Single amino acid substitution in human platelet glycoprotein Ibβ is responsible for the formation of the platelet-specific alloantigen Iyª

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We recently described a new low-frequency platelet alloantigen on the human platelet glycoprotein (GP) Ib-IX complex, termed Iyª, which was implicated in a severe case of neonatal alloimmune thrombocytopenia. Immunoprecipitation studies with trypsin-treated platelets indicated that the Iyª alloantigenic determinants are formed by the membrane-associated remnant moiety of GP Ibα (GP Ibα$_r$) together with GP Ibβ and GP IX. To elucidate the molecular basis underlying the Iyª alloantigen, we amplified *GPIbα$_r$*, *GPIb*β, and *GPIX* genes by polymerase chain reaction (PCR). Nucleotide-sequence analysis of these 3 genes showed a G to A transition at position 141 on *GPIb*β gene in a subject positive for Iyª. This transition resulted in a Gly$_{15}$Glu dimorphism on the N-terminal domain of *GPIb*β. This finding was confirmed by genotyping analysis of 6 Iyª-positive subjects by restriction fragment length polymorphism (RFLP) studies using *Nar*I endonuclease. In 300 randomly selected healthy blood donors, one Iyª-positive individual was found. Phenotypes determined by monoclonal antibody-specific immobilization of platelet antigens assay and genotypes determined by RFLP were identical in this population. Analysis of Iyª-positive platelets showed that the point mutation affected neither the degree of surface expression nor the function of the GP Ibα-GP Ibβ-IX complex on the platelet surface. Transient expression of the GP Ib-IX complex in CHO cells using wild-type GP Ibβ (Gly$_{15}$) or mutant GP Ibβ (Glu$_{15}$) allowed us to demonstrate that this single amino acid substitution is sufficient to induce Iyª epitope(s). (Blood. 2000;95:1849-1855)

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Introduction

Human platelet glycoproteins (GP) are carriers of alloantigenic determinants that can elicit an alloimmune response leading to platelet destruction, such as occurs in neonatal alloimmune thrombocytopenia (NAIT), posttransfusion purpura, and platelet transfusion refractoriness.[1] Four GP subunits (GP Ia, GP Ibα, GP IIb, and GP IIIa) on the platelet surface are known to be polymorphic and immunogenic in humans.[2,3] Two allelic variants have been found for GP Ia and GP Ibα, bearing human platelet alloantigens (HPA) 5a/5b (Brᵇ/Brª) and 2a/2b (Koᵇ/Koª), respectively.[4,5] GP IIb exists in 3 allelic variants carrying HPA-3a/3b (Bakª/Bakᵇ) and HPA-9bW (Maxª).[6,7] GP IIIa is the most polymorphic molecule. Ten allelic variants encoding GP IIIa have been found in the human gene pool so far, 9 of which are immunogenic as carriers of HPA-1a (Plᴬ1) and HPA-4a (Yukᵇ or Penª), HPA-1b (Plᴬ2), HPA-4b (Yukª or Penᵇ), HPA-6bW (Caª), HPA-7bW (Moª), HPA-8bW (Srª), HPA-10bW (Laª), HPA-11bW (Groª), and Oeª alloantigenic determinants.[8-15] Most of these alloantigens result from point mutations in wild-type DNA that produce single amino acid substitutions and lead to the expression of the offending alloantigenic determinants. The Oeª variant is an exception because it results from a deletion of a codon of the mutated GP IIIa (Plᴬ2) isoform.[15]

We recently described a new low-frequency platelet alloantigen on the GP Ib-IX complex, termed Iyª, that was responsible for a case of severe NAIT.[16] GP Ib-IX complex is a receptor for both von Willebrand factor and thrombin and plays an essential role in adhesion of platelets to the subendothelium. GP Ib is a heterodimer consisting of a large α chain (molecular weight [MW], 143 kilodaltons [kd]) and a smaller disulfide-linked β chain (MW, 27 kd). In the platelet membrane, GP Ib forms a noncovalent complex with GP IX (MW, 22 kd).[17] GP Ib is also weakly associated with GP V (MW, 82 kd) in a noncovalent manner.[18] All 4 GP are members of the leucine-rich GP family containing a variable number of leucine repeats.[19-22] GP Ibα, GP Ibβ, GP IX, and GP V are known to be derived from distinct genes, with the entire open reading frame of the mature protein located within a single exon.[23-26] The *GPIbα* gene is located on chromosome 17,[27] whereas the *GPIb*β gene is on chromosome 22.[24] The *GPIX* and *GPV* genes are on distinct sites of the long arm of chromosome 3, on band q21 and band q29, respectively.[28]

We here report the first molecular variant of GP Ibβ responsible for the formation of a clinically important alloantibody in NAIT. Because this new alloantigen was found in 1 of 300 healthy blood donors tested, it may be involved in other cases of alloimmune thrombocytopenia.

Materials and methods

Monoclonal antibodies (MAB)

MAB Gi10 and Gi27 against the remnant moiety of GP Ibα and GP Ibβ, respectively, were raised and characterized in our laboratory.[12,29] MAB SZ2

and AN51 specific for GP Ibα[30,31] were purchased from Dianova and Dako (both Hamburg, Germany). MAB FMC25 against GP IX[32] was provided by H. Zola, Adelaide, Australia.

