Evidence That an Abnormality in the Glycoprotein Ib alpha Gene Is Not the Cause of Abnormal Platelet Function in a Family With Classic Bernard-Soulier Disease

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The underlying molecular basis for Bernard-Soulier Disease (BSD) is currently unknown. Platelets from patients with this autosomal recessive bleeding disorder have multiple abnormalities, including a markedly reduced von Willebrand factor-dependent adhesiveness due to a deficiency of the platelet membrane glycoprotein (GP) Ib/IX complex. In the present studies, we have used an intragenic restriction fragment length polymorphism (RFLP) for Tag I in the GPIba gene to study linkage between this gene and the inheritance of BSD in a family with two affected siblings. Whereas the proband was heterozygous, showing both the 0.7 and 4.0 kb bands of this polymorphism (A/B), her affected brother was homozygous for the 0.7 kb band (A/A). Accordingly, these siblings did not inherit the same pair of GPIba alleles from their parents. Additionally, one child of the proband was A/A, while the second studied child was A/B, with neither showing any evidence of BSD. No construct of heterozygosity or homozygosity for GPIba alleles in this family is consistent with a model in which one or more defective GPIba alleles could produce BSD. RFLP analysis with BamHI or HindIII showed entirely normal patterns in the patients, indicating the absence of any gross deletion of the GPIba gene. GPIba mRNA from patient platelets was reverse transcribed and subsequently amplified by the polymerase chain reaction, demonstrating the presence of GPIba transcript. Furthermore, trace amounts of GPIb could be shown on the surface of patient platelets. Based on these results, a defect in the GPIba gene is unlikely to be the cause of BSD in this family.

CLASSIC BERNARD-SOULIER disease (BSD) is a rare autosomal recessive bleeding disorder, originally described in 1948 by J. Bernard and J.P. Soulier. It is characterized by prolonged bleeding time, thrombocytopenia, giant platelets, and markedly decreased platelet aggregation with ristocetin, but normal aggregation with other agents. The primary functional abnormality in BSD results from decreased adhesiveness of these platelets to exposed subendothelium as a result of their decreased ability to bind von Willebrand factor (vWF). Studies of platelet membrane glycoproteins (GP) by Nurden and Caen in 1975 and Phillips et al. in 1976 demonstrated a deficiency of GPIb in BSD patients. Later studies have also demonstrated lack of or markedly deficient of GPIX, GPIb, which is composed of a large (GPIba) and a small (GPIbβ) subunit, and GPIX appear to form a noncovalent heterodimeric complex that serves as the vWF receptor. In subsequent years, additional glycoprotein deficiencies have been identified in BSD patients, including GPV3 and a 210,000 Kd membrane glycoprotein, which may serve as a platelet Fc receptor.

Lopez et al. isolated a full-length cDNA for GPIba from human erythroleukemia (HEL) cells. Subsequently, two groups have sequenced the GPIba gene and shown that the entire coding region is contained within a single exon. In the present work, we have studied a BSD family with two affected siblings, and have used GPIba cDNA probes to study possible abnormalities of the GPIba gene and of gene expression in the affected patients.

METHODS

Patients. A previously unreported BSD family with two affected siblings, the proband (patient II-1) and her brother (patient II-4), were studied. The proband was a 30-year-old female with a history of excessive bleeding after tonsillectomy, menorrhagia, epistaxis continuing into adulthood, and profuse bleeding associated with ear piercing. This patient’s youngest brother (patient II-4) had frequent episodes of epistaxis, one severe enough to require hospitalization. Her two other siblings (patients II-2 and II-3) and her five children (patients III-1, III-2, III-3, III-4, III-5) were all asymptomatic. The father and the mother of the proband were not available for study; however, both had negative histories for an abnormal bleeding tendency.

