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Research Article

The human GPIa/IIa complex, also known as integrin alpha 2 beta 1, serves as a major receptor for collagen in platelets and other cell types. In addition to its role in platelet adhesion to extracellular matrix, GPIa/IIa is also known to bear the clinically important Br(a) and Brb alloantigenic determinants, which can result in antibody-mediated platelet destruction. Immunochemical studies showed that the Br antigenic epitopes reside solely on the GP Ia subunit and do not depend on sialic acid residues. To define the polymorphism responsible for the Br alloantigen system platelet RNA PCR technique, was used to amplify GPIa mRNA transcripts. Nucleotide sequence analysis of the amplified platelet GPIa cDNA from Br(a/a) and Brb/b individuals revealed a single A<-->G polymorphism at base 1648. MnlI RFLP analysis of cDNA from serologically determined individuals confirmed that this polymorphism segregates with Br phenotype. This single base change results in a substitution of Lys (AAG) in Br(a) to Glu (GAG) in Brb at amino acid residue 505. In spite of the reversal in charge at this position, however, we found no difference in the ability of Bra and Brb homozygous platelets to adhere to collagens types I, III, or V, nor did anti-Bra or anti-Brb alloantibodies interfere with platelet adhesion to any of these fibrillar collagens. The identification of the nucleotide substitution that defines [...]

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The Human Platelet Alloantigens Br^a and Br^b Are Associated with a Single Amino Acid Polymorphism on Glycoprotein Ia (Integrin Subunit α_2)

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Abstract

The human GPIa/IIa complex, also known as integrin $\alpha_2\beta_1$, serves as a major receptor for collagen in platelets and other cell types. In addition to its role in platelet adhesion to extracellular matrix, GPIa/IIa is also known to bear the clinically important Br^a and Br^b alloantigenic determinants, which can result in antibody-mediated platelet destruction. Immunochemical studies showed that the Br antigenic epitopes reside solely on the GP Ia subunit and do not depend on sialic acid residues. To define the polymorphism responsible for the Br alloantigen system platelet RNA PCR technique, was used to amplify GPIa mRNA transcripts. Nucleotide sequence analysis of the amplified platelet GPIa cDNA from Br^{a/a} and Br^{b/b} individuals revealed a single A \leftrightarrow G polymorphism at base 1648. MnlI RFLP analysis of cDNA from serologically determined individuals confirmed that this polymorphism segregates with Br phenotype. This single base change results in a substitution of Lys (AAG) in Br^a to Glu (GAG) in Br^b at amino acid residue 505. In spite of the reversal in charge at this position, however, we found no difference in the ability of Br^a and Br^b homozygous platelets to adhere to collagens types I, III, or V, nor did anti-Br^a or anti-Br^b alloantibodies interfere with platelet adhesion to any of these fibrillar collagens. The identification of the nucleotide substitution that defines the Br^a/Br^b alloantigen system will now permit both pre- and postnatal diagnosis for Br phenotype. (*J. Clin. Invest.* 1993. 92:2427–2432.) Key words: neonatal alloimmune thrombocytopenia • platelet RNA • polymerase chain reaction • platelet adhesion • DNA typing

Introduction

The human platelet membrane glycoprotein Ia/IIa (GPIa/IIa)¹ complex, also known as $\alpha_2\beta_1$, is a member of the integrin family that is involved in adhesive interactions (for review, see reference 1). In unstimulated platelets, the GPIa/IIa heterodimer mediates Mg²⁺-dependent platelet adhesion to collagen (2).

In addition to its physiological role, this complex bears clinically important alloantigens, the platelet alloantigen system

Br^a/Br^b (3, 4). The gene frequencies for these two alleles have been calculated to be 0.1110 for Br^a and 0.8890 for Br^b in the Caucasian population (3). Br alloantigens have also been found on activated T lymphocytes (5) and on endothelial cells (6), consistent with the known tissue distribution of $\alpha_2\beta_1$.

Alloimmunization against these antigens can either cause neonatal alloimmune thrombocytopenia (7–9), can be responsible for refractoriness to platelet transfusion (10), or may also be involved in posttransfusion purpura (11). Anti-Br^a is the second most frequent alloantibody involved in neonatal alloimmune thrombocytopenia in the Caucasian population (12, 13).

Platelet RNA PCR technology (14) has made it possible to assign single nucleotide substitutions to each of the currently recognized biallelic platelet alloantigen systems, Pl^A, Ko, Bak, and Pen (15–18).

Recently, the complete amino acid sequence of GPIa has been deduced from the nucleotide sequences of human lung fibroblast (19). The mature GPIa polypeptide consists of 1,152 amino acids and contains a transmembrane domain and a short cytoplasmic segment. Although the overall sequence homology in comparison with other integrin α subunits is 18–25%, GPIa comprises a similar distribution of cysteine residues and cation-binding domains. A major characteristic of GPIa is the presence of a 191-amino acid insert (I domain) that contains potential sites for interaction with collagen (for review, see reference 1).

In this present study, we have further characterized the biochemical and molecular biological properties underlying the polymorphism of human platelet GPIa that are responsible for the immunogenicity of Br^a/Br^b alloantigen system.