Phenotyping

Phenotyping of human platelets for the presence of Iy^a alloantigenic determinants was performed by using the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay as described previously.[33] MAB FMC 25 was used as capture antibody.[16,32]

Immunoprecipitation analysis

Washed platelets from ACD–anticoagulated blood were labeled with biotin hydrazide (Pierce, Munich, Germany) as described by Fabris et al, with minor modifications.[34] Briefly, 10^9 platelets in 900 μL of phosphate-buffered saline (PBS)-EDTA (3.72 g/L of EDTA, 1 μmol/L of leupeptin, 1 mmol/L of phenylmethylsulfonyl fluoride (PMSF), 4 mmol/L of N-ethylmaleimide in PBS; pH 7.4) were exposed to 100 μL of 12 mmol/L sodium metaperiodate at 4°C for 10 minutes; 0.6 mol/L of glycerol was then added. After being washed twice with 500 μL of PBS-EDTA, the platelets were incubated with 3 mmol/L of biotin hydrazide at room temperature for 2 hours. Labeled platelets were washed 4 times and lysed in 1 mL solubilization buffer (25 mmol/L of Tris, 10 mmol/L of EDTA, 100 mmol/L of sodium chloride (NaCl) containing 1% Triton X-100, 2 mmol/L of PMSF, 1 mmol/L of leupeptin, and 2 mmol/L of N-ethylmaleimide) for 30 minutes at 4°C.

Aliquots of 10^9 labeled platelets were digested with 100 μL of trypsin (1 mg/mL; Sigma, Deisenhofen, Germany) for 5 minutes at 37°C. Digestion was stopped by adding 200 μL of soybean trypsin inhibitor (1 mg/mL; Sigma). Trypsin-treated platelets were washed twice and then solubilized as described above.

After centrifugation (30 minutes at 16 000g at 4°C), immunoprecipitation was performed as described previously.[12] Immunoprecipitates were separated by using 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and visualized with use of streptavidin-peroxidase and chemiluminescence substrate. Colored protein MW markers (Rainbow; Amersham, Braunschweig, Germany) were used as the standard.

Immunoblotting

Aliquots of 10^9 washed platelets were lysed in 1 mL of solubilization buffer. After centrifugation (30 minutes at 16 000g at 4°C), proteins were separated with SDS-PAGE and transferred to nitrocellulose membrane. Membrane strips were blocked with 1.5% bovine serum albumin (BSA) in PBS and then incubated with an MAB dilution (20 μg/mL) or serum for 30 minutes at room temperature. The strips were washed twice with Tris buffer (pH 7.4) containing 0.05% Tween 20 and then incubated with peroxidase-conjugated rabbit antimouse or antihuman antibodies (1:200 000 dilution; Dianova). After washing, the recognized protein was visualized by using the enhanced chemiluminescence substrate system (Amersham).

Determination of GP Ib-IX binding sites

Aliquots of 2×10^7 washed platelets were incubated with increasing amounts of MAB Gi10 (50-100 μg) at 37°C for 30 minutes. The sensitized platelets were washed 3 times with 0.2% BSA in isotonic saline before resuspension in 80 μL of isotonic saline. Bound MAB were eluted with 40 μL of 100 mmol/L of NaCl (pH 2.2; adjusted with acetic acid) containing 1.5% BSA for 10 minutes at room temperature. After centrifugation, eluates were neutralized with a predetermined volume (about 3.3 μL) of 2.5 mmol/L of Tris buffer. A sandwich enzyme-linked immunoassay (ELISA) using purified normal mouse IgG (m-IgG) as the standard was used to quantify the number of binding sites, as reported previously.[12] In brief, microtiter wells were coated overnight with 100 μL of goat antimouse IgG F(ab′)$_2$ (1:1000 dilution in coating buffer; Dianova) at 4°C. After being washed 3 times with 200 μL of 1% BSA in PBS (PBS-BSA), wells were

blocked with 200 μL of PBS-BSA for 30 minutes at 4°C and then incubated with either 100 μL of eluates or 100 μL of various dilutions of m-IgG (2500-50 pg) for 1 hour at 37°C. Afterward, the wells were washed 3 times, and 100 μL of alkaline phosphatase-labeled goat antimouse IgG Fc (1:1000 dilution, Dianova) was added. After 1 hour of incubation at 37°C, the wells were washed 5 times. Finally, 100 μL of p-nitrophenylphosphate substrate solution (Sigma) was added and the plate was incubated at room temperature for 30 minutes. The color reaction was stopped by adding 50 μL of 3 mol/L of sodium hydroxide and was read at 405 nm in a Titertek photometer (Helsinki, Finland). All samples were assayed in duplicate.

Platelet function studies

Platelet-rich plasma (PRP) was obtained by centrifugation (200g for 15 minutes) of ACD–anticoagulated blood collected from Iy^a-positive and Iy^a-negative individuals. The platelet count was adjusted to 3×10^5 per μL by dilution with autologous plasma. To aliquots of 180 μL of PRP, 20 μL of various dilutions of ristocetin (2.5, 5, 10, and 15 μg/mL) were added and the change in optical density was monitored by using an aggregometer, with continuous stirring, at 37°C.

To evaluate the functional effect of anti-Iy^a antibodies, aliquots of 180 μL of PRP derived from Iy^a-positive individuals were mixed with either 20 μL of isotonic saline, MAB SZ2 (20 μg/mL), or 20 μL eluates of heat-inactivated serum (normal human serum [NHS] or anti-Iy^a) and incubated at 37°C for 30 minutes in an atmosphere supplemented with 5% carbon dioxide (CO_2). After stimulation with ristocetin (15 μg/mL), platelet aggregation was recorded as described above.

