β3 was slightly higher than in other mutants tested (see Figure 1). This low level expression could be correlated to the 10 % of β3 expression at the surface of the patients' platelets (see Supp Table S1).

Surprisingly, the αIIb p.Q595H substitution identified in a GT type I patient did not affect the expression of the mutant complex which is similar to that obtained with the wild type form.

As control, untransfected cells did not significantly bind any of the Moabs tested and irrelevant mouse IgG gave less than 1 % of positive cells whatever the transfectant tested.

In silico analyses of mRNA processing for missense allelic variations and for the intronic deletion c.1125+3_1125+6del

Some exonic single point mutations can alter normal processing of the mRNA and lead to the synthesis of an aberrant mRNA, associated or not with its degradation. Genscan,

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NNSPLICE 0.9 and ESEFinder web servers were used to predict the effect of the missense mutations responsible for the substitutions that have been tested in flow cytometry. From this panel, the c.1878G>C responsible for the p.Q595H substitution was the unique variation predicted to modify normal splicing and to affect ESE binding sites. Splice site prediction by NNSPLICE 0.9 cannot identify the normal donor splice site of intron 18 in the mutant. Genscan analysis did not predict splice anomalies, but scores were lowered for both exons 18 (0.987 to 0.791) and 19 (0.995 to 0,849) implying a probability of correct splicing of 75 % instead of 99 % for the normal sequence. Furthermore ESEFinder shows mutation 1878G>C to increase the score of an SF2/ASF binding site from 2.67 to 3.28 and to create a new one with a score of 2.50 (threshold 1.95). Finally an SRp55 binding site that presents a high score (4.33 - threshold 2.67) encompassing the normal splice site is also created.

In the β3 gene, the homozygous 4 bp deletion c.1125+3_1125+6del (patient 25) was the single intronic mutation identified which does not directly affect a splice site. However Genscan analysis predicts the use of a cryptic donor splice site located 70 bp downstream the normal one. If this transcript is not degraded, this would lead to an in frame introduction of 23 amino-acids between β3 β-I and hybrid domains. ESEFinder shows no modifications but NNSPLICE 0.9 suggests the contiguous donor splice site would be ignored in the mutant.

Western-blot analysis of the p.C38Y β3 mutation effect on the complex expression.

All the β3 mutants tested strongly affect complex expression (Figure 1) but the p.C38Y substitution also strongly and specifically affects that of β3 when tested with anti-β3 Moab SZ21. To understand this specific effect, synthesis of the Y38β3 form of the complex in the transfected cells was analyzed by using a Western-blot technique with Moabs SZ22 to αIIb and SZ21, XIIF9 and AP3 to β3 (see Figure 2). SZ22 showed that αIIb is clearly detectable in the lysate of the wild type αIIbβ3 transfectant (lane c) as in the control platelet lysate (lane a).

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For the αIIb-Y38β3 mutant transfectant, only a band of slightly greater relative mobility which might correspond to a degradation product of αIIb was detected (lane d). Moab XIIF9 revealed the presence of the Y38β3 mutant in the αIIb-Y38β3 transfectant (lane d). However, this mutant glycoprotein presents a lower relative mobility when compared to the wild type forms detected in lysates from platelets or wild type αIIbβ3 transfectant (lane c). Both SZ21 and AP3 failed to react with the Y38β3 mutant (lanes d) but reacted with normal β3 expressed in platelet and wild type αIIbβ3 transfectant (lanes a and c respectively). XIIF9 and AP3 but not SZ21 reacted with the endogenous β3 subunit of the vitronectin receptor expressed in Cos-7 cells as shown by the faint band detected in non transfectant cells (lane b). These results suggested that the p.C38Y mutation should impair the complex formation, or at least its normal processing, as no αIIb of normal mobility could be detected.

Role of the Lys253 of the β–I domain of β3 in the interaction with the αIIb β-propeller

To understand precisely the impact of the p.K253M mutation, we have analyzed the interface between αIIb (residues 1-452) and β3 (residues 109-352) subunits using the protein structures obtained by the group of Springer (PDB code 2VDL [Springer et al., 2008]). From a simple visualization, the side chain of the β3 K253 protrudes from the β–I domain toward the αIIb beta-propeller surface and makes contact with different residues of the αIIb subunit (see Figure 3A). Table 3 summarizes the different results concerning the estimations of the direct electrostatic and desolvation interaction free energy between the αIIb and β3 chains. Interestingly, K253 has a key role in terms of binding free energy with the most negative contribution (-5.5 kcal/mol) and also in terms of electrostatics (-9.7 kcal/mol). Inversely, desolvation free energy is high (+4.1 kcal/mol). The desolvation of charged and polar amino

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acid is unfavorable from an energy point of view. This penalty of desolvation is greatly compensated by Coulomb interactions and by the hydrogen bonds which are formed in the complex.

Analysis of important interactions of by K253 underlines its weight in the interaction as it has the 1st (-6.7 kcal/mol with D232 of αIIb), the 6th (-2.0 kcal/mol with F231) and the 13th (-0.8 kcal/mol with P238) most negative interactions of the interface. One interaction with W262 is slightly unfavorable. Concerning free energy, the same observations are made, with two strong interactions with D232 (-5.5 kcal/mol) and F231 (-1.2 kcal/mol). One unfavorable interaction is observed again with W262. These results highlight the significance of this residue, the most important in terms of electrostatic and free energy computation.

A structural model of M253 mutant was constructed. Regarding the whole αIIb and β3 interface, the substitution provokes a striking loss of electrostatic energy (+9.1 kcal/mol) but a gain in desolvatation free energy (-5.72 kcal/mol) that cannot counter-balance the decrease of electrostatic energy. As shown in Table 3, the diminution of the length of the side-chains and the change from a charged polar amino acid to a hydrophobic amino acid has a deep impact. Residue 253 has no more (negative) interaction with either D232 or F231, or P238. Only a single interaction with W262 is conserved (see Figure 3B).

From a visual point of view, the decrease of electrostatic energy between K253 (see Figure 3C) and M253 (see Figure 3D) is striking. Overall, the most important electrostatic energy implicated in the interaction becomes negligible. All these contacts are lost in presence of the methionine, except for W262 that only preserves an electrostatic link. These results indicate that it is rather the loss of K253 lost than the presence of the methionine which is responsible for the defect of complex expression.

DISCUSSION

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The aim of the study was to identify mutations responsible for GT platelet phenotype. SDS-PAGE, western-blot and flow cytometry analyses carried out on patients' platelets confirmed GT clinical diagnosis. Twenty-nine mutations were identified in both αIIb and β3 genes. Most of these mutations (47 %) correspond to missense mutations but nonsense, insertion, deletion, and single point mutations affecting splice sites were also found.

Mutations previously reported in the literature (see tables 1 and 2) have not been further studied in-vitro for their involvement in GT. It can be noted that their presence in the patients studied herein and in one or more additional unrelated patients would confirm their role in GT phenotype. New mutations directly affecting splice sites, creating nonsense codon or insertion / deletion associated with frame shift anomaly were considered as known cause of GT. The pathological effects of new allelic variations responsible for missense substitutions and one new allelic variation leading to a splicing anomaly are discussed below.

The substitutions p.V903F (patient 6 and propositus 15) and p.S926L (patient 16) affect the extra-cellular N-terminal part of the αIIb β-subunit that composes the calf-2 domain (see Figure 4). In both cases they prevent αIIbβ3 complex expression at the Cos-7 cell surface. They are predicted to impair the correct folding of the Calf-2 domain as the amino-acids V903 and S926 are located in its core (see Figure 4). To estimate their structural importance, the human αIIb primary sequence has been aligned with several corresponding integrin sequences - marsupial, fish, amphibian, prochordates and insect (Supp Figure S1A). These sequences share at least 45 % of identity with the human αIIb (Psi-blast data from http://www.ncbi.nlm.nih.gov/BLAST) and only 17 % of these residues are conserved through all the seven organisms tested. It is noteworthy that V903 and S926 belong to these few amino-acids, indicating that their conservation could be linked to a strong structural evolutionary constraint. Furthermore, their poor conservation in other human integrin α-subunits (data not shown) reinforces their α-IIb specificity.