Platelet function studies. Blood from normal volunteers or from patients was collected into ½ volume of 3.8% sodium citrate, and platelet-rich plasma (PRP) was prepared by centrifugation at room temperature (22°C) for 50 to 90 seconds at 900g Platelet aggregation and secretion studies were done on this citrated PRP as previously described. For immunoprecipitation studies, 8 platelet equivalents of NP-40 lysate were incubated overnight at 4°C with 3.75 to 7.5 µg of the anti-GPIb MoAb AP-1 (a gift of Dr Thomas Kunicki, Milwaukee, WI) or AS-7 together with 150 µL of goat anti-mouse IgG-agarose (Sigma). Immunoprecipitate complexes were then eluted from the agarose beads in 5% sodium dodecyl sulfate (SDS) buffer. Platelet lysates or immunoprecipitates were electrophoresed nonreduced on a 5% to 15% exponential SDS-polyacrylamide gel electrophoresis (PAGE) gel, and labeled polypeptides were detected by autoradiography. From the Department of Pathology, SUNY Health Science Center, Syracuse, NY; and the Hematology Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA. Submitted September 26, 1989; accepted February 26, 1990. Supported by Grant No. HL32853 from the National Heart, Lung, and Blood Institute. Address reprint requests to Jonathan L. Miller, MD, PhD, Department of Pathology, SUNY Health Science Center, 750 E Adams St, Syracuse, NY 13210.

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fluorography. Densitometric analysis of bands was done using a Helena Laboratories (Beaumont, TX) gel scanner.

**Southern blot analysis of genomic DNA.** Human genomic DNA was isolated from white blood cells by modification of the method of Bell et al. From 20 mL of blood, 100 to 600 pg of DNA was obtained. Genomic DNA was digested with restriction endonucleases BamHI, HindIII, BglII, or TaqI (all from Boehringer Mannheim Biochemicals, Indianapolis, IN), the resulting DNA fragments were electrophoresed in a 0.8% agarose gel and transferred to a nylon membrane (Gene Screen Plus; NEN Research Products, Boston, MA), and the membrane was then hybridized at 42°C for 16 hours with GPIbα cDNA probe labeled with [3H]dCTP. The membrane was then taken through a series of washes (two washes at room temperature for 5 minutes each in 0.3 mol/L NaCl-0.03 mol/L sodium citrate; two washes at 65°C for 30 minutes each in 0.3 mol/L NaCl-0.03 mol/L sodium citrate-1% SDS; two washes at room temperature for 30 minutes each in 0.015 mol/L sodium chloride-0.0015 mol/L sodium citrate), as recommended by the manufacturer of the membrane (NEN Research Products). The membrane was then exposed to Kodak X-Omat AR X-ray film for 1 to 3 days at -70°C.

**Analysis of platelet RNA.** From ACD anticoagulated blood of patients or of normal volunteers, PRP was prepared by multiple centrifugations, as described above. After several washes with PBS containing 2 mmol/L EDTA, platelet suspensions (1 to 10 x 10⁹ platelets/mL) were centrifuged at room temperature for 10 minutes at 1,000g. The resulting cell pellet was lysed by adding 3.6 mL of a solution containing 4 mol/L guanidinium isothiocyanate, 5 mmol/L Na₂EDTA, 0.1 mol/L 8-mercaptoethanol and 0.5% N-lauroylsarcosine (sarkosyl). RNA was isolated using a guanidium/cesium chloride gradient essentially as described by Maniatis et al. From 100 mL of patient blood or 50 mL of blood from normal controls, 10 to 14 pg of total RNA was obtained. To this RNA was added 1 unit of RQI RNase-free DNase (Promega Corp, Madison, WI) in a final volume of 20 μL. After incubation at 37°C for 15 minutes, phenol/chloroform/isoamyl alcohol extraction, followed by ethanol precipitation, was done for the recovery of RNA. For first-strand cDNA synthesis and subsequent amplification of the cDNA by the polymerase chain reaction (PCR), two DNA 17-mer primers were obtained from Genetic Designs, Inc (Houston, TX). J9 (5'-TCCTGCTGCTGGCTGAG-3') included bases 737 to 753 of the cDNA sense strand sequence published by Lopez et al. and J10 (5'-GGCTGCTCTAGAGAAAGCA-3') corresponded to the anti-sense strand from bases 1035 to 1051. First strand GPIbα cDNA was synthesized by adding oligonucleotide primer J10 (50 pmol) to 1 μg of total platelet RNA and 500 U of Moloney marine leukemia virus (M-MLV) reverse transcription (BRL) in a final volume of 50 μL, essentially as described by Newman et al. After the addition of 100 pmol of primer J9 and 50 pmol more of primer J10, together with KCl and gelatin, the reaction mix (100 μL) was heated to 94°C for 2 minutes and cooled; amplification was then started by adding 5 U of Taq polymerase (Perkin-Elmer Cetus Corp, Norwalk, CT). Amplification was