Isolation and amplification of genomic DNA

Genomic DNA was isolated from 10 mL of EDTA–anticoagulated blood from Iy^a-phenotyped donors as described previously.[12] Primers used to amplify the *GPIb*α$_r$, *GPIb*β, and *GPIX* genes (Table 1) were constructed according to published DNA sequences.[20,23,25] The coding regions of the *GPIb*α gene encompassing nucleotides 826 to 1965 were amplified in 2 overlapping fragments (bases 826 to 1518 and bases 1192 to 1965) by using primer pairs GP Ibα 1-GP Ibα 2 and GP Ibα 3-GP Ibα 4, respectively. Amplification was performed in a total volume of 50 μL containing 10 μL of genomic DNA (400-600 ng), 0.3 μmol/L of each primer, 200 μmol/L of each dNTP, 1.5 mmol/L of magnesium chloride ($MgCl_2$), and 1.5 U of *Taq* GOLD polymerase on a GeneAmp 9600 DNA thermal cycler (Perkin Elmer, Weiterstadt, Germany). After heating at 97°C for 5 minutes, polymerase chain reaction (PCR) was performed under the following conditions. For amplification of the first region (bases 826-1518), denaturation was done for 75 seconds at 94°C, annealing for 120 seconds at 52°C, and extension for 180 seconds at 72°C. For the second region (bases 1192-1965), denaturation was done for 60 seconds at 95°C, annealing for 90 seconds at 55°C, and extension for 120 seconds at 72°C. Both amplifications proceeded for 36 cycles. In the final cycle, all samples were kept at 72°C for 10 minutes and then chilled to 4°C.

The entire coding region of *GPIb*β (nucleotides 47-939) was amplified by using 0.5 μmol/L each of GP Ibβ 1 and GP Ibβ 2 primer, 200 μmol/L

Table 1. Sequences and positions of primers used in the polymerase chain reaction amplification of GPIbα$_r$, GPIbβ, and GPIX genes

Primer	Position	Sequence (5′ → 3′)
GP Ibα 1	826-843	GTGCAGTGTGACAATTCA
GP Ibα 2	1518-1501	GTCATTTCTGGAGCTCTC
GP Ibα 3	1192-1220	CCAAGCCCGACCACCTCAGAGCCC
GP Ibα 4	1965-1942	AGCCCACAGGCTCTTCTCTCAAGG
GP Ibβ 1	47-72	CTGAGCTTACTGCTCCTGCTGCTGGC
GP Ibβ 2	939-920	GGGTTTATTCAGCACCAGAG
GP Ibβ 3	378-359	ACGCAACGCAGGTCGCGGTA
GP IX 1	271-288	ACCAAGGACTGCCCCAGC
GP IX 2	750-733	ATCCAGGGCCTCTGTGGT

Numbering is according to published complementary DNA sequences.[20,23,25]

each of dNTP, 1.5 mmol/L of $MgCl_2$, 10% dimethyl sulfoxide (DMSO), 1.5 U µL of *Taq* GOLD polymerase, and 5 µL of 10 × PCR buffer. A cycle consisted of denaturation at 94°C for 75 seconds, annealing at 48°C for 90 seconds, and primer extension at 72°C for 120 seconds and was repeated 35 times.

The coding region of *GPIX* (nucleotides 271-750) was amplified by using 0.5 µmol/L each of GP IX 1 and GP IX 2 primer, 200 µmol/L each of dNTP, 1.2 mmol/L of $MgCl_2$, 0.01% gelatin, 1.5 U of *Taq* GOLD polymerase, and 5 µL 10 × PCR buffer under the same PCR conditions used for the *GPIb*β gene except that the annealing was done at 52°C.

Subcloning and sequencing

All PCR products were purified on 1.5% SeaKem agarose gel (FMC, Hessisch Oldendorf, Germany) by using Geneclean (Dianova). Purified DNA was flushed with Klenow DNA polymerase (Biolabs, Schwalbach, Germany) for blunt-end ligation into the *Eco*RV site of the pGEM5 plasmid and then transformed into DH5α high-efficiency competent *Escherichia coli* (Gibco BRL, Eggenstein, Germany). Before ligation, the GP Ibβ PCR product was shortened by digestion with *Sma*I endonuclease (Biolabs). Recombinant colonies were selected by blue-white screening on indicator plates. For single-strand nucleotide sequencing, plasmid DNA from 8 positive clones of each region was amplified with use of biotinylated forward primer 5′-CGC CAG GGT TTT CCC AGT CAC GAC G-3′ and nonbiotinylated reverse primer 5′-GCT TCC GGC TCG TAT GTT GTG TGG-3′ or vice versa. Biotinylated single-strand DNA was isolated by magnetic beads (Dynal, Norway), sequenced with SP6 and T7 primers using a fluorescence DNA-sequencing kit (Perkin Elmer), and then analyzed on ABI Prism 373 DNA Sequencers (Applied Biosystems, Weiterstadt, Germany).

Genotyping by restriction fragment length polymorphism (RFLP)

Genomic DNA was amplified by using primer pair GP Ibβ 1-GP Ibβ 3 as described above, except that ''hot start'' PCR was saved by application of *Taq* GOLD polymerase (1.5 U, Perkin Elmer). Aliquots of 7 µL of PCR products were subjected to RFLP using 2 U of *Nar*I endonuclease (Biolabs) and then analyzed on 3.0% NuSieve agarose gel (Gibco).