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The missense mutation c.1878G>C (patient 10), contiguous with the donor splice site of intron 18, was predicted to be responsible for a p.Q595H substitution in αIIb. However this p.Q595H mutation identified in a type I GT patient does not prevent the expression of the mutant complex at the surface of transfected Cos-7 cells. Study of the patient's platelet mRNA revealed full normal β3 mRNA synthesis but αIIb mRNA remained undetectable. A splicing anomaly can lead to mRNA decay and explain the lack of detection of the mRNA coding for αIIb in patient 10. *In silico* splicing testing by using the Genscan and_NNSPLICE 0.9 web servers effectively predicted splicing anomalies. Similarly Jayo and coworkers [Jayo et al., 2006] reported that the αIIb c.2829C>T mutation (exon 27) predicted to induce a p.L912P substitution, partially leads to exon 28 skipping. The authors pointed out that rather than the disruption of an ESE site, the aberrant mRNA processing would result from the strengthening of the SR protein SC35 binding score by 0.65 units (ESEFinder analysis). Interestingly the ESEFinder web server predicted the mutation c.1878G>C to increase the score by 0.61 of an existing SF2/ASF binding site and to create two new binding sites for SF2/ASF and SRp55 factors with significant high scores (respectively 2.5 and 4.33). It can therefore be hypothesized that the 1878G>C mutation alters the normal splice factor pattern necessary to accurately splice intron 18. This was confirmed by a minigene study that showed the c.1878G>C alteration to provoke exon 18 skipping during mRNA splicing (data not shown).

In the β3 gene, the homozygous 4 bp deletion c.1125+3_1125+6del (patient 25) was the single intronic mutation identified that does not directly affect a splice site but its contiguous intronic sequence. Genscan analysis predict an altered splicing that correlates with the partial loss of a sequence necessary to hybridize a small nuclear ribonucleoprotein involved in the splicing process [Wang and Cooper, 2007].

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The mutation c.191G>A (patient 21) responsible for a p.C38Y substitution, is located in the PSI domain of the β3. Transient co-transfection of the β3 mutated cDNA and with normal αIIb cDNA showed the mutation to reproduce a GT type I phenotype as no complex was detected on the cell surface by any of the anti-αIIbβ3 Moabs tested herein and including testing with a panel to β3 (XIIF9, AP3 and Y2-51, data not shown). Computational analysis (Genscan, ESEFinder and NNSPLICE 0.9) indicated that the c.191G>A mutation would not induce splicing modification of the pre-mRNA. However, western-blot analyses showed that αIIb and the mutated β3 were synthesized in transfected COS cells. The mutated Y38 β3 presents a lower relative mobility than the wild type form which could result from structural modifications induced by the C16 – C38 disulfide bond disruption. A similar observation has been reported by Valentin and co-workers [Valentin et al., 1995], who have shown that alanine substitution of C38 lowered the relative mobility of β3 expressed in COS cells. The structural modification of the PSI domain induced by the p.C38Y mutation was evidenced by the lack of reactivity of Moabs AP3 and SZ21 whose epitopes are respectively located in amino-acids stretches 49-98 [Peterson et al., 2003] and 28-35 [Honda et al., 1995]. So the structural anomaly of the PSI domain impairs the complex association and / or its post-translational maturation leading to its retention inside the cell.

The missense mutations p.M118R, p.G221D and p.K253M are located in the β-I domain of β3 and are responsible for Type I GT. M118 and G221 are highly conserved in most integrin β subunits (data not Shown), and through invertebrates to vertebrates (see Supp Figure S1B). Most of the β-I domain mutations previously described, are responsible for Variant [Bajt et al., 1992; D'Andrea et al., 2002; D'Andrea et al., 2008; Lanza et al., 1992; Loftus et al., 1990] or type II GT [Jackson et al., 1998; Morel-Kopp et al., 2002; Nair et al., 2004; Tadokoro et al., 2002; Ward et al., 2000]. For comparison, Figure 5 locates the mutated amino-acids in the primary sequence (panel A) and in the β-I domain structure (panel B). Whatever the variant or

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type II GT phenotype, the mutated complexes are located completely or partially at the surface of the β-I domain, and some of them (see Figure 5B, underlined amino-acids) present significant electrostatic or / and free energies interactions with the β-propeller (data not shown). Variant mutations concern the MIDAS or SyMBS sites [Zhu et al., 2008] directly affecting amino-acids involved in metal coordination (p.D119Y, p.S123P) or their very nearby environment (p.R217W or Q) (see Figure 5A). Both variant and GT Type II mutations support at least a 10 % expression of αIIbβ3 at the platelet surface. This contrasts with the failure to detect the β3-R118 and -D221 forms of the complex. Both the p.M118R and the p.G221D mutations, located inside the β-I domain, should deeply alter its proper folding, preventing complex expression at the platelet surface. Due to the nature of the amino-acid involved, these mutations induce important changes of steric hindrance and charge. However, in one case, the mutation p.L117W which affects a 100 % buried amino-acid still permits a 10 % expression of αIIbβ3 at the platelet surface [Basani et al., 1997]. We could hypothesize that the characteristics of these residues, both hydrophobic and aliphatic, could explain this behavior. In contrast the p.G221D mutation, changes of volume and charge of the side-chains has a too deep impact. Similarly, such a steric hindrance change could explain the type I phenotype induced by the p.M124V mutation [Gonzalez-Manchon et al., 2004], as the M124 side chain pointed inward the β-I domain.

Unlike for M118 and G221, K253 is not conserved in other integrin β subunits and if it is present in β3 from fish to primates it is replaced by an arginine in amphibian and absent from invertebrate integrins (see Supp Figure S1B). However the p.K253M substitution results in type I GT. The 3-dimensional structure of the αIIbβ3 headpiece revealed that the side chain of K253 protrudes from the β-I domain toward a pocket at the surface of the αIIb β-propeller (see Figure 3A and 5B). Analyses of electrostatic, desolvation and free energies revealed that K253 is one of the most important amino-acids involved in the β-I domain interaction with

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the β-propeller of αIIb. Furthermore, a mutant model of the αIIbβ3 headpiece shows that the p.K253M substitution does not introduce steric and energy clashes but only suppresses important contacts with the αIIb β-propeller. So, the p.K253M mutation revealed K253 as a key amino-acid for the association of the β-I domain with the β-propeller. The last mutation reported so far of the β-I domain that leads to type I GT was a substitution of the I325-P326-G327 sequence by a methionine. This mutation impairs the αIIbβ3 association [Morel-Kopp et al., 1997]. However, the mutated amino-acids locate between the α6 helix and the β7 sheet and do not directly face the αIIb β-propeller surface (see Figure 5B). Furthermore the I325-P326-G327 sequence does not seem to have significant if any, interaction with the β-propeller as indicated by free and electrostatic energy analysis (data not shown). It might indirectly impair the interaction(s) of the α5 and / or α6 helix with the β-propeller. The last four mutations described, p.K137Q [Peterson et al., 2008], p.T140I (HPA-16bw) [Jallu et al., 2002], p.R143Q (HPA-4) [Wang et al., 1992] and p.T195N (HPA-17bw) [Stafford et al., 2008] only lead to allelic variants involved in alloimmune thrombocytopenia that are not related to GT phenotype. All these amino-acids locate at the surface of the β-I domain with their side chains directed outside.

In conclusion, we have identified 29 mutations in a panel of 24 GT patients and 2 GT carriers. Seventeen of these mutations have not been described so far. We have confirmed by *in vitro* studies that the missense mutations identified impaired normal expression of the mutant complex (except for one case). Nonsense and splice site mutations were considered to be related to the GT phenotype. This study emphasizes the high allelic heterogeneity of both the αIIb and β3 genes involved in GT phenotype. Finally the GT variant p.K253M of β3 unveiled the key role of K253 in the β-propeller / β-I association of the αIIbβ3 headpiece.

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Reference List

Bajt ML, Ginsberg MH, Frelinger III AL, Berndt MC, Loftus JC. 1992. A spontaneous mutation of integrin αIIbβ3 (platelet glycoprotein IIb-IIIa) helps define a ligand binding site. J Biol Chem 267:3789-3794.