Methods

Serological platelet typing. The Br and Bak phenotypes of platelet donors used in this study were determined by glycoprotein-specific immunoassay, mAb-specific immobilization of platelet antigens (MAIPA), as previously described (20).

Antibodies. The platelet-specific alloantibodies Br^a, Br^b, and Bak^a were obtained from mothers of children with neonatal alloimmune thrombocytopenia and from polytransfused patients. mAbs Gi14 and Gi9, which recognize two different epitopes on the GPIa/IIa complex, were produced in our laboratory (21). The mAb K20 directed against GPIIa was purchased from Dianova (Hamburg, Germany). IgG antibodies were isolated from human sera or mouse ascites by protein G-Sepharose chromatography (Pharmacia, Freiburg, Germany). Purified IgG antibodies were coupled to cyanogen bromide-activated Sepharose 4B as recommended by the manufacturer (Pharmacia).

Adsorption. Aliquots of 8×10^7 washed platelets in PBS buffer, pH 6.2, supplemented with 2% BSA were incubated in the absence or presence of 40 μ U protease-free neuraminidase from *Arthro bacter ureafaciens* (Boehringer Mannheim GmbH, Mannheim, Germany) for 6 h at 37°C and washed twice with PBS. Sera containing anti-Br^a, -Br^b, and -Bak^a antibodies were diluted up to 75-fold to obtain an optical density of 1.0 in MAIPA before adsorption experiments. 70 μ l of diluted sera

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1. Abbreviations used in this paper: GPIa/IIa, glycoprotein Ia/IIa; MAIPA, mAb-specific immobilization of platelet antigens; RCD, Ringer's citrate-dextrose buffer; TB, Tyrode buffer.

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were adsorbed twice with 4×10^7 untreated or neuraminidase-treated platelets for 30 min at 37°C. After centrifugation (13,000 g for 3 min), the remaining specific alloantibodies in the supernatants were tested in MAIPA assay using mAbs Gi14 or Gi5 as previously described (20). The results were expressed as percent adsorption of specific antibody.

Radioimmunoprecipitation. Aliquots of 10^9 washed platelets were radiolabeled with ^{125}I using the lactoperoxidase-catalyzed method of Phillips and Agin (22). Aliquots of 10^8 labeled platelets were incubated with neuraminidase as described above. Untreated and neuraminidase-treated platelets were then immunoprecipitated with 50 μl of anti-Br^a serum as previously described (4). For dissociation experiments, 10^9 labeled platelets were solubilized in 1 ml 10 mM Tris buffer, pH 8.0, containing 1% NP40, 1% leupetin (Sigma Immunochemicals, München, Germany) at 4°C for 30 min. After centrifugation at 13,000 g for 30 min at 4°C, platelet lysates were precleared on the nutator overnight with 100 μl of a Sepharose 4B beads (Pharmacia) coupled with rabbit anti-human IgG (Dako, Hamburg, Germany). To dissociate GPIa/IIa complexes, aliquots of 500 μl precleared platelet lysates were acidified with citrate buffer to pH 2.0 for 30 min at 4°C (23). In control experiments, 500 μl aliquots of platelet lysates were kept at pH 8.0 at 4°C. After neutralization with 1 M Tris buffer, pH 8.0, lysates were incubated with mAb K20 coupled to Sepharose 4B beads to remove the remaining undissociated GPIa/IIa complex, and they were subsequently immunoprecipitated with alloantibodies covalently linked to Sepharose 4B beads. After incubation for 30 min at 4°C, the beads were washed three times with 10 mM Tris buffer, pH 8.0, containing 0.1% NP40. The pellet was resuspended in 100 μl sample buffer containing 2% SDS and boiled for 5 min. The immunoprecipitated proteins were analyzed on 7.5% SDS-PAGE. Finally, the gels were dried and autoradiographed on Kodak X-O-Mat S film using an intensifying screen (Cronex Hi-Plus; Du Pont, Frankfurt, Germany). Rainbow protein mixture (Amersham Buchler, Braunschweig, Germany) was used as molecular mass standards (myosin, 200 kD; phosphorylase b, 92.5 kD; bovine serum albumin, 69 kD; ovalbumin, 46 kD; carbonic anhydrase, 30 kD; and lysozyme, 14.3 kD).

Isolation and amplification of platelet RNA. Platelet RNA was isolated from EDTA anticoagulated blood of Br-typed donors as previously described (17). 10- μl aliquots of platelet mRNA were heated to 68°C for 10 min and quickly cooled on ice water before reverse transcription. First-strand cDNA was then synthesized using 10 μM oligo dT, 40 U RNasin (Boehringer Mannheim GmbH), 2 mM of each dNTP (Pharmacia) 500 U of cloned Moloney murine leukemia virus reverse transcriptase and 5 \times enzyme buffer (Gibco, Eggenstein, Germany) in a total volume of 30 μl . cDNA synthesis was carried out at 40°C for 45 min and was stopped by chilling to 0°C.