Construction of allele-specific GP Ibβ expression vectors

A full-length complementary DNA (cDNA) encoding wild-type GP Ibβ in pDX plasmid was removed with *Eco*RI (Biolabs) and ligated into the mammalian pcDNA3.1 Zeo expression vector (Invitrogen, Leek, Holland). Specific mutation G→A at position 141 was induced in the wild-type GP Ibβ construct by site-directed mutagenesis with use of a QuickChange Mutagenesis Kit (Strategene, Heidelberg, Germany). For PCR amplification, single nucleotide-mismatched sense primer 5′-GGG ACG CTC GTG GAC TGC GAG CGC CGC GGG CTG ACT TGG-3′ and antisense primer 5′-CCA AGT CAG CCC GCG GCG CTC GCA GTC CAC GAG CGT CCC-3′ corresponding to base 122 to 160 of GP Ibβ cDNA were constructed. After 12 cycles of amplification (denaturation for 30 seconds at 95°C, annealing for 60 seconds at 55°C, and extension for 12 minutes at 68°C) in the presence of 10% DMSO, PCR product was digested with *Dpn*I and transformed into DH5α high-efficiency competent *E coli*. Plasmid DNA from positive clones was amplified by PCR using GP Ibβ 1 and GP Ibβ 3 primers, and subjected to RFLP analysis with *Nar*I as described above. Purified GP Ibβ allele-specific constructs used for subsequent transfection were validated by nucleotide-sequence analysis.

Cell culture and transfection

CHO cells stably expressing GP Ibα and GP IX[35] were transiently transfected by liposome-mediated delivery of plasmid DNA with use of a commercially available kit (Lipofectamine, Gibco BRL, Grand Island, NY). Cells were grown to approximately 70% confluence on 50-mm² tissue culture dishes. Twelve microliters of liposome suspension and 2 µg of plasmid DNA (either pcDNA 3.1 Zeo vector containing the wild-type or mutated cDNA of GP Ibβ, or the vector alone) were separately mixed in 200 µL of serum-free medium. The 2 suspensions were then combined, mixed gently, and allowed to form DNA-liposome complexes for 30 minutes at room temperature. The mixture was diluted in 1.6 mL of serum-free medium and added to the cells, which had been washed twice with the same medium. The cells were exposed to the mixture for 5 hours under standard culture conditions (37°C in 5% CO_2), after which 2 mL of medium containing 10% fetal-calf serum was added. Twenty-four hours later, the medium was changed.

Flow cytometry

Forty-eight hours after transfection, cells were detached from the dishes with 0.54 mmol/L of EDTA and washed twice with PBS. Cells were fixed in 1% paraformaldehyde and washed twice. A total of 400 000 cells were counted by hemacytometer, resuspended in 200 µL of PBS, and incubated with 50 µL of serum (either anti-Iyᵃ serum or NHS) for 30 minutes at 37°C. The cells were washed twice with PBS and resuspended in 200 µL of PBS. They were incubated with 40 µL of a 1:40-diluted fluorescein isothiocyanate-conjugated rabbit antihuman antibody (Dianova) for 30 minutes at 37°C. To remove unbound antibody, cells were washed twice. A total of 10 000 cells from each transfection were analyzed with an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ).

Results

Immunochemical characterization

To characterize the Iyᵃ antigen, immunoprecipitation studies with surface-labeled biotinylated platelets were performed (Figure 1). When anti-Iyᵃ immunoprecipitate was electrophoresed under reducing conditions, 3 bands—GPIbα, GPIbβ, and GP IX—could be detected, with apparent MW of 145 kd, 27 kd, and 22 kd, respectively (Figure 1, left panel, lane 1). In the control experiments, these bands could not be precipitated from Iyᵃ-negative platelets (Figure 1, lane 2).

To analyze the surface expression of the GP Ib-IX complex in Iyᵃ-positive platelets, we compared the levels of GP Ib-IX precipitates from Iyᵃ-positive and Iyᵃ-negative platelets by immunoprecipitation (Figure 1, right panel). When MAB Gi27 directed against GP Ibβ was used, similar amounts of GP Ibα, GP Ibβ, and GP IX were precipitated from both platelet phenotypes. In addition, in both platelet types, GP Ibα, GP Ibβ, and GP IX subunits migrated with similar mobility. Similar results were obtained with MAB SZ2 directed against the GP Ibα subunit and with MAB FMC25 directed against the GP IX subunit (data not shown). These observations indicate that normal amounts of GP Ib-IX complex are expressed on the surface of Iyᵃ-positive platelets and that the Iyᵃ antigen is not associated with an MW polymorphism.

To further localize the epitope recognized by anti-Iyᵃ antibodies, we took advantage of the fact that trypsin cuts off the amino-terminal part of GP Ibα, leaving GP Ibα$_r$ as the remnant moiety, which is associated with GP Ibβ and GP IX on the platelet surface.[36] After trypsin treatment, anti-Iyᵃ antibodies (Figure 2, lane 4) still precipitated the GP Ibα$_r$-Ibβ complex (MW, 65 kd) and GP IX subunit (MW 22, kd) under nonreducing conditions, as did MAB Gi10 and Gi27 (Figure 2, lanes 2 and 3). In contrast, MAB SZ2 (Figure 2, lane 1), directed against the glycocalicin moiety, did not precipitate any platelet proteins.