Basani RB, Brown DL, Vilaire G, Bennett JS, Poncz M. 1997. A Leu^{117}--> Trp mutation within the RGD-peptide cross-linking region of β3 results in Glanzmann thrombasthenia by preventing αIIbβ3 export to the platelet surface. Blood 90:3082-3088.

Basani RB, French DL, Vilaire G, Brown DL, Chen F, Coller BS, Derrick JM, Gartner TK, Bennett JS, Poncz M. 2000. A naturally occurring mutation near the amino terminus of αIIb defines a new region involved in ligand binding to αIIbβ3. Blood 95:180-188.

Burge CB, Karlin S. 1998. Finding the genes in genomic DNA. Curr Opin Struct Biol 8:346-354.

Caen JP. 1972. Glanzmann thrombasthenia. J Clin Invest 1:388-391.

Caen JP. 1989. Glanzmann's thrombasthenia. Baillieres Clin Haematol 2:609-625.

Camacho CJ, Zhang C. 2005. FastContact: rapid estimate of contact and binding free energies. Bioinformatics 21:2534-2536.

Canutescu AA, Shelenkov AA, Dunbrack RL, Jr. 2003. A graph-theory algorithm for rapid protein side-chain prediction. Protein Sci 12:2001-2014.

Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. 2003. ESEfinder: A web resource to identify exonic splicing enhancers. Nucleic Acids Res 31:3568-3571.

Champ PC, Camacho CJ. 2007. FastContact: a free energy scoring tool for protein-protein complex structures. Nucleic Acids Res 35:W556-W560.

D'Andrea G, Bafunno V, Del VL, Amoriello A, Morabito P, Vecchione G, Grandone E, Margaglione M. 2008. A beta3 Asp217-->Val substitution in a patient with variant Glanzmann Thrombasthenia severely affects integrin alphaIIBbeta3 functions. Blood Coagul Fibrinolysis 19:657-662.

D'Andrea G, Colaizzo D, Vecchione G, Grandone E, Di Minno G, Margaglione M, on behalf of the Glanzmann's thrombastenia Italian team (GLATIT). 2002. Glanzmann's thrombasthenia : Identification of 19 new mutations in 30 patients. Thromb Haemost 87:1034-1042.

French DL, Coller B. 1997. Hematologically important mutations: Glanzmann thrombasthenia. Blood Cells, Molecules, and Diseases 23:39-51.

Gonzalez-Manchon C, Butta N, Larrucea S, Arias-Salgado EG, Alonso S, Lopez A, Parrilla R. 2004. A variant thrombasthenic phenotype associated with compound heterozygosity of integrin β_3-subunit : (Met124Val)β3 alters the subunit dimerization rendering a decreased number of constitutive active αIIbβ3 receptors. Thromb Haemost 92:1377-1386.

Gonzalez-Manchon C, Fernandez-Pinel M, Arias-Salgado EG, Ferrer M, Alvarez MV, Garcia-Munoz S, Ayuso MS, Parrilla R. 1999. Molecular genetic analysis of a compound heterozygote for the glycoprotein (GP) IIb gene associated with Glanzmann's thrombasthenia : disruption of the 674-687 disulfide bridge in GPIIb prevents surface exposure of GPIIb-IIIa complexes. Blood 93:866-875.

Honda S, Honda Y, Bauer B, Ruan C, Kunicki TJ. 1995. The impact of three-dimensional structure on the expression of Pl^{A} alloantigens on human integrin β_3. Blood 86:234-242.

Jackson DE, White MM, Jennings LK, Newman PJ. 1998. A ser $_{162}$--> Leu mutation within glycoprotein (GP) IIIa (integrin β_3) results in an unstable $\alpha_{IIb}\beta_3$ complex that retains partial function in a novel form of type II Glanzmann thrombasthenia. Thromb Haemost 80:42-48.

Jallu V, Diaz-Ricart M, Ordinas A, Pico M, Vezon G, Nurden AT. 1994. Two human antibodies reacting with different epitopes on integrin β_3 of platelets and endothelial cells. Eur J Biochem 222:743-751.

Jallu V, Meunier M, Brement M, Kaplan C. 2002. A new platelet polymorphism Duv(a+), localized within the RGD binding domain of glycoprotein IIIa, is associated with neonatal thrombocytopenia. Blood 99:4449-4456.

Jayo A, Pabon D, Lastres P, Jimenez-Yuste V, Gonzalez-Manchon C. 2006. Type II Glanzmann thrombasthenia in a compound heterozygote for the alphaIIb gene. A novel missense mutation in exon 27. Haematologica 91:1352-1359.

Jin Y, Dietz HC, Montgomery RR, Bell WR, Mcintosh I, Coller B, Bray PF. 1996. Glanzmann thrombastenia. Cooperative between sequence variants in *Cis* during splice site selection. J Clin Invest 98:1745-1754.

Jin Y, Dietz HC, Nurden A, Bray PF. 1993. Single-strand conformation polymorphism analysis is a rapid and effective method for the identification of mutations and polymorphisms in the gene for GP IIIa. Blood 82:2281-2288.

Kato A, Yamamoto K, Miyazaki S, Jung SM, Maoi M, Aoki N. 1992. Molecular basis for Glanzmann's thrombasthenia (GT) in a compound heterozygote with glycoprotein IIb gene : a proposal for the classification of GT based on the biosynthetic pathway of glycoprotein IIb-IIIa complex. Blood 79:3212-3218.

Lanza F, Stierlé A, Fournier D, Morales M, André G, Nurden AT, Cazenave JP. 1992. A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb-IIIa. Complexes and a glycoprotein $IIIa^{214}Arg$-->^{214}Trp mutation. J Clin Invest 89:1995-2004.

Laskowski RA, Mac Arthur MW, Moss DS, Thornton JM. 1993. PROCHECK : A program to check the stereochemical quality of protein structures. J Appl Cryst 26:286-291.

Loftus JC, O'Toole TE, Plow EF, Glass A, Frelinger III AL, Ginsberg MH. 1990. A $\beta 3$ integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. Science 249:915-918.

For Peer Review

Mitchell WB, Li JH, Singh F, Michelson AD, Bussel J, Coller BS, French DL. 2003. Two novel mutations in the αIIb calcium-binding domains identify hydrophobic regions essential for αIIbβ3 biogenesis. Blood 101:2268-2276.

Morel-Kopp MC, Kaplan C, Proulle V, Jallu V, Melchior C, Peyruchaud O, Aurousseau MH, Kieffer N. 1997. A three amino acid deletion in glycoprotein IIIa is responsible for type I Glanzmann's thrombasthenia : Importance of residues IIe325 Pro326 Gly327 for B3 integrin subunit association. Blood 90:669-677.

Morel-Kopp MC, Melchior C, Chen P, Ammerlaan W, Lecompte T, Kaplan C, Kieffer N. 2002. A naturally occuring point mutation in the β3 integrin MIDAS-like domain affects differently αvβ3 and αIIbβ3 receptor function. Thromb Haemost 86:1425-1434.

Nair S, Ghosh K, Shetty S, Mohanty D. 2004. A novel Ser123Pro substitution in the MIDAS domain of integrin 3 associated with variant Glanzmann's thrombasthenia in an Indian patient. Haematologica 89:1529-1530.

Negrier C, Vinciguerra C, Attali O, Grenier C, Larcher ME, Dechavanne M. 1998. Illegitimate transcription : its use for stuying genetic abnormalities in lymphoblastoid cells from patients with Glanzmann thrombasthenia. Br J Haematol 100:33-39.

Newman PJ, Seligsohn U, Lyman S, Coller BS. 1991. The molecular genetic basis of Glanzmann thrombasthenia in the iraqi-jewish and arab populations in Israel. Proc Natl Acad Sci USA 88:3160-3164.

Nurden AT. 2005. Qualitative disorders of platelets and megakaryocytes. J Thromb Haemost 3:1773-1782.

Nurden AT, Breillat C, Jacquelin B, Combrie R, Freedman J, Blanchette VS, Schmugge M. 2004. Triple heterozygosity in the integrin α_{IIb} subunit in a patient with Glanzmann's thrombasthenia. J Thromb Haemost 2:813-819.

