

NH₂-Terminal Globular Domain of Human Platelet Glycoprotein Ib α Has a Methionine¹⁴⁵/Threonine¹⁴⁵ Amino Acid Polymorphism, Which Is Associated with the HPA-2 (Ko) Alloantigens

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Abstract

The glycoprotein (GP) Ib/IX complex, a prominent platelet GP complex, is the primary receptor for vWF. Previously, we have established that an antigenic polymorphism of platelets, the HPA-2 or Ko alloantigen system, is located on the 45-kD amino-terminal globular domain of GPIb α . With the polymerase chain reaction, we have amplified two segments of the GPIb α gene coding for the first 382 amino acids of two HPA-2a and two HPA-2b homozygous individuals. Nucleotide sequence analysis revealed as the only difference a C-T polymorphism at position 434 of the coding region for the mature protein. This base change results in a substitution of threonine (ACG) in HPA-2a (Ko^b) to methionine (ATG) in HPA-2b (Ko^a) at amino acid position 145. The C-T polymorphism is reflected in a difference in restriction enzyme recognition, resulting in an Aha 2-site in the HPA-2b allele and a SfaNI site in the HPA-2a allele. Restriction fragment length polymorphism analysis of the amplified DNA of 3 HPA-2(a⁻,b⁺), 2 HPA-2(a⁺,b⁺), and 11 HPA-2(a⁺,b⁻) donors showed that these restriction sites were associated with the HPA-2 alleles. DNA-typing for the HPA-2 alloantigen system on genomic DNA obtained from a small number of cells may be applied for determining the genotype of a fetus from an immunized mother or of severely thrombocytopenic patients. (*J. Clin. Invest.* 1992. 89:381–384.) **Key words:** platelets • von Willebrand factor receptor • DNA polymorphism • RFLP analysis • thrombocytopenia • HPA-2 system • platelet antigen

Introduction

The platelet-specific biallelic alloantigen systems HPA-1 (Zw/P1^A) and HPA-3 (Bak) are localized on glycoprotein (GP)^I IIIa

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1. *Abbreviations used in this paper:* GP, glycoprotein; NAITP, neonatal alloimmune thrombocytopenia; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

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and IIb, respectively. It has been shown that single amino acid substitutions are associated with these polymorphic systems (1, 2). We have obtained evidence that the platelet-specific HPA-2 (Ko) alloantigens are localized on the elastase-sensitive 45-kD amino-terminal globular domain of the GP Ib α molecule (3, 3a). This biallelic platelet-specific alloantigen system, with gene frequencies of 0.926 for HPA-2a and 0.074 for HPA-2b in the Caucasian population, was first described by van der Weerd et al. (4). Alloantibodies against these antigens can either be responsible for refractoriness to platelet transfusions (5) or can cause neonatal alloimmune thrombocytopenia (NAITP) (6).

The nucleotide sequence of the cDNA encoding the GPIb α chain has been determined (7). Elucidation of the structure of the GPIb α gene revealed a coding region within a single exon (8). This offers the unique opportunity to perform polymerase chain reaction (PCR) directly on genomic DNA of leukocytes to detect molecular differences in the coding region. Two sets of primers were selected for the amplification of two overlapping fragments of the GPIb α gene coding for the amino acids 1–382.

In this study, we provide evidence that a C-T substitution of bp 434 in the gene coding for the GPIb α protein results in threonine¹⁴⁵/methionine¹⁴⁵ replacement and is related to the HPA-2 phenotype of the donor. Moreover, we show that the substitution leads to associated restriction-site differences that make it possible to type for HPA-2 at the genomic level.

Methods

Determination of the HPA-2 (Ko) phenotype on platelets was performed by the indirect platelet immunofluorescence test on chloroquine-treated platelets as described before (9, 10) with HPA-2a- and HPA-2b-specific alloantisera.