In immunoblotting analysis, no reactivity of anti-Iyᵃ antibodies with any platelet proteins was detectable (Figure 3, lane 1) under either nonreducing or reducing conditions. In the control
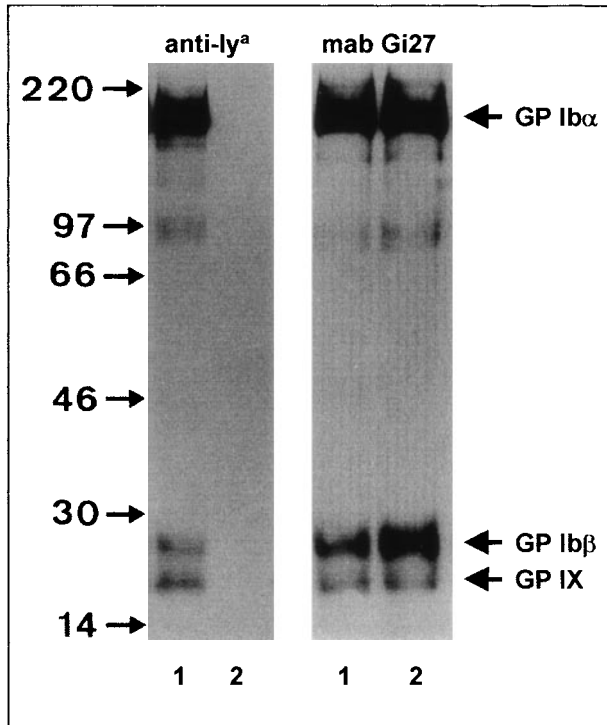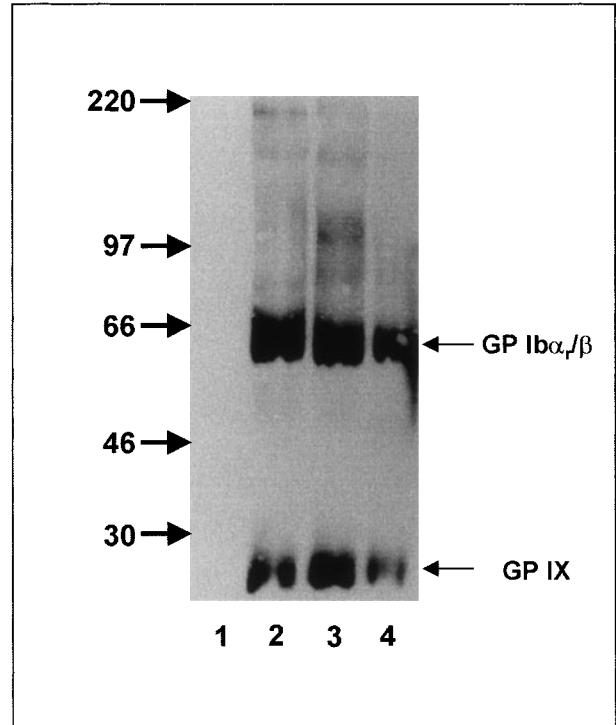
Figure 1. Immunoprecipitation analysis of the GPIbα-Ibβ-IX complex of Iyᵃ-phenotyped platelets. Platelets from Iyᵃ-positive (lane 1) and Iyᵃ-negative (lane 2) individuals were surface labeled with biotin, lysed, and immunoprecipitated with anti-Iyᵃ (left panel) and monoclonal antibody (MAB) Gi27 (right panel). Immunoprecipitates were analyzed with 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to nitrocellulose membrane, and visualized by using a streptavidin-horseradish peroxidase–chemiluminescence substrate system.

Figure 2. Immunoprecipitation analysis of the GPIbα-Ibβ-IX complex of Iyᵃ-phenotyped platelets after trypsin treatment. Aliquots of 10^9 biotinylated platelets from an Iyᵃ-positive individual were treated with trypsin, washed, lysed, and immunoprecipitated with MAB SZ2 (lane 1), MAB Gi10 (lane 2), MAB Gi27 (lane 3), and anti-Iyᵃ antibodies (lane 4). Immunoprecipitates were analyzed with 7.5% SDS-PAGE under nonreducing conditions.

experiments, MAB SZ2 (Figure 3, lane 2) reacted with GP Ib (both α and β subunits) under nonreducing conditions. Under nonreducing conditions, MAB Gi27 (Figure 3, lane 3) showed binding to GP Ib and to its proteolytic fragment, GP Ibr. Under reducing conditions, MAB SZ2 recognized the GP Ibα subunit and MAB Gi27 recognized the GP Ibβ subunit.

Amplification and analysis of *GPIbα*, *GPIbβ*, and *GPIX* genes

Because anti-Iyᵃ antibodies bind to trypsin-treated platelets, we predicted that the region formed by amino acid residues 450 to 610 (nucleotides 1440-1920) of *GPIbα*, together with *GPIbβ* and *GPIX*, would carry the Iyᵃ epitope(s). To analyze this region, we amplified the *GPIbα* gene in 2 overlapping fragments (nucleotides 826-1518 and 1192-1965) and the entire coding regions of the *GPIbβ* (nucleotides 47-939) and *GPIX* genes (nucleotides 271-750). PCR products of *GPIbα*, *GPIbβ*, and *GPIX* genes from an Iyᵃ-positive individual migrated with the same electrophoretic mobility as PCR products derived from an Iyᵃ-negative individual (data not shown). All PCR products were subcloned, and 6 independent clones from each fragment were subjected to nucleotide-sequence analysis.

Nucleotide-sequence analysis of *GPIbα* and *GPIX* fragments from an Iyᵃ-positive individual and an Iyᵃ-negative individual did not show any differences in nucleotides (data not shown). However, nucleotide-sequence analysis of the 517-base-pair (bp) fragment of *GPIbβ* encoding nucleotides 47-563 from an Iyᵃ-positive individual revealed a single G to A substitution at base 141 in 3 of 6 subclones examined (Figure 4). In contrast, all clones from an

Iyᵃ-negative individual encoded a G at this position (data not shown). These results are consistent with the idea that Iyᵃ-positive individuals are usually heterozygous for this low-frequency antigen. The G to A substitution changes a GGG codon for glycine to GAG, which encodes for glutamic acid at amino acid 15 of the mature GP Ibβ.