Nurden AT, Ruan J, Pasquet JM, Gauthier B, Combrié R, Kunicki T, Nurden P. 2002. A novel ^{196}Leu to Pro substitution in the β3 subunit of the αIIbβ3 integrin in a patient with a variant form of Glanzmann thrombasthenia. Platelets 13:101-111.

Peretz H, Rosenberg N, Landau M, Usher S, Nelson EJ, Mor-Cohen R, French DL, Mitchell BW, Nair SC, Chandy M, Coller BS, Srivastava A, Seligsohn U. 2006. Molecular diversity of Glanzmann thrombasthenia in southern India: new insights into mRNA splicing and structure-function correlations of alphaIIbbeta3 integrin (ITGA2B, ITGB3). Hum Mutat 27:359-369.

Peterson JA, Gitter M, Kanack A, Curtis B, McFarland J, Aster RH. 2009. New low-frequency platelet glycoprotein polymorphisms associated with neonatal alloimmune thrombocytopenia. Transfusion published online : 10 Oct 2009

Peterson JA, Nyree CE, Newman PJ, Aster RH. 2003. A site involving the "hybrid" and PSI homology domains of GPIIIa (beta 3-integrin subunit) is a common target for antibodies associated with quinine-induced immune thrombocytopenia. Blood 101:937-942.

For Peer Review

Quintanar A, Jallu V, Legros Y, Kaplan C. 1998. Human platelet antigen genotyping using a fluorescent SSCP technique with an automatic sequencer. Br J Haematol 103:437-444.

Reese MG, Eeckman FH, Kulp D, Haussler D. 1997. Improved splice site detection in Genie. J Comput Biol 4:311-323.

Ruan J, Peyruchaud O, Alberio L, Valles G, Clemetson K, Bourre F, Nurden AT. 1998. Double heterozygosity of the GPIIb gene in a Swiss patient with Glanzmann's thrombastenia. Br J Haematol 102:918-925.

Schlegel N, Gayet O, Morel-Kopp MC, Wyler B, Hurtaud-Roux MF, Kaplan C, Mc Gregor J. 1995. The molecular genetic basis of Glanzmann thrombasthenia in a Gipsy population in France. Identification of a new mutation on the α-IIb gene. Blood 86:977-982.

Scott JP, Chao YL, Newman PJ, Ward CM. 1998. A frameshift mutation at Gly975 in the transmembrane domain of GPIIb prevents GPIIb-IIIa expression. Analysis of two novel mutations in a kindred with type I Glanzmann thrombasthenia. Thromb Haemost 80:546-550.

Smith PJ, Zhang C, Wang J, Chew SL, Zhang MQ, Krainer AR. 2006. An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. Hum Mol Genet 15:2490-2508.

Springer TA, Zhu J, Xiao T. 2008. Structural basis for distinctive recognition of fibrinogen gammaC peptide by the platelet integrin alphaIIbbeta3. J Cell Biol 182:791-800.

Stafford P, Garner SF, Rankin A, Kekomaki R, Watkins NA, Ouwehand WH. 2008. A single-nucleotide polymorphism in the human ITGB3 gene is associated with the platelet-specific alloantigen Va (HPA-17bw) involved in fetal maternal alloimmune thrombocytopenia. Transfusion 48:1432-1438.

Tadokoro S, Tomiyama Y, Honda S, Arai M, Yamamoto N, Shiraga M, Kosugi S, Kanakura Y, Kurata Y, Matsuzawa Y. 1998. A Gln747 -->Pro substitution in the αIIb subunit is responsible for a moderate $\alpha_{IIb}\beta_3$ deficiency in Glanzmann thrombasthenia. Blood 92:2750-2758.

Tadokoro S, Tomiyama Y, Honda S, Kashiwagi H, Kosugi S, Shiraga M, Kiyoi T, Kurata Y, Matsuzawa Y. 2002. Missense mutations in the β_3 subunit have a different impact on the expression and function between $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. Blood 99:931-938.

Valentin N, Visentin GP, Newman PJ. 1995. Involvement of the cystein-rich domain of glycoprotein IIIa in the expression of the human platelet alloantigen, Pl^{A1}: Evidence for heterogeneity in the humoral response. Blood 85:3028-3033.

Wang GS, Cooper TA. 2007. Splicing in disease: disruption of the splicing code and the decoding machinery. Nat Rev Genet 8:749-761.

Wang R, Furihata K, McFarland JG, Friedman K, Aster RH, Newman PJ. 1992. An amino acid polymorphism within the RGD binding domain of platelet membrane

For Peer Review

glycoprotein IIIa is responsible for the formation of the Pena/Penb alloantigen system. J Clin Invest 90:2038-2043.

Ward CM, Kestin AS, Newman PJ. 2000. A Leu262Pro mutation in the integrin β3 subunit in an αIIb-β3 complex that binds fibrin but not fibrinogen. Blood 96:161-169.

Xiao T, Takagi J, Coller BS, Wang JH, Springer TA. 2004. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. Nature 432:59-67.

Zhu J, Luo BH, Xiao T, Zhang C, Nishida N, Springer TA. 2008. Structure of a complete integrin ectodomain in a physiologic resting state and activation and deactivation by applied forces. Mol Cell 32:849-861.

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Figure legends :

Figure 1 : Flow cytometry analysis of the expression of the mutant forms of αIIbβ3. Transiently transfected COS-7 cells expressing the wild type (WT) or the following mutants of αIIbβ3, p.S926L, p.Q595H, p.V903F (αIIb) and p.C38Y, p.M118R, p.G221D, p.K253 (β3) were tested in flow cytometry by using Moabs SZ22 (hatched bar), SZ21 (dot bar) and P2 (white bar) or irrelevant mouse IgG (black bar). As control, untransfected cells (NT) were also tested. Following Moab incubation, bound IgGs were detected by using a polyclonal goat anti-mouse IgG conjugated to FITC. Washed cells were analyzed on an Epic-XML flow cytometer. Except for the αIIb p.Q595H one, all mutants showed a markedly decreased expression of the complex when compared to the wild type. Results represent the mean ± SD of 3 experiments.

Figure 2 : Western blot analysis of the Y38 β3 form of the αIIbβ3 complex expressed in COS-7 cells. SDS lysates from normal platelets (lane a), non-transfected COS-7 cells (lane b), or COS-7 cells expressing the wild-type (lane c) or the Y38 β3 (lane d) forms of the complex were subjected to SDS-polyacrylamide gel electrophoresis under non reduced conditions and transferred to nitrocellulose membrane. Following incubation with Moabs SZ22, XIIF9, SZ21 and AP3, membrane bound IgGs were revealed by using anti-mouse IgG conjugated to peroxidase and the enhanced chemiluminescence detection technique.

Figure 3 : Location and modeling of the electrostatic effect of the p.K253M substitution. (A) and (B) Ribbon diagram of the αIIb β-propeller (in blue) and β3 βI (in red) complexed domains. For the βI domain, The residue 253 is shown in green with (A) a lysine and (B) a methionine, while for β-propeller partners the P228 is shown in yellow, the F231 in

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grey, the W262 in pink and the D232 in orange. (C) and (D) Electrostatics visualization of the single point mutation p.K253M with (C) wild K253 β3 and (D) mutated M253 β3 subunits. The visualizations are done using PyMol software (http://www.pymol.org).

Figure 4 : Localization of the V903 and S926 on a ribbon diagram of αIIb. This αIIb cartoon shows the internal location of the V903 and S926 side chains (orange, ball and stick representation) in the calf-2 composed by the N-terminal part of the β-subunit of αIIb (blue) and the C-terminal part of the α-subunit (yellow and red respectively for β-sheet and α-helix). This ribbon diagram was obtained from the resolved structure of αIIb (PDB code 3FCS) using the Swiss-PdbViewer software (version 4.0.1).