Genetic characterization. Genomic DNA was isolated from leukocytes after proteinase-K treatment and chloroform extraction using standard methods (11). Oligonucleotides for the PCR are the following: primer 1, ATAAGCTTGCTGCTCCTGCTGCCAAGCC (nucleotides 551–568 [8] extended with a HindIII site at its 5' end); primer 2, TAGAATTCAGCATTGTCTCCTGCAGCCAGCG (nucleotides 1,236–1,259 [8] extended with an EcoRI site at its 5' end); primer 3, ATAAGCTTCGCTGTATACAATACCAAAGGG (nucleotides 1,139–1,153 [8] extended with a HindIII site at its 5' end); and primer 4, TAGAATTCGGAGTGGGCTCCAGGGTGGTCATG (nucleotides 1,730–1,753 [8] extended with an EcoRI site). PCR was done with 0.7 μ g DNA and 20 pmol of each of the PCR primers using 2 U Taq-DNA polymerase (Promega Corp., Madison, WI) in a buffer recommended by the manufacturer, in a total volume of 50 μ l. 33 amplification cycles were carried out, with denaturation for 1 min at 95°C, annealing of primers for 2 min at 62°C, and extension for 2.5

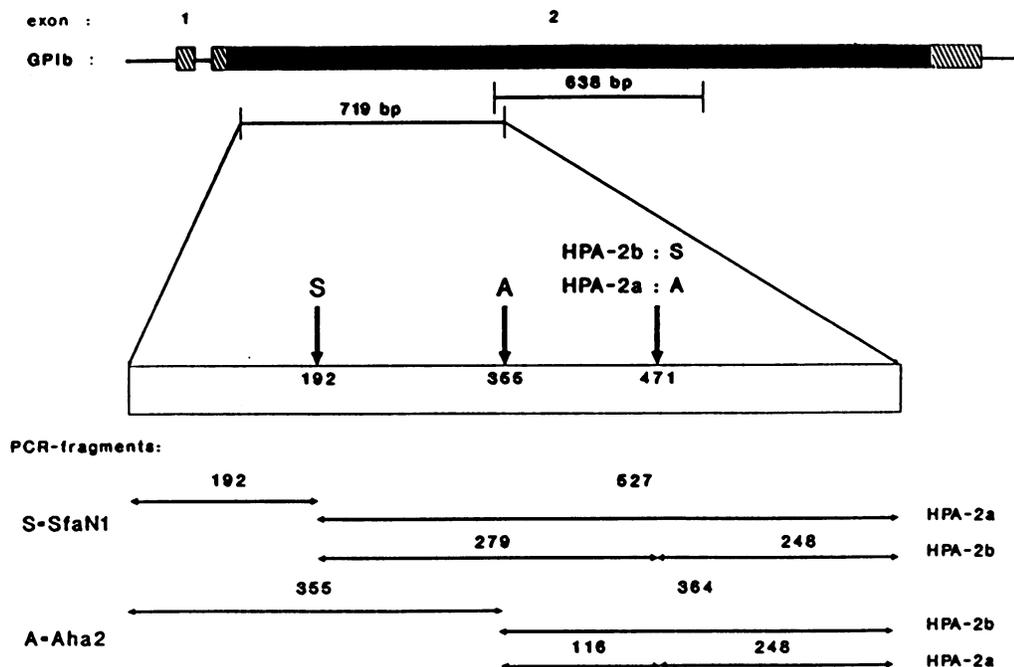


Figure 1. Restriction-site map of the PCR-amplified 719-bp fragment of GPIb. The filled bar represents the coding region and the shaded areas the additionally transcribed parts of the DNA. The arrows indicate the positions of the restriction-enzyme sites. The length of the fragments after SfaNI and Aha2 digestion is given in basepair numbers for both the HPA-2a and HPA-2b gene PCR products.

min at 72°C. Amplified fragments were purified by agarose electrophoresis, digested with HindIII and EcoRI, and subsequently cloned into pUC18. Nucleotide sequences were determined by the dideoxy-chain-termination method using Sequenase according to the manufacturer's recommendations (United States Biochemical Corp., Cleveland, OH). SfaNI and Aha2 restriction digestions were performed under conditions recommended by the manufacturers (Biolabs, New England Nuclear, Boston, MA).