Overlapping sets of primers were constructed based on the published sequence of VLA-2 (19) to amplify the entire coding region of platelet GPIa. The following oligonucleotides were used for the PCR to amplify a region encompassing bases 1486–1900: primer no. 1, 5'-CAATATCACGGTTATTCAGGCTCACC-3' (nucleotides 1470–1495); primer no. 2, 5'-CCCATTTAAATCTCCATAGCCATCC-3' (nucleotides 1938–1914); primer no. 3, 5'-CAGGCTCACCGAGGTGAC-CAGATTGGC-3' (nucleotides 1486–1512); and primer no. 4, 5'-AGTACTGGAGATGGCTCCTA-3' (nucleotides 1900–1881). 5 μl of cDNA were diluted with 5 μl 10 \times PCR buffer, 0.3 μM of each primer (no. 1 and 2) and 175 μM dNTP in a total volume of 50 μl . After heating at 96°C for 5 min, 3 μl *Taq* polymerase dilution (1:10, 1.5 U) (Boehringer Mannheim) was added at 85°C and amplification was performed on a DNA thermal cycler (Biometra, Göttingen, Germany) for 15 cycles. Each cycle consisted of denaturation at 93°C for 55 s, annealing at 52°C for 55 s, and extension at 72°C for 95 s. In the final cycle, the samples were kept at a temperature of 72°C for 10 min and then chilled to 4°C. 2- μl aliquots of PCR products were amplified again for 30 cycles using nested primers no. 3 and 4 under following conditions: denaturation at 93°C for 55 s, annealing at 50°C for 55 s, and at 72°C for 85 s.

Analysis of PCR products. 5 μl of the PCR-amplified products were analyzed on 1.2% agarose gel containing ethidium bromide (Dianova).

Amplified DNA was isolated from the gel, purified (GeneClean; Dianova), and subcloned into pcRTM 1000 (ITC, Heidelberg, Germany). The inserts were sequenced by the dideoxy chain termination method using Sequenase 2.0 (United States Biochemical, Bad Homburg, Germany). In some cases, amplified cDNA was subjected to restriction digestion using MnlI endonuclease (New England Biolabs, Schwalbach, Germany) and analyzed on 4% NuSieve agarose gel using a Tris borate EDTA buffer system (Biozym, Hameln, Germany).

Platelet adhesion assay. The adhesion of nonactivated human platelets to collagen was measured as described by Kunicki et al. (24) with minor modifications. Platelets were obtained from acid citrate dextrose-anticoagulated blood by differential centrifugation and washed with Ringer's citrate-dextrose buffer (RCD). Aliquots of 2×10^9 washed platelets in isotonic saline were labeled with 400 μCi ^{51}Cr at room temperature for 30 min. Platelets were then washed once with RCD, once with RCD without Ca^{2+} , Mg^{2+} , and resuspended in tyrode buffer (TB) (without Ca^{2+} , Mg^{2+}) at a final concentration of 2×10^8 platelets/ml. For adhesion, microtiter wells were coated overnight at 4°C with 100 μl collagen type I, III, or V (25 $\mu\text{g}/\text{ml}$ in PBS; Sigma Immunochemicals). Additional control wells were coated with 100 μl BSA (5 mg/ml in PBS) (Serva, Heidelberg, Germany). Each well was then washed twice with 200 μl 5% BSA in PBS (PBS/BSA) and blocked for 90 min with PBS/BSA. For adhesion, 100 μl labeled platelets (10^7) in TB containing 4 mM MgCl_2 were added to coated wells and the trays were incubated in a humidified 37°C CO_2 incubator for 90 min. Unbound platelets were removed by gentle aspiration onto absorptive pads and by washing of the wells five times with TB. Bound platelets were solubilized twice with 150 μl 2% SDS, and the radioactivity of bound ^{51}Cr was determined. Inhibition of adhesion by mAbs or by alloantibodies was tested by previous incubation of platelets with purified antibodies for 1 h at room temperature.

Results

Localization of the Br antigenic determinants to GPIa. Previous studies have localized the Br epitopes to the GPIa/IIa heterodimer complex. To further localize the epitope of Br alloantigens, dissociation experiments with GPIa/IIa complex were performed. It has been demonstrated that treatment of the human fibroblast lysates with citrate buffer at pH 3.0 leads to dissociation of the β_1 integrin subunit from other α chains (23). Our preliminary experiments showed that under these conditions, platelet GPIa/IIa chains did not completely dissociate from each other. Therefore, the remaining undissociated GPIa/IIa was removed by adsorption with mAb K20 specific for GPIIa. After this treatment, GPIa could be precipitated with anti-Br^a and anti-Br^b antibodies without coprecipitation of GPIIa from platelet lysates (Fig. 1), demonstrating that the Br antigenic determinants are carried by GPIa, and are not

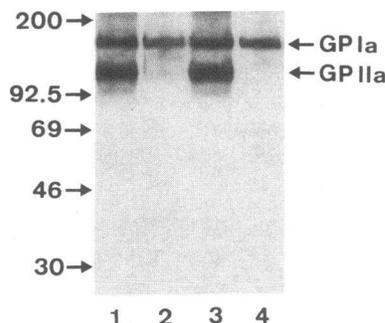


Figure 1. Immunoprecipitation of ^{125}I -radio-labeled platelet proteins from a Br^a- and Br^b-homozygous donors with anti-Br^a (lanes 1 and 2) and anti-Br^b (lanes 3 and 4), respectively. Lanes 1 and 3, GPIa/IIa complex precipitated from untreated platelets. Lanes 2 and 4, GPIa precipitated from

platelets treated with citrate buffer to pH 2.0 as described in Methods. Immunoprecipitates were analyzed on 7.5% SDS-PAGE under nonreduced conditions.