Figure 5 : Localization of the allelic variants of the β-I domain of β3. Residues identified in this study and from the literature as involved in GT or neonatal alloimmune thrombocytopenia and that locate in the β-I domain of β3 are reported on its primary sequence (panel A) and a ribbon diagrams showing the β-I domain and a partial view of the β-propeller of αIIb (panel B). Allelic variants of residues highlighted in magenta, blue, green are respectively responsible for variant, II and I GT phenotypes or yellow for those concerning alloimmunisation. The GT type induced by the allelic variant of the H192 (grey) is not reported [Peretz et al., 2006]. Amino-acids identified by the energy analyze as in contact with the αIIb β-propeller are underlined. The primary sequence (panel A) identifies the secondary structures [Xiao et al., 2004] and amino-acids responsible for MIDAS (★), ADMIDAS (●) and SyMBS (♦) sites [Springer et al., 2008]. As known, variant GT mutations GT specifically affect metal ion binding sites but other mutants concern residues evenly distributed along the primary sequence. Boxed residues have been identified in this study. The ribbon diagram (panel B) was obtained from the resolved structure of αIIb (PDB file 2VDL) using the Swiss-

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PdbViewer software (version 4.0.1). α-helix (red) and β-sheet (yellow) are shown. All residues whose mutants are involved in type II GT lay on the β-I surface except for the L117, some of them (underlined amino-acids) directly contacting the β-propeller. The Met 118 and Gly 221 locate inside the β-I domain and the Lys 253 points out to the β-propeller surface however their identified allelic variants completely prevent the αIIbβ3 complex expression at the platelet surface.

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Table 1 : αIIb mutations identified by genomic analysis[a]

N°	Proband	GT[b]	Ex/IVS[c]	Sequence alteration[d]	Status[e]	Restriction enzyme	Predicted HGVS[f]	Predicted Classical[g]	Accession number[h]
1	CabGT-1	I	Ex 4	c.531T>A	Hom.	Hga I	p.C177*	p.C146*	ss131010618
2	CabGT-2	I	Ex 10	c.917dupA	Het.	Alw I	p.R307Efs*22	p.R276E + FS[k]	ss131007603
			Ex19	c.1912dup T	Het.	No enzyme	p.C639Mfs*22	p.C608M + FS[k,l]	ss 131007606
3	CabGT-3	I	Ex 13	c.1214T>C	Het.	Sfc I	p.I405T	p.I374T[l]	ss 131007609
			IVS14	c.1442-13_1442-1del	Het.	Hph I	Splice[i]	Splice	ss 131007612
4	CabGT-4	I (car[w])	IVS14	c.1442-13_1442-1del	Het.	Hph I	Splice	Splice	ss 131007612
5	CabGT-5	I	IVS14	c.1442-13_1442-1del	Het.	Hph I	Splice	Splice	ss 131007612
				NI[j]					
6	CabGT-6	I	Ex 14	c.1413C>G	Het.	Dde I	p.Y471*	p.Y440*[m]	ss 131007615
			Ex 27	c.2800G>T	Het.	Mae III / Tsp45 I	p.V934F	p.V903F	ss 131007617
7	CabGT-7	I	Ex 16	c.1563T>A	Het.	No enzyme	p.C521*	p.C490*	ss 131007620
				NI					
8	CabGT-8	NC[x]	Ex 17	c.1672C>T	Hom.	Mse I	p.Q558*	p.Q527*	ss 131007623
9	CabGT-9	I	IVS18	c.1879-2A>G	Hom.	Xba I	Splice	Splice	ss 131007625
10	CabGT-10	I	Ex 18	c.1878G>C	Hom.	Hpy CH IV	p.Q626H / Splice	p.Q595H / Splice	ss 131007628
11	CabGT-11	I	Ex 18	c.1787T>C	Hom.	No enzyme	p.I596T	p.I565T[n]	ss 131008441
12	CabGT-12	II	Ex 21	c.2113T>C	Het.	Fok I	p.C705R	p.C674R[l,o,p]	ss 131007632
			IVS29	c.3060+2T>C	Het.	Hph I	p.V982_K1020del	p.V951_K989del[q]	ss 131007634
13	CabGT-13	I	Ex 23	c.2333A>C	Hom.	Bsr I / TspR I	p.Q778P	p.Q747P[r]	ss 131007637
14	CabGT-14	I	Ex 27	c.2748_2757del	Hom.	Rsa I	p.T917Sfs181	p.T886S + FS	ss 131007639
15	CabGT-15	I (car)	Ex 27	c.2800G>T	Het.	Mae III / Tsp45 I	p.V934F	p.V903F	ss 131007617
16	CabGT-16	I	Ex 28	c.2870C>T	Hom.	Hpy CH4 V	p.S957L	p.S926L	ss 131007642
17	CabGT-17	II	Ex 28	c.2929C>T	Het.	Ava I	p.R977*	p.R946*[l,s,t]	ss 131007644
				NI					
18	CabGT-18	NC	Ex 29	c.2944G>A	Het.	Bst NI	p.V982M	p.V951M[u,v]	ss 131007647
			Ex 29	c.2965G>A	Het.	Nci I	p.A989T	p.A958T[u,v]	ss 131007649
				NI					

[a] cDNA ITGA2B GenBank accession number NM_000419.3

[b] GT : Glanzmann type

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[c] Ex : Exon ; IVS : intronic sequence
[d] Nomenclature according to HGVS : +1 corresponds to the A of ATG translation initiation codon
[e] Status : Het. : heterozygous; Hom. : Homozygous
[f] Predicted effect, nomenclature according to HGVS : +1 corresponds to the initiating Met (signal peptide included)
[g] Predicted effect, classical nomenclature : +1 corresponds to the first amino acid of the mature αIIb
[h] NCBI dSNP accession number
[i] Splice defect based upon modeling with GENSCAN / NNSPLICE and ESEFinder
[j] NI : 2nd mutation not Identified
[k] FS : Frame shift
References : [l] D'Andrea et al., 2002 [D'Andrea et al., 2002]; [m] Scott et al., 1998 [Scott et al., 1998]; [n] Ruan et al., 1998 [Ruan et al., 1998]; [o] Gonzalez-Manchon et al., 1999 [Gonzalez-Manchon et al., 1999]; [p] Mitchell et al., 2003 [Mitchell et al., 2003]; [q] French et al., 1997 [French and Coller, 1997]; [r] Tadokoro et al., 1998 [Tadokoro et al., 1998]; [s] Basani et al., 2000 [Basani et al., 2000]; [t] Peretz et al., 2006 [Peretz et al., 2006]; [u] Nurden et al., 2004 [Nurden et al., 2004]
[v] V951M is responsible for GT but not A958T [Nurden et al., 2004]
[w] Car : carrier
[x] NC : Not communicated

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Table 2 : β3 mutations identified by genomic analysis[a]

N°	Proband	GT[b]	Ex/IVS[c]	Sequence alteration[d]	Status[e]	Restriction enzyme	Predicted HGVS[f]	Predicted Classical[g]	Accession number[h]
19	CabGT-19	NC[j]	IVS2	c.166-2A>G	Hom.	Apa I	Splice[i]	Splice	ss 131007652
20	CabGT-20	I	Ex 2	c.100C>T	Hom.	Hpy CH4 IV	p.R34*	p.R8*[l]	ss 131007655
21	CabGT-21	I	Ex 3	c.191G>A NI[k]	Het.	Tsp 45I	p.C64Y	p.C38Y	ss 131007657
22	CabGT-22	I	Ex 4	c.431T>G	Hom.	Bsm F1	p.M144R	p.M118R	ss 131007660
23	CabGT-23	II	Ex 5	c.665 T>C	Hom.	Acy I	p.L222P	p.L196P[m]	ss 131007662
24	CabGT-24	I	Ex 5	c.740 G>A	Het.	Fok I	p.G247D	p.G221D	ss 131007665
			Ex 6	c.836 A>T	Het.	Fat I	p.K279M	p.K253M	ss 131007667
25	CabGT-25	NC	IVS8	c.1125+3_1125+6del NI	Het.	No enzyme	Splice	Splice	ss 131007670
26	CabGT-26	I	Ex 9	c.1143C>A	Het.	Taq I	p.K376_T420del	p.K350_T394del[n]	ss 131007672
			Ex 9	c.1260G>A	Het.	Hph I			ss 131007674

[a] cDNA ITGB3 GenBank accession number NM_000212.2

[b] GT : Glanzmann type I or II (< 5% or 10 – 25 % of normal platelet αIIbβ3[Caen, 1989]). See Supp_mat tableS1 for detailed data.