Results

Amplification by PCR and sequence analysis. Two segments of the gene coding for GPIb α were amplified. The PCR products were subcloned in pUC 18. From each subcloned amplified fragment, three independent clones were subjected to nucleotide sequence analysis. The complete sequences of the first 1,146 bp (382 amino acids) were obtained from the amplified DNA. The nucleotide sequences of two HPA-2(a+,b-) donors were identical with the two published sequences of GPIb α . However, the sequence determined from the lymphocytes of two HPA-2(a-,b+) donors showed a C-T substitution of nucleotide 434. The C-T substitution changes an ACG codon for threonine into ATG, which codes for methionine at amino acid 145 of the mature GPIb α .

RFLP analysis. The C-T substitution converts an Aha2 restriction site into a SfaNI restriction site in the gene segment amplified by primers 1 and 2. The position of the Aha2 and SfaNI restriction sites on the 719-bp PCR product and the length of the fragments generated after digestion by these enzymes are schematically shown in Fig. 1. The actual digestion products are shown in Fig. 2 and are consistent with the predicted sizes from Fig. 1. Subsequent analysis of the DNA of 11 HPA-2(a+,b-), 2 HPA-2(a+,b+), and 3 HPA-2(a-,b+) individuals revealed fragments that were concordant with the serologically determined phenotype. From this, we conclude that the C-T polymorphism is uniquely related with the HPA-2 phe-

notypes ($P < 0.0002$ for the association of the ATG codon and the HPA-2b phenotype [Fisher exact test]).

Inheritance of HPA-2 genes. A family with a child suffering from NAITP due to anti-HPA-2b alloantibodies (6) was genotyped by RFLP analysis using the restriction enzyme SfaNI (Fig. 3). HPA-2 typing by alloantibodies was concordant with the genotyping, showing that the HPA-2 alleles are inherited together with the HPA-2 phenotype.

Discussion

GPIb, in complex with GPIX, is one of the most prominent glycoproteins on the platelet membrane (12, 13). GPIb/IX is essential for platelet interaction with the damaged subendothelium through vWF (14). It is one of the receptors for vWF (15, 16) and also has binding sites for thrombin (17). Its deficiency

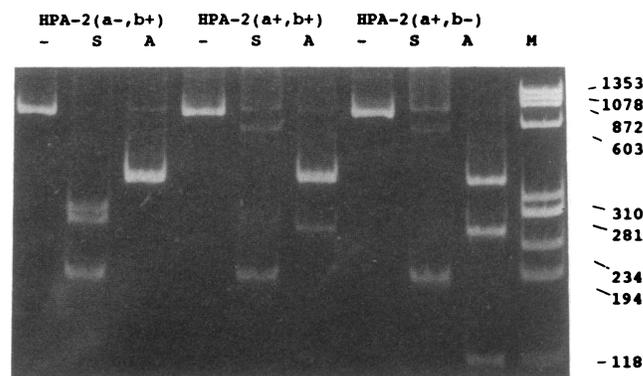


Figure 2. Analysis of SfaNI (S)- and Aha2 (A)-digested PCR products from DNA of HPA-2-phenotyped individuals. Size markers (M) are indicated in basepair numbers on the right of the figure. Undigested PCR products are shown in the lanes signed (-) for each donor.