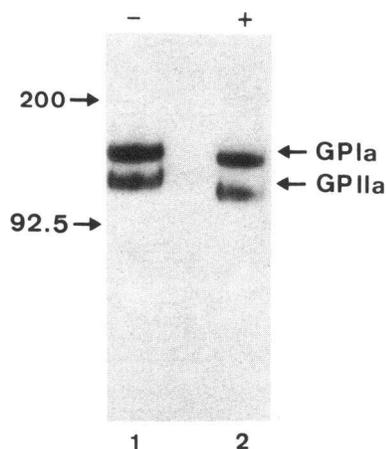


Figure 2. Immunoprecipitation of ^{125}I -radio-labelled platelets proteins from untreated (lane 1) and neuraminidase-treated Br^a -homozygous platelets (lane 2) with anti- Br^a alloantibody. Immunoprecipitates were analyzed on 5% SDS-PAGE under nonreduced conditions.

complex dependent. To examine whether sialic acid residues contribute to the formation of Br epitopes, we immunoprecipitated lysates from neuraminidase-treated platelets. As shown in Fig. 2, anti- Br^a , could still precipitate the desialylated GPIa/IIa complex with lower apparent molecular weights (lane 2). To expand this analysis, different Br^a , Br^b and also Bak^a antisera were adsorbed with untreated and neuraminidase-treated, phenotyped platelets. The amount of specific alloantibodies remaining in the supernatants were then analyzed in MAIPA assay. The results were calculated as percent adsorption (Table I). All 18 Br antibodies were equally adsorbed with untreated and neuraminidase-treated platelets. In contrast, 3/6 Bak^a antibodies were adsorbed by untreated platelets, but not by neuraminidase-treated platelets (83.3% vs 10.4% adsorption). This implicates that Bak epitopes, unlike Br, are influenced by neuraminidase treatment, confirming earlier report (25, 26).

Amplification and analysis of GPIa mRNA from $\text{Br}^{a/a}$ and $\text{Br}^{b/b}$ individuals. To analyze the nucleotide sequence coding for GPIa, platelet mRNA was sequentially amplified by reverse transcription PCR using eight sets of primers, subcloned and sequenced. Comparison of the 414-bp nucleotide sequence (nucleotides 1486–1900) of GPIa mRNA derived from two Br^a and two Br^b homozygous individuals (Fig. 3) revealed a single nucleotide difference at base 1648 (Fig. 4). Whereas the $\text{Br}^{a/a}$ individual has an A at this position, the $\text{Br}^{b/b}$ individual has a G. This results in the substitution of a lysine for glutamic acid at amino acid residue 505 of the mature GPIa. Analysis of the other seven regions of GPIa mRNA revealed no other nu-

Table I. Adsorption (Percent) of Anti- Br^a , - Br^b , and - Bak^a with Untreated and Neuraminidase (N)-treated Platelets

Alloantibodies	Adsorption (percent)		n
	Untreated	N-treated	
Br^a	88.5±11.8	89.7±10.2	5
Br^b	78.6±7.5	82.9±6.3	5
Bak^a	93.1±11.4	93.7±5.6	3
Bak^a (N+)	83.3±19.7	10.4±10.0	3

After adsorption, Br and Bak alloantibodies were tested in MAIPA assay using mab Gi14 (anti GPIa/IIa) or Gi5 (anti GPIIb/IIIa) as capture antibodies. N+, neuraminidase sensitive; n, number of sera tested.

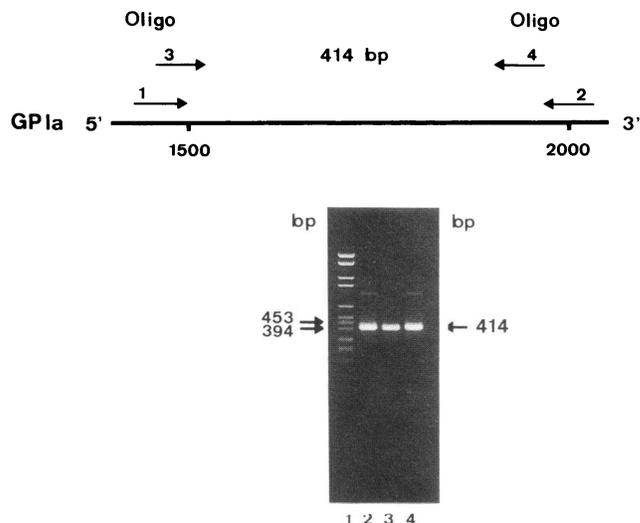


Figure 3. Amplification of nucleotides 1486–1900 of human platelets GPIa mRNA. The locations of the two oligonucleotide primer sets (arrows 1 and 2 and arrows 3 and 4) used for PCR-amplification of GPIa mRNA transcript and the expected 414-bp PCR product are illustrated above. cDNA synthesized from platelet RNA derived from a known $\text{Br}^{a/a}$, $\text{Br}^{a/b}$, and $\text{Br}^{b/b}$ individuals (lanes 2, 3, and 4) was amplified and analyzed on 1.2% agarose gel stained with ethidium bromide. DNA size standards (pBr 328 DNA.Bgl I + pBr 328 DNA.Hinf I) (Boehringer Mannheim GmBH) are shown in lane 1. The resulting 414-bp products (arrow) from homozygous Br^a and Br^b individuals (lanes 2 and 4) were isolated from preparative gels and subcloned for nucleotide sequence analysis.