Correlation of the $G_{141}A$ dimorphism with the Iyᵃ phenotype

Along with the Iyᵃ-positive subjects from the index family,[16] one Iyᵃ-positive individual (Kr) was identified among 300 German blood donors phenotyped for the Iyᵃ antigen with the MAIPA assay. The pedigrees of both families are shown in Figure 5. No case of NAIT was observed in the Kr family. To determine whether the G
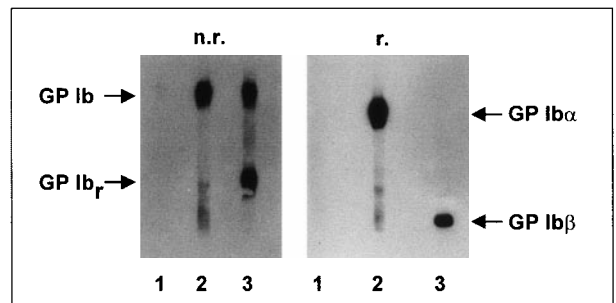
Figure 3. Immunoblotting studies. Aliquots of 10^9 washed platelets from an Iyᵃ-positive individual were lysed, and proteins were separated by using 7.5% SDS-PAGE under nonreducing (n.r.) and reducing (r.) conditions. After proteins were transferred to a nitrocellulose membrane, membrane strips were incubated with anti-Iyᵃ antiserum (lane 1), MAB SZ2 (lane 2), and MAB Gi27 (lane 3). Antibody binding was detected by using corresponding peroxidase-conjugated antibodies and chemiluminescence substrate.
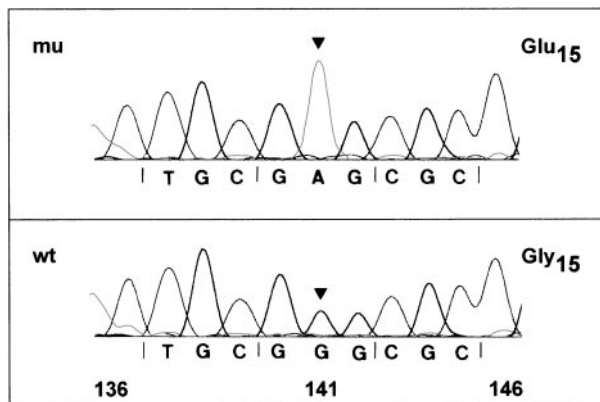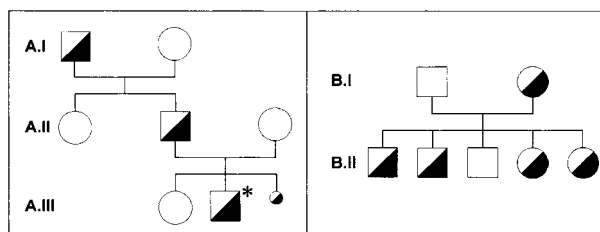
Figure 4. DNA-sequence analysis of amplified *GPIb*β gene of an Iyᵃ-positive individual. Polymerase chain reaction (PCR) products of the *GPIb*β gene encompassing nucleotides 47-563 were subcloned in the plasmid vector pGEM-5Zf and sequenced on both strands. Nucleotide-sequences of 2 positive clones are shown. (A) The wild-type G in position 141 is changed to an A (arrow), predicting a glycine to glutamic acid (GGG→GAG) polymorphism at position 15 of the mature glycoprotein. (B) The sequence is identical to the published wild-type sequence for *GPIb*β.[20]

Figure 6. PCR-restriction fragment length polymorphism analysis of Iyᵃ-phenotyped individuals with use of *Nar*I. (A) A 332-base-pair product encompassing nucleotides 47-378 of the *GPIb*β gene was obtained from genomic DNA by using primers GPIbβ 1 and GP Ibβ 3. The arrows indicate the cleavage sites recognized by the restriction endonuclease *Nar*I. The length of the expected digested fragments from Iyᵃ-positive and Iyᵃ-negative alleles is also shown. (B) Analysis of *Nar*I-digested PCR products from genomic DNA of the members of the Iy family (left panel) and the Kr family (right panel). Lanes are inscribed according to the pedigrees (Figure 4). Undigested products from samples obtained from 3 individuals are shown in lanes 1 to 3 (left panel). In lane M, pBr 322 *Hae*III DNA fragments are shown as standards.
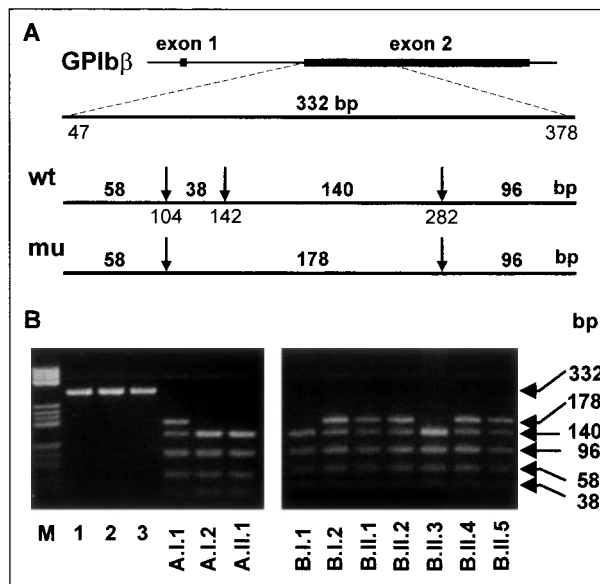
to A substitution at position 141 of the *GPIb*β gene segregates with the Iyᵃ phenotype, we established genomic DNA typing with a PCR-RFLP technique. The G to A substitution abolishes a cleavage site for the restriction endonuclease *Nar*I, which cleaves at 5′-GG↓CGCC-3′ but not at 5′-AGCGCC-3′ sequences (Figure 6A). We used this technique to genotype 3 members of the index family (A.I.1, A.I.2, and A.II.1 in Figure 5) and all members of the Kr family (Figure 5B and Figure 6B). After amplification of genomic DNA by using primer pair GP Ibβ 1 and GP Ibβ 3, the 332-bp PCR product was digested with *Nar*I. All Iyᵃ-negative individuals had 140-bp, 96-bp, 58-bp, and 38-bp restriction fragments. All Iyᵃ-heterozygous individuals could be differentiated from the Iyᵃ-negative subjects by the presence of an additional 178-bp fragment. The results of the genotyping of 300 unrelated individuals correlated with the phenotyping results.