[c] Ex : Exon ; IVS : intronic sequence

[d] Nomenclature according to HGVS : +1 corresponds to the A of ATG translation initiation codon

[e] Status : Het. : heterozygous; Hom. : Homozygous

[f] Nomenclature according to HGVS : +1 corresponds to the initiating Met (signal peptide included)

[g] Classical nomenclature : +1 corresponds to the first amino acid of the mature β3

[h] NCBI dSNP accession number

[i] Splice defect based upon modeling with GENSCAN, NNSPLICE and ESEFinder

[j] NC : Not comunicated

[k] NI : 2nd mutation not Identified

References : [l] Negrier et al., 1998 [Negrier et al., 1998]; [m] Nurden et al., 2002 [Nurden et al., 2002]; [n] Jin et al., 1996 [Jin et al., 1996]

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Table 3 : Energetic effects of the Lys 253 Met substitution in the αIIb/ β3 interaction

		Lys 253			Met 253		
Energy of :		**Rank**	**Value (kcal/mol)**	**αIIb contact**	**rank**	**Value (kcal/mol)**	**αIIb contact**
αIIbβ3 Interface	**Electrostatic (4r) Energy**	/	-25.14	/	/	-16.08	/
	Desolvation Free Energy	/	-1.09	/	/	-6.81	/
	Van der Waals (CHARMm19)	/	-4894.63	/	/	-4903.95	/
Residue	**Binding free energy**	1st negative	-5.541	/	12th negative	-1.309	/
	Desolvation free energy	1st positive	4.124	/	6th negative	-1.347	/
	Electrostatic energy	1st negative	-9.666	/	/	*low*	/
Contact	**Electrostatic**	1st negative	-6.715	D232	/	/	/
		6 th negative	-2.076	F231	/	/	/
		13th negative	-0.788	P228	/	/	/
		10th positive	0.670	W262	6th negative	-0.935	W262
	Free energy	1st negative	-5.517	D232	/	/	/
		8th negative	-1.166	F231	/	/	/
		2th positive	2.057	W262	/	/	/

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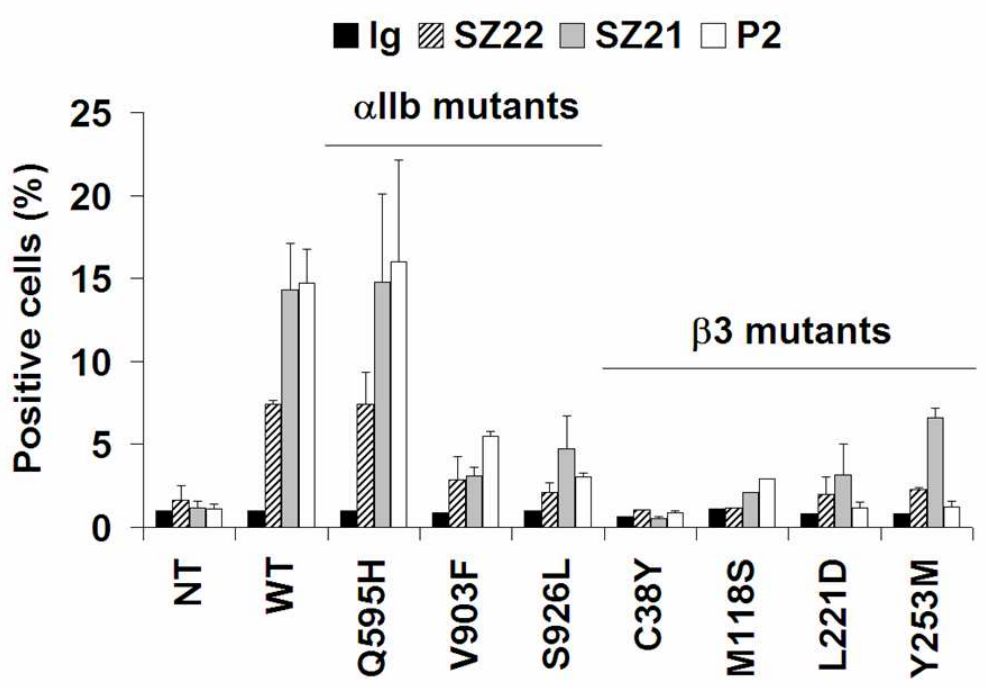

Figure 1 : Flow cytometry analysis of the expression of the mutant forms of αIIbβ3.
235x165mm (96 x 96 DPI)