cleotide differences. Platelet GPIa cDNA from four different individuals sequenced so far have a C at nucleotide 195 (data not shown), whereas the published cDNA clone from a fibroblast line has G (19). This silent C ↔ G polymorphism, which is independent from Br system, represents the third base in the codon for Gly₂₀ and creates a new restriction site for Cvi JI endonuclease (Table II).

RFLP analysis. To determine whether the A_{1648}G substitution noted in the pair of individuals above segregates with Br phenotype, we took advantage of the fact that the A ↔ G substitution created a recognition site for the restriction enzyme MnlI, which cleaves at 5'-TATCAAAGAGG-3' but not 5'-TATCAAAAAGG-3' sequences. Analysis of the 414-bp PCR

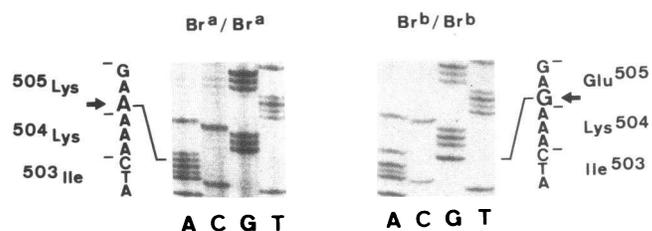


Figure 4. DNA sequence analysis of amplified GPIa cDNAs from Br^a and Br^b homozygous individuals. The region of the autoradiograph shown here includes the sequence from base 1642–1662. The sequence of the $\text{Br}^{a/a}$ individual is identical to the published sequence for VLA-2, having an A at base 1648. The base changes to a G in the $\text{Br}^{b/b}$ individuals, resulting in a lysine (AAG) ↔ glutamic acid (GAG) polymorphism at position 505 of mature GPIa. The polymorphic nucleotide is indicated with arrows.

Table II. Integrin $\alpha 2$ Subunit Polymorphism

cDNA position	Fibroblast cell line*	Platelets		RFLP	Amino acid change
		Br ^{a/a}	Br ^{b/b}		
195	GGG	GGC	GGC	Cvi JI	Gly ₂₀ Gly
1648	AAG	AAG	GAG	Mnl I	Lys ₅₀₅ Glu

* Sequence from Takada and Hemler (19).

products from 10 additional Br-phenotyped donors (three Br^a/Br^a, three Br^a/Br^b, and four Br^b/Br^b individuals) yielded the fragments of the predicted size and corresponded to serological typing results. RFLP analysis of one representative amplified cDNA sample from each possible Br phenotype is shown in Fig. 5.

Effect of the Br polymorphism and Br alloantibodies binding on platelet function. GPIa/IIa has been shown to mediate platelet/collagen interactions in platelet and other cell types (1, 2). To examine whether the amino acid substitution underlying the Br polymorphism affects the function of GPIa/IIa, we studied platelet adhesion to collagens in the absence and presence of purified Br^a, Br^b human alloantibodies, and mAbs spe-

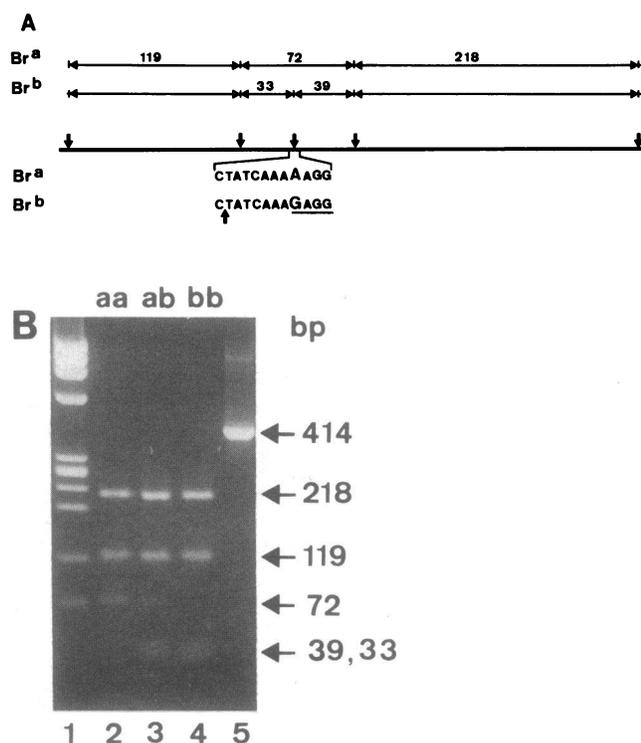


Figure 5. (A) Restriction map of the 1486–1900 (414 bp) PCR fragment of GPIa. The arrows indicate the positions of the recognition sites for MnlI. The length of the fragments after digestion is shown above (in basepairs) for both Br^a and Br^b gene products. The recognition site (GAGG) of MnlI endonuclease is underlined. (B) Analysis of MnlI digested 414-bp products from DNA of Br^{a/a} (lane 2), Br^{a/b} (lane 3), and Br^{b/b} (lane 4) phenotyped individuals on 4% NuSieve agarose gel stained with ethidium bromide. Lane 5 represents the undigested 414 bp PCR product. DNA size standards (Φ X 174 RF DNA.Hae III) (Gibco BRL, Eggenstein, Germany) are shown in lane 1.