Expression of the wild-type and mutated GP Ibα-Ibβ-IX complex in CHO cells

To demonstrate that the G to A substitution at nucleotide 141 of GPIbβ cDNA is sufficient to induce formation of the epitope recognized by anti-Iyᵃ serum, we performed transient transfection of CHO cells expressing GP Ibα and GP IX with eucaryotic expression vectors carrying wild-type or mutated GP Ibβ cDNA. Two days after transfection, surface expression of Iyᵃ epitope(s)

Figure 5. Pedigrees of the index family and the Kr family. In the Iy (index) family (A), the family member with the index case of neonatal alloimmune thrombocytopenia (NAIT) is marked with an asterisk (A.III.2). A third pregnancy was interrupted because of massive intraventricular bleeding in the fetus (A.III.3). All individuals were phenotyped with use of the monoclonal antibody-specific immobilization of platelet antigens assay. Material for genotyping was available only for individuals A.I.1, A.I.2, and A.II.1. One member of the Kr family (B) was found in assessing 300 healthy blood donors. All individuals were phenotyped and genotyped. No case of NAIT was observed in the Kr family.

was examined by flow cytometry after staining with anti-Iyᵃ serum. Sham-transfected CHO cells solely expressing GP Ibα and GP IX failed to bind anti-Iyᵃ antibodies (data not shown). CHO cells expressing the wild-type complex also did not bind anti-Iyᵃ antibodies (Figure 7A, dark curve), whereas those expressing the mutated GP Ib-IX complex showed antibody binding (Figure 7B, arrow). NHS was used as a negative control (Figure 7A and 7B, bright curves). These results were reproduced in independent transfection experiments in 2 different laboratories.

Effect of G to A mutation on the expression and function of GP Ib-IX complex

To determine whether the G to A mutation influences the efficiency of expression of the GP Ib-IX complex on the platelet surface, binding isotherms were generated by using MAB Gi10 in a quantitative sandwich ELISA.

In accordance with the findings from our immunoprecipitation analysis, platelets from Iyᵃ-positive individuals bound amounts of MAB Gi10 ($22\ 753 \pm 200$ molecules per platelet; n = 3) similar to the amounts bound by platelets from Iyᵃ-negative individuals ($23\ 000 \pm 300$ molecules per platelet; n = 3).

To determine the possible effect of the point mutation on the ability of the GP Ib-IX complex to bind von Willebrand factor, platelets from an Iyᵃ-positive individual were compared with those from 3 Iyᵃ-negative individuals in standard platelet aggregation assays. The ristocetin-induced agglutination of Iyᵃ-positive platelets was indistinguishable from that of Iyᵃ-negative platelets (data not shown). All these results suggest that neither the expression nor the function of the GP Ib-IX complex is affected by the Gly₁₅Glu mutation.

When ristocetin-induced agglutination was performed in the
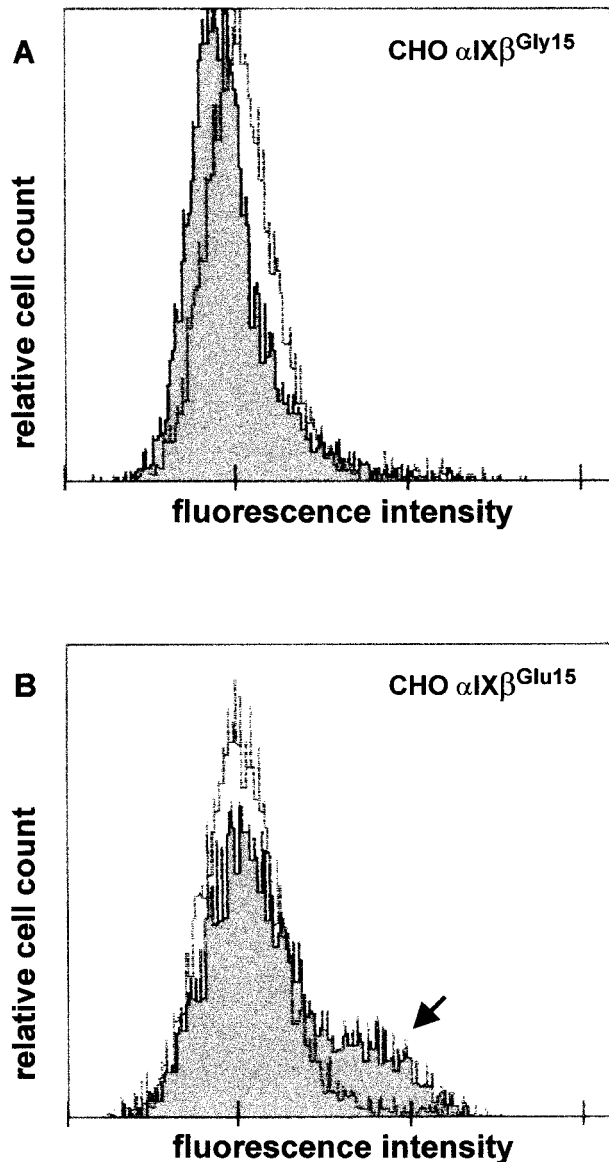
Figure 7. Detection of expression of Iyᵃ epitope on recombinant GP Ibα-Ibβ-IX complexes by flow cytometry. CHO cells stably expressing GP Ibα and GP IX were transiently transfected with eucaryotic expression vectors carrying wild-type (A) or mutated (B) complementary DNA for GPIbβ. Forty-eight hours after transfection, expression of the Iyᵃ epitope on the cell surface was determined by using anti-Iyᵃ antibodies (dark). Normal human serum was used as a negative control (bright). The arrow indicates the subpopulation of cells showing antibody binding.