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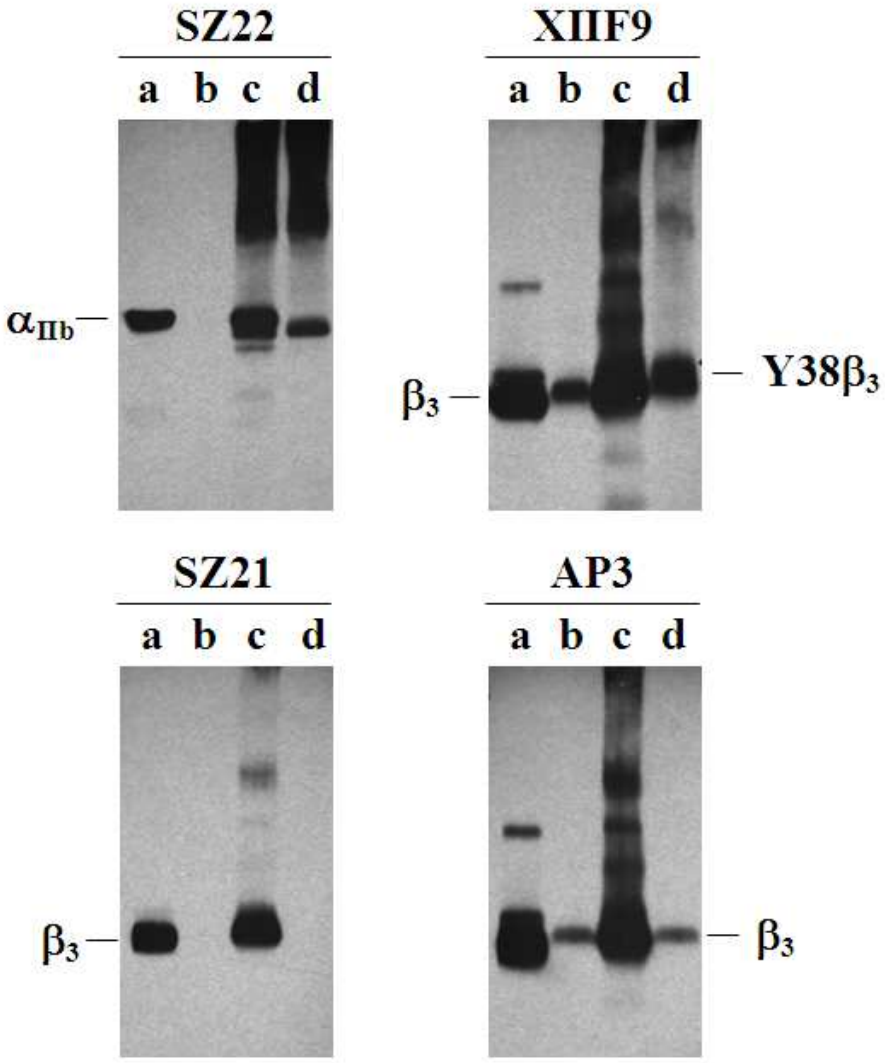
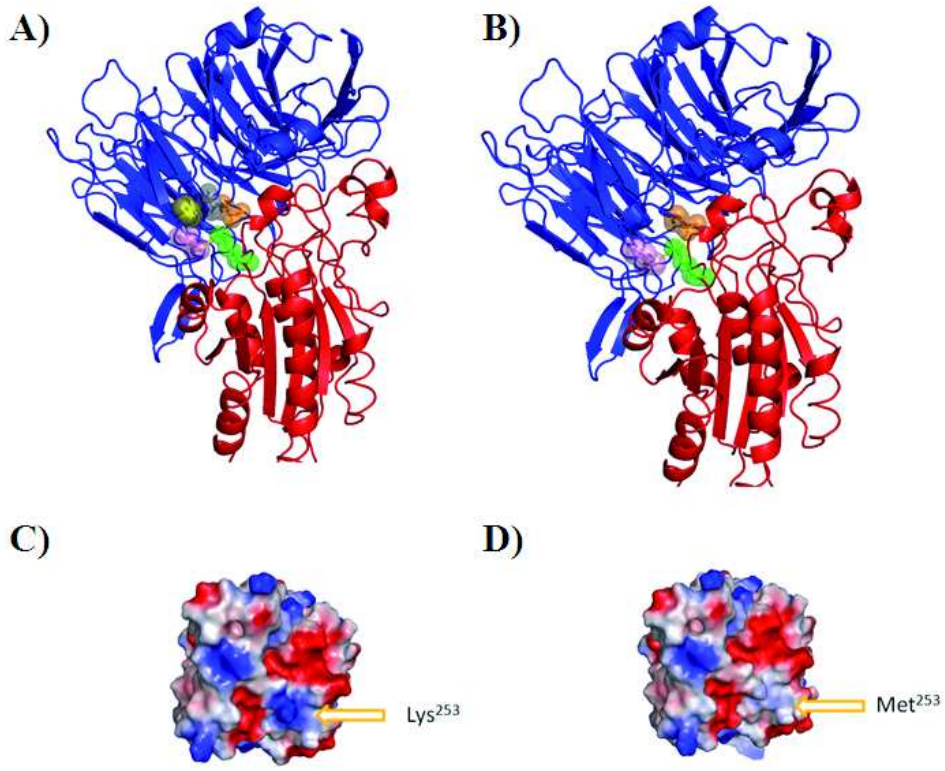
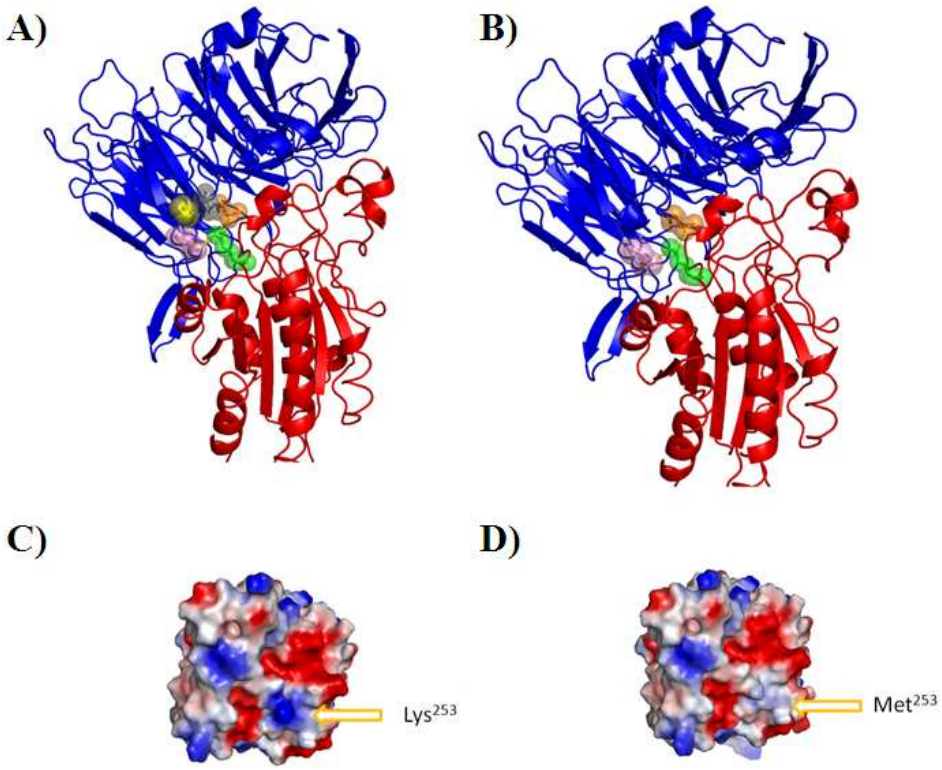


Figure 2 : Western blot analysis of the Y38 β3 form of the αIIbβ3 complex expressed in COS-7 cells.
155x177mm (96 x 96 DPI)



CMYK color scale

Figure 3 : Location and modeling of the electrostatic effect of the p.K253M substitution.
193x158mm (96 x 96 DPI)



RGB color scale

Figure 3 : Location and modeling of the electrostatic effect of the p.K253M substitution.
193x158mm (96 x 96 DPI)

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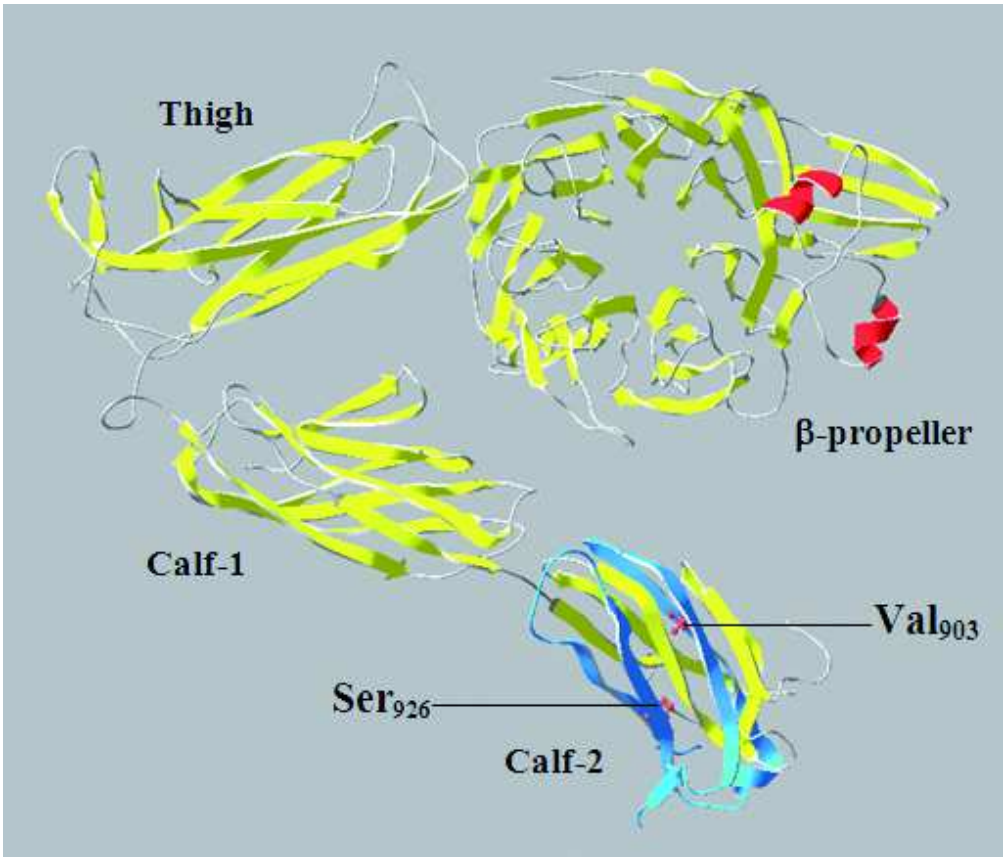
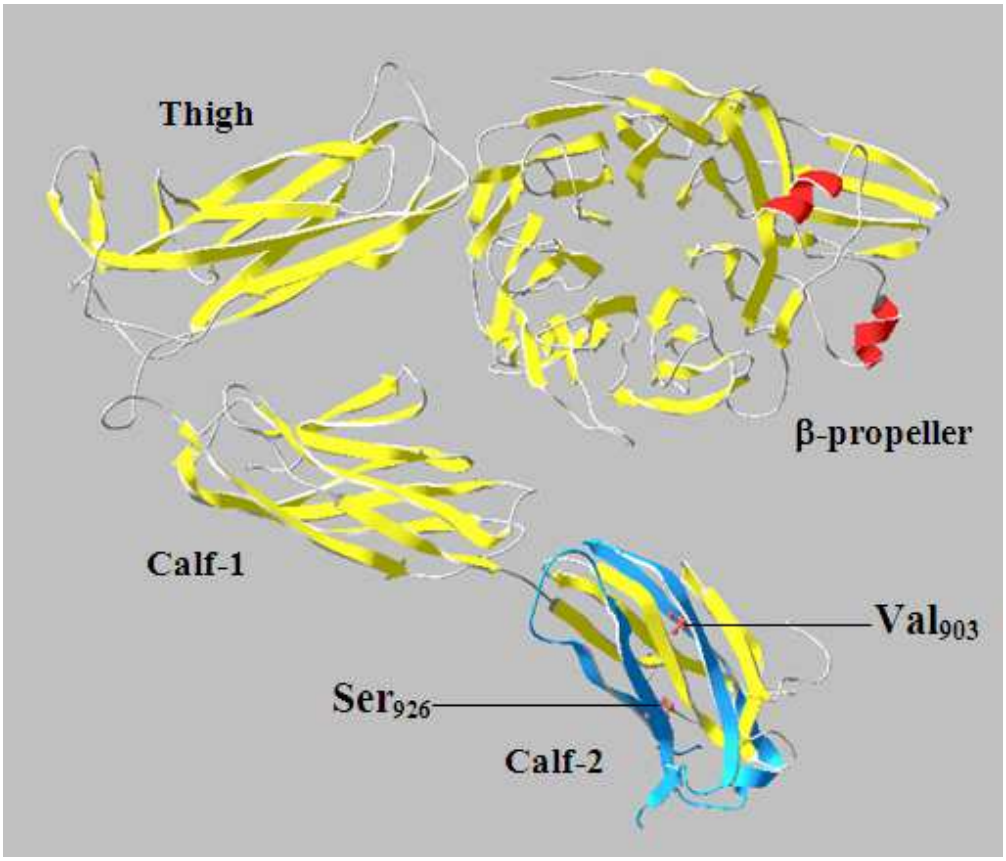