Table III. Platelet Adhesion

Platelets phenotype	Antibodies	Specificity	Platelets bound (10 ⁵)
Br ^{a/a}	None	—	16.1±5.0
Br ^{b/b}	None	—	16.4±4.8
Br ^{a/a}	Anti-Br ^a	Br ^a	17.8±4.4
Br ^{b/b}	Anti-Br ^b	Br ^b	17.1±4.6
Br ^{b/b}	Human IgG	—	17.4±4.7
Br ^{b/b}	mAb Gi9	GPIa/IIa	0.3±0.3
Br ^{b/b}	mAb Gi14	GPIa/IIa	16.8±4.2

The effect of monoclonal antibodies (5 μ g/ml) and human IgG alloantibodies (500 μ g/ml) on the adhesion of ⁵¹Cr-labeled platelets to microtiter trays coated with type I collagen was assessed. The variance of triplicate did not exceed 5%. The number of platelets adherent to wells coated with BSA was routinely < 1% of that observed in collagen coated wells. Number of donors tested = 3.

cific for GPIa/IIa complex. The results of these experiments are summarized in Table III. The platelet adhesion response of Br^{a/a} platelets was indistinguishable from Br^{b/b} platelets, and neither anti-Br^a nor anti-Br^b alloantibodies inhibited platelet adhesion to type I collagen. In contrast, mAb Gi9, directed against a functional epitope of the GPIa/IIa complex, inhibited completely platelet adhesion, whereas mAb Gi14, directed against a different epitope on GPIa/IIa complex, did not. Similar results were obtained with type III and V collagens (data not shown).

Discussion

In our previous studies, we have demonstrated that the Br antibodies precipitated GPIa/IIa ($\alpha_2\beta_1$), but not GPIc/IIa ($\alpha_3\beta_1$) or GPIc'/IIa ($\alpha_6\beta_1$) from platelets, suggesting that the alloantigenic determinants of Br might reside on the GPIa molecule. However, we were unable to rule out that Br alloantigens might be complex-specific, perhaps residing on the GPIIa chain and inducing the antibody binding site only by its specific association with GPIa, but not with GPIc or GPIc'.

To elucidate the molecular basis of the Br alloantigens, a more precise assignment of their epitope to GPIa or GPIIa is essential. Since our efforts to sublocalize the Br alloantigenic determinants using immunoblotting techniques failed (unpublished observation), we took the advantage of the fact that GPIa/IIa complex is partially dissociated under acidic conditions. After treatment of platelet lysates with acidic buffer to pH 2.0, Br antibodies precipitated only GPIa indicating that the Br epitopes reside solely on GPIa, and are not complex specific.

Recently, Take et al. (25) demonstrated that the expression of Bak^a alloantigens is not uniform, since the binding of some Bak^a antisera to GPIIb is sensitive to removal of sialic acid residues. These results are in agreement with the data of Goldberger et al. (26), who showed that posttranslational processing of pro-GPIIb was required for efficient expression of Bak alloantigens. In this regard, the Br alloantigens differ from those of the Bak system, since desialylation did not affect the binding of multiple anti-Br^a and -Br^b alloantibodies to their GPIa target epitopes, and does not depend on the presence of sialic acid.

Based on these data and availability of the complete nucleotide sequence of integrin α_2 cDNA (19), we applied platelet RNA PCR techniques to amplify platelet GPIa mRNA transcripts from Br^{a/a} and Br^{b/b} individuals. Nucleotide sequence analysis of the entire coding region revealed a single A \leftrightarrow G base change at position 1648. This mutation changes an AAG codon for lysine into GAG that encodes for glutamic acid at amino acid position 505 of the mature GPIa polypeptide chain. RFLP analysis, using the enzyme MnlI, which is capable of discriminating between the two isoforms of amplified cDNA, demonstrated that this nucleotide substitution segregated with the serologically determined phenotype of 10 additional Br^{a/a}, Br^{a/b}, and Br^{b/b} individuals. The Lys₅₀₅ \leftrightarrow Glu₅₀₅ polymorphism represents the only difference between the Br^a and Br^b allelic forms within the coding region of the mature GPIa, indicating that this single amino acid substitution is sufficient to direct the formation of Br alloantigens, as has been similarly shown for the PI^A, Bak, and Pen systems (26, 18). However, the actual antibody combining site on GPIa remains to be determined.