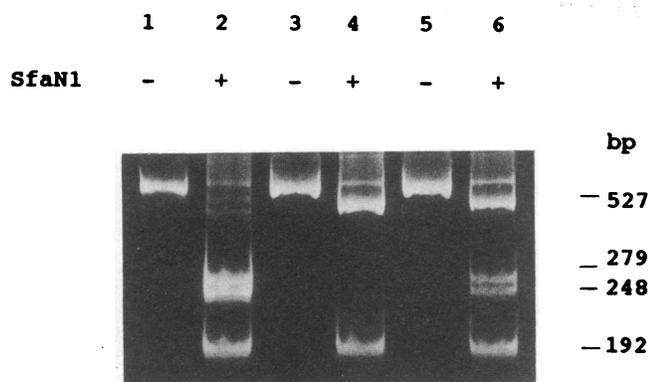


Figure 3. RFLP analysis of SfaNI-digested PCR-amplified DNA of a family with a child suffering from NAITP due to anti-HPA-2b antibodies. Lane 2 shows the products from DNA of the father, lane 4 those of the mother, and lane 6 those of the child. The number of basepairs of the fragments is indicated on the right (lanes 1, 3, and 5 show the undigested PCR products).

in the Bernard Soulier syndrome is associated with a bleeding tendency, indicative of its crucial role in the maintenance of normal hemostasis (13). The vWF-binding site is localized on the 45-kD amino-terminal globular domain, which can be removed by elastase (18). In blocking studies with synthetic oligopeptides, the domain, which interacts with vWF, has been located between amino acids 234 and 262 of the GPIb α chain (19).

Recently, we have obtained evidence that the biallelic platelet-specific HPA-2 antigen system is located on this globular domain. HPA-2a alloantibodies inhibit the ristocetin-induced aggregation of HPA-2a+ but not of HPA-2a- platelets, suggesting that the epitopes recognized by these antibodies are in the proximity of the vWF-binding site.² Thus, more knowledge about the precise location of these antigens could lead to new strategies to modify platelet function in pathological situations.

To investigate the molecular basis of the HPA-2 antigens, we applied the PCR with two sets of primers (set I, amino acids 4–217; set II, amino acids 191–382) on genomic DNA from two HPA-2(a+,b-) and two HPA-2(a-,b+) individuals. The results presented here, obtained from the gene fragment coding for the 217 NH₂-terminal amino acids of the GPIb α molecule from HPA-2(a-,b+) donors, disclose a C-T substitution in base pair 434. This polymorphism causes a threonine¹⁴⁵/methionine¹⁴⁵ polymorphism, and we suggest that this polymorphism is associated with the HPA-2 (Ko) alloantigens.

Alloantigens present on a particular membrane glycoprotein may be due to single nucleotide substitutions, as has been shown for HPA-1 and HPA-3 antigens (1, 2); but multiple nucleotide differences may also exist. In the case of the HPA-2, a single nucleotide substitution is probably responsible for the alloantigens as well. Previously, we could show that HPA-2 alloantigens are located on the 45-kD globular domain of GPIb α , possibly in proximity of the vWF-binding site, mapped between amino acids 232 and 262 (20). The GPIb α chain DNA fragment in which we found the nucleotide difference spanned the coding region for the whole 45-kD globular domain.

The C-T substitution in the gene for the GPIb α chain does not change the number of potential glycosylation sites. This is

in agreement with an identical molecular weight of GPIb α from HPA-2(a+,b-) or HPA-2(a-,b+) individuals when analyzed on SDS polyacrylamide gels (unpublished observations). A molecular-weight polymorphism has been reported in the Caucasian and Japanese population (20, 21). Recently, it has been shown that this size heterogeneity is probably caused by variations in the length of the rod-shaped COOH-terminal part of GPIb α (22). From this, we conclude that the HPA-2 polymorphism and the size polymorphism of the GPIb α chain are probably independent genetic variations.

The RFLP analysis performed with the amplified gene segment coding for the 217 NH₂-terminal amino acids obtained from 21 phenotyped donors and from the family with a child with NAITP due to HPA-2 antagonism gives additional support to the theory that this C-T polymorphism at bp 434 is uniquely associated with the HPA-2 phenotypes. The change in restriction site implies that RFLP analysis from amplified genomic DNA can be used for the determination of the HPA-2 genotypes. This method is a valuable additional tool to conventional serological typing, e.g., for severely thrombocytopenic patients and for determining the genotype of a fetus in case of previous alloimmunization of a mother against one of the HPA-2 alloantigens with a HPA-2(a+,b+) heterozygous father (23).

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