presence of purified anti-Iyᵃ antibodies, no inhibition was observed in platelets from either Iyᵃ-positive or Iyᵃ-negative individuals. In the control experiments, MAB SZ2 (20 μg/mL) completely inhibited the ristocetin-induced agglutination (data not shown).

Discussion

We report the characterization of the new platelet-specific alloantigen, Iyᵃ, that was responsible for a severe case of NAIT. Our immunochemical studies demonstrated that anti-Iyᵃ precipitated GP Ibαᵣ, GP Ibβ, and GP IX from trypsin-treated platelets. Because we were unable to detect reactivity of the Iyᵃ antibody with either serum or affinity-purified antibody in immunoblot analysis, we

predict that the alloantigenic determinants of Iyᵃ are dependent on protein conformation sensitive to denaturation by SDS.

To elucidate the molecular basis underlying the Iyᵃ antigen, we amplified *GPIbαᵣ*, *GPIbβ*, and *GPIX* genes from genomic DNA derived from Iyᵃ-positive and Iyᵃ-negative individuals. Nucleotide-sequence analysis of the *GPIbα*, *GPIbβ*, and *GPIX* genes showed a single G to A transition at nucleotide 141 in the *GPIbβ* gene, changing glycine to glutamic acid at residue 15 of the mature GP Ibβ protein. RFLP analysis using the restriction endonuclease *Nar*I, which is capable of discriminating between these 2 alleles, demonstrated that this nucleotide substitution correlated with the serologic phenotypes of 6 Iyᵃ-positive individuals from 2 independent families and 300 Iyᵃ-negative unrelated blood donors.

The $Gly_{15}Glu$ dimorphism of the GP Ibβ protein represents the only difference between Iyᵃ-positive and Iyᵃ-negative individuals. Flow cytometry analysis that used CHO cells expressing the wild-type (Gly_{15}) or mutated (Glu_{15}) GP Ibα-Ibβ-IX complex on their surface showed that this single amino acid substitution is directly responsible for formation of the Iyᵃ alloantigenic determinant(s).

The actual Iyᵃ-antibody binding sites are probably complex-specific, formed by GP Ibβ and other GP Ib subunits. Which GP Ib subunit—GP Ibα, GP IX, or both—is required for the alloantigenic formation remains unclear. However, expression of recombinant mutated GP Ibβ in CHO cells confirmed that this single amino acid substitution is sufficient to induce formation of the epitope(s) recognized by anti-Iyᵃ serum.

Bernard-Soulier syndrome (BSS) is an extremely rare autosomal recessive bleeding disorder in which patients have a low platelet count and large platelets that are unable to adhere to subendothelium. The functional defect lies in the inability of the platelets to bind von Willebrand factor.[37] In patients with classic BSS, the level of GP Ib-IX complex is greatly reduced because of abnormalities (mutations and deletions) in the *GPIb-IX* genes that lead to a biosynthetic defect affecting expression, processing, or synthesis of the complex. In contrast, patients with variant BSS have normal amounts of GP Ib-IX complex, but the complex is dysfunctional. Recently, BSS variants due to point mutations in a leucine-rich domain of GP Ibα and GP IX have been identified.[38-40] So far, only 2 GP Ibβ mutations have been reported to be associated with BSS. The first involved a giant-platelet syndrome caused by point mutations that led to amino acid substitutions Tyr to Cys at residue 88 and Ala to Pro at residue 108 of the mature glycoprotein.[41] The patient was heterozygous for these mutations. It was suspected that the Tyr to Cys transition affected the disulfide linkage between GP Ibα and GP Ibβ. The second mutation was in a patient with velocardiofacial syndrome who had a point mutation at position −133 ($C_{133}G$) and deletion of the other allele.[42]

Studies of Iyᵃ-positive platelets showed that the $Gly_{15}Glu$ point mutation of the GP Ibβ gene does not impede expression or function of the GP Ib-IX complex. Wright et al[39] observed variable band shifts within the GP Ibβ coding region in a few patients with BSS and healthy blood donors by means of single-stranded conformation polymorphism. However, this sequence variation did not show any linkage to the BSS phenotype. Our RFLP analysis of these patients with BSS showed that this polymorphism is not related to the $Gly_{15}Glu$ polymorphism (data not shown).

So far, all the alloantigenic epitopes responsible for the observed cases of NAIT, posttransfusional purpura, and refractoriness to platelet transfusion have been found to be on GP Ia, GP Ibα, GP IIb, or GP IIIa. The elucidation of the Iyᵃ alloantigen represents

the first characterization of an immunologically important polymorphism of GP Ibβ.

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