There is evidence that the $\alpha_2\beta_1$ integrin is the main receptor mediating the adhesion of platelets to collagen (for review, see reference 1). Its deficiency in patient platelets is associated with a specific defect in platelet responsiveness to collagen (27, 28). The Glu₅₀₅ \leftrightarrow Lys₅₀₅ polymorphism associated with Br alloantigens is located between the first and second divalent cation-binding domains of GPIa. Since GPIa/IIa receptor-mediated platelet adhesion to collagen requires Mg²⁺ cation (2), we examined whether the Br-dependent GPIa structural variants affect platelet adhesion to collagen. No differences were observed in the ability of Br^a versus Br^b platelets to adhere to collagen, nor were there any effects of anti-Br alloantibodies on platelet adhesion to collagen. Thus, it would appear that both allelic forms of GPIa are able to function equally well.

While it has been widely supposed that the cDNA sequence of platelet GPIa would be identical to that of other cells, no data have so far been published. During the course of this investigation, we derived the first complete nucleotide sequence of the GPIa (integrin α_2) coding region exclusively from platelets, and found that it is identical to that previously obtained from fibroblast and endothelial cells. These data indicate that there are no megakaryocyte-specific alternative splicing events distinguishing platelet GPIa mRNA from that of fibroblasts.

Interestingly, although the calculated gene frequency of Br^a in the human gene pool is only 0.1110 (3), comparison of the polymorphic base 1648 pointed out that the cDNA sequence of GPIa published by Takada and Hemler (19) represents the rarer Br^a (AAG \rightarrow Lys₅₀₅) allelic form. In addition to this polymorphic position, we also noted one other sequence difference, a silent G₁₉₅ \leftrightarrow C₁₉₅ in the codon for Gly₂₀ in the GPIa polypeptide chain. Though the cDNA published sequence was derived from a fibroblast cell line, whereas our sequences were derived from platelet RNA, this dimorphism likely represents a simple allelic variation, rather than a cell-type specific difference, as no evidence for multiple copies of the GPIa gene expressed by different cells currently exists.

In conclusion, we have elucidated the molecular basis underlying the Br^a/Br^b polymorphism of GPIa. The single point mutation (AAG \leftrightarrow GAG) that distinguishes the Br^a and Br^b allelic forms can be discriminated by the use of the MnlI restriction enzyme. Based on these data, DNA typing for Br genotypes can now be developed. This will be of particular utility for

typing of thrombocytopenic fetuses of Br-alloimmunized pregnant women, similar to that which has already been realized for several of the other clinically important platelet alloantigen systems (29).

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References

- Hemler, M. H. 1991. Structure and function of VLA proteins and related integrins. *In* Receptors for Extracellular Matrix. J. A. McDonald and R. P. Mecham, editors. Academic Press, San Diego, CA. pp. 256-287.
- Staatz, W. D., S. M. Rajpara, E. A. Wayner, W. G. Carter, and S. A. Santoro. 1989. The membrane glycoprotein Ia-IIa (VLA-2) complex mediates the Mg²⁺-dependent adhesion of platelets to collagen. *J. Cell Biol.* 108:1917-1924.
- Kiefel, V., S. Santoso, M. Weisheit, and C. Mueller-Eckhardt. 1989. The Br^a/Br^b alloantigen system on human platelets. *Blood.* 73:2219-2223.
- Santoso, S., V. Kiefel, and C. Mueller-Eckhardt. 1989. Immunochemical characterization of the new platelet alloantigen system Br^a/Br^b. *Br. J. Haematol.* 72:191-198.
- Santoso, S., V. Kiefel, and C. Mueller-Eckhardt. 1989. Human platelet alloantigens Br^a/Br^b are expressed on the very late activation antigen 2 (VLA-2) of T lymphocytes. *Hum. Immunol.* 25:237-246.
- Giltay, J. C., H. J. M. Brinkman, A. Vlekke, V. Kiefel, J. A. van Mourik, and A. E. G. Kr. von dem Borne. 1990. The platelet glycoprotein Ia-IIa-associated Br-alloantigen system is expressed by cultured endothelial cells. *Br. J. Haematol.* 75:557-560.
- Kiefel, V., S. Santoso, B. Katzmann, and C. Mueller-Eckhardt. 1987. A new platelet-specific alloantigen Br^a. Report of 4 cases with neonatal alloimmune thrombocytopenia. *Vox Sang.* 54:101-106.
- Kiefel, V., Y. Schechter, D. Atias, S. Santoso, and C. Mueller-Eckhardt. 1991. Neonatal alloimmune thrombocytopenia due to anti-Br^b (HPA-5a). *Vox Sang.* 60:244-245.
- Bettaieb, A., P. Fromont, M. Rodet, B. Godeau, N. Duedari, and P. Bierling. 1991. Br^b, a platelet alloantigen involved in neonatal alloimmune thrombocytopenia. *Vox Sang.* 60:230-234.
- Bierling, P., P. Fromont, A. Bettaieb, and N. Duedari. 1989. Anti-Br^a antibodies in the French population. *Br. J. Haematol.* 73:428-429.
- Christie, D. J., S. Pulkrebek, J. L. Putnam, M. L. Slatkoff, and K. D. Pischel. 1991. Posttransfusion purpura due to alloantibody reactive with glycoprotein Ia/IIa (anti-HPA-5b). *Blood.* 77:2785-2789.
- Mueller-Eckhardt, C., V. Kiefel, A. Grubert, H. Kroll, M. Weisheit, S. Schmidt, G. Mueller-Eckhardt, and S. Santoso. 1989. 348 cases of suspected neonatal alloimmune thrombocytopenia. *Lancet.* i:363-366.
- Kaplan, C., M. C. Morell-Kopp, H. Kroll, V. Kiefel, N. Schlegel, N. Chesnel, and C. Mueller-Eckhardt. 1991. HPA-5b (Br^a) neonatal alloimmune thrombocytopenia: clinical and immunological analysis of 39 cases. *Br. J. Haematol.* 78:425-429.
- Newman, P. J., J. Gorski, G. C. White II, S. Gidwitz, C. J. Cretney, and R. H. Aster. 1988. Enzymatic amplification of platelet-specific messenger RNA using the polymerase chain reaction. *J. Clin. Invest.* 82:739-744.
- Newman, P. J., R. S. Derbes, and R. H. Aster. 1989. The human platelet alloantigens, PI^{A1} and PI^{A2}, are associated with a leucine³³/proline³³ amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *J. Clin. Invest.* 83:1778-1781.
- Kuijpers, R. W. A. M., N. M. Faber, H. Th. M. Cuypers, W. H. Ouwehand, and A. E. G. Kr. von dem Borne. 1992. NH₂-terminal globular domain of human platelet glycoprotein Iba has a methionine¹⁴⁵/threonine¹⁴⁵ amino acid polymorphism, which is associated with the HPA-2 (Ko) alloantigens. *J. Clin. Invest.* 8:381-384.
- Lyman, S., R. H. Aster, G. P. Visentin, and P. J. Newman. 1990. Polymorphism of human platelet membrane glycoprotein IIb associated with the Bak^a/Bak^b alloantigen system. *Blood.* 75:2343-2348.
- Wang, R., K. Furihata, J. G. McFarland, K. Friedman, R. H. Aster, and P. J. Newman. 1992. An amino acid polymorphism within the RGD binding