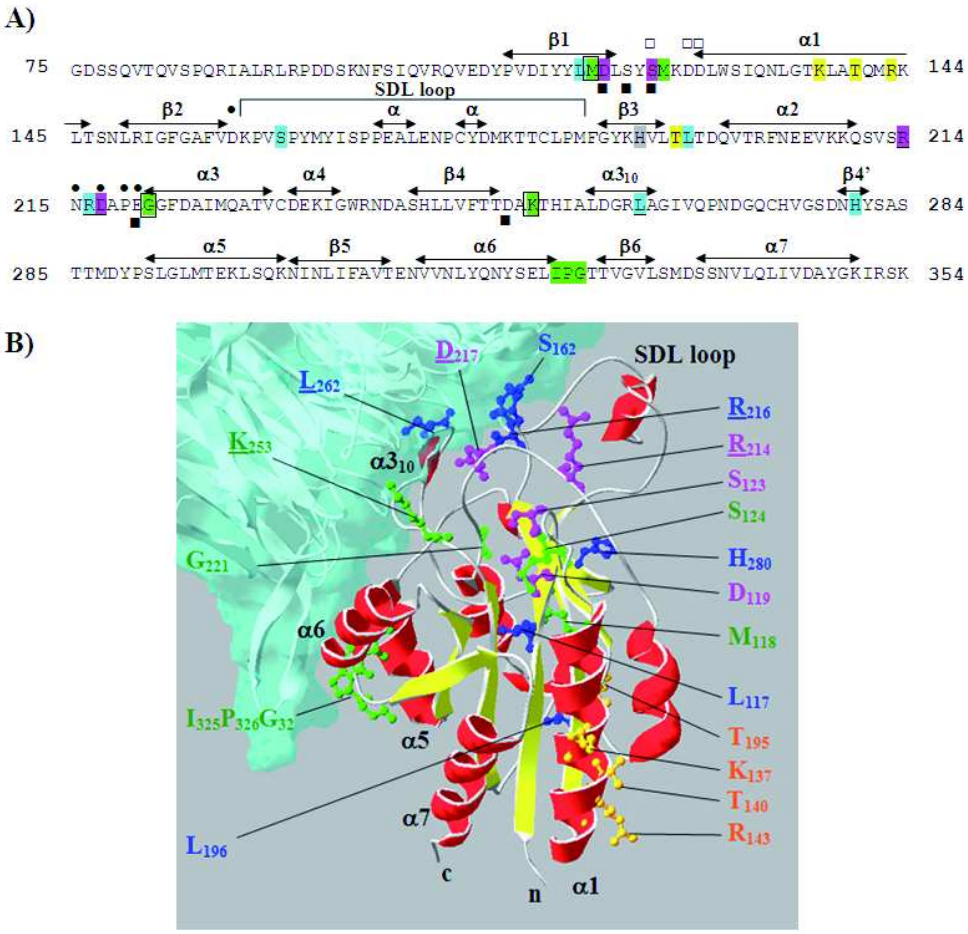
CYMK color scale

Figure 4 : Localization of the V903 and S926 on a ribbon diagram of αIIb.
135x115mm (96 x 96 DPI)



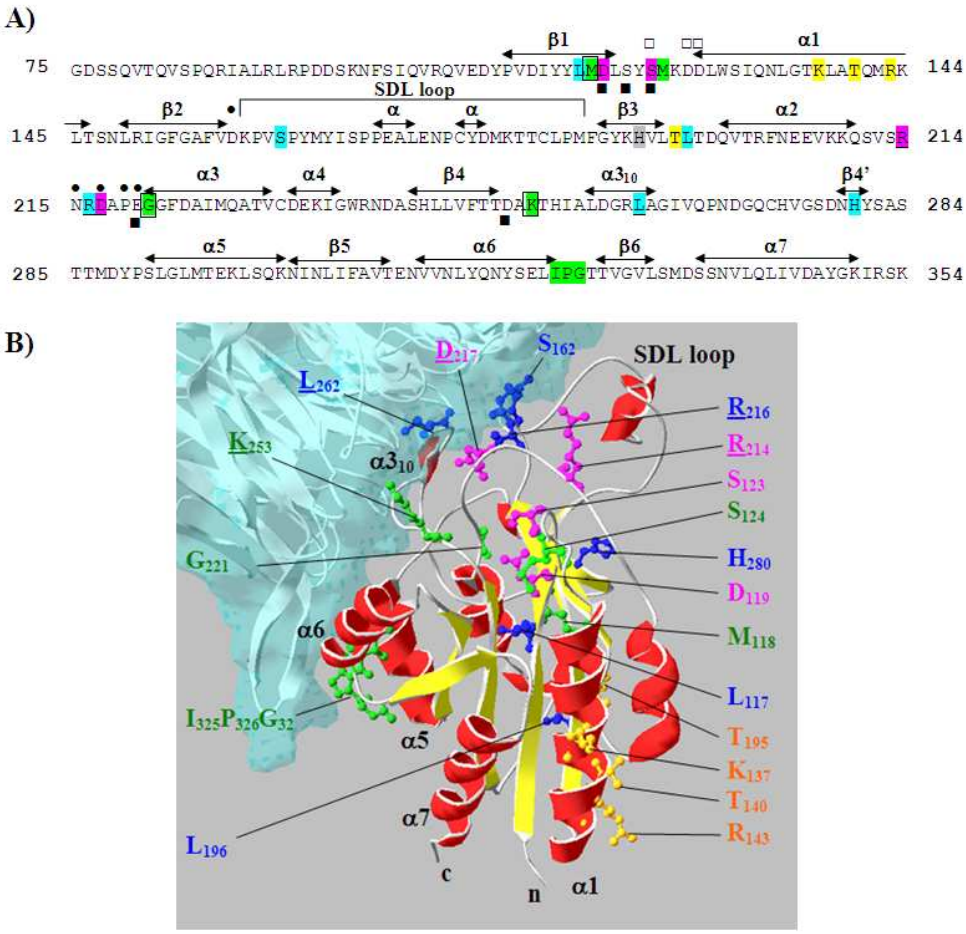
RGB color scale

Figure 4 : Localization of the V903 and S926 on a ribbon diagram of αIIb.
135x115mm (96 x 96 DPI)



CMYK color scale

Figure 5 : Localization of the allelic variants of the β-I domain of β3.
212x200mm (96 x 96 DPI)



RGB color scale

Figure 5 : Localization of the allelic variants of the β-I domain of β3.
212x200mm (96 x 96 DPI)