domain of platelet membrane glycoprotein IIIa is responsible for the formation of the Pen^a/Pen^b alloantigen system. *J. Clin. Invest.* 90:2038-2043.

19. Takada, Y. and M. E. Hemler. 1989. The primary structure of the VLA-2/collagen receptor α^2 subunit (platelet GPIa): homology to other integrins and the presence of a possible collagen-binding domain. *J. Cell Biol.* 109:397-407.

20. Kiefel, V., S. Santoso, M. Weisheit, and C. Mueller-Eckhardt. 1987. Monoclonal antibody-specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. *Blood.* 70:1722-1726.

21. Santoso, S., V. Kiefel, and C. Mueller-Eckhardt. 1991. Monoclonal antibodies recognize a heterogeneity of platelet glycoprotein Ia molecules. *Thromb. Haemost.* 65:678. (Abstr.)

22. Phillips, D. R. and P. P. Agin. 1977. Platelet plasma membrane glycoproteins: evidence for the presence of nonequivalent disulfide bonds using non-reduced reduced two-dimensional gel electrophoresis. *J. Biol. Chem.* 252:2121-2126.

23. Gullberg, D., A. Tingström, A. C. Thuresson, L. Olsson, L. Terracio, T. K. Borg, and K. Rubin. 1990. $\beta 1$ Integrin-mediated collagen gel contraction is stimulated by PDGF. *Exp. Cell Res.* 186:264-272.

24. Kunicki, T. J., D. J. Nugent, S. J. Staats, R. P. Orzechowski, E. A. Wayner, and W. G. Carter. 1988. The human fibroblast class II extracellular

matrix receptor mediates platelet adhesion to collagen and is identical to the platelet glycoprotein Ia-IIa complex. *J. Biol. Chem.* 263:4516-4519.

25. Take, H., Y. Tomiyama, Y. Shibata, T. Furubayashi, S. Honda, H. Mizutani, T. Nishiura, T. Tsubakio, Y. Kurata, T. Yonesawa, and S. Tarui. 1990. Demonstration of the heterogeneity of epitopes of the platelet-specific alloantigen, Bak^a. *Br. J. Haematol.* 76:395-400.

26. Goldberger, A., M. Kolodziej, M. Ponz, J. S. Benett, and P. J. Newman. 1991. Effect of single amino acid substitutions on the formation of the PIA and Bak alloantigenic epitopes. *Blood.* 78:681-687.

27. Nieuwenhuis, H. K., J. W. N. Akkermann, W. P. M. Houdijk, and J. J. Sixma. 1985. Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. *Nature (Lond.)* 318:470-472.

28. Kehrel, B., L. Balleisen, R. Kokott, R. Mesters, W. Stenzinger, K. J. Clemetson, and J. van de Loo. 1989. Deficiency of intact thrombospondin and membrane glycoprotein Ia in platelets with defective collagen-induced aggregation and spontaneous loss of disorder. *Blood.* 71:1074-1078.

29. McFarland, J. G., R. H. Aster, J. B. Bussel, J. G. Gianopoulos, R. S. Derbes, and P. J. Newman. 1991. Prenatal diagnosis of neonatal alloimmune thrombocytopenia using allele-specific oligonucleotide probes. *Blood.* 78:2276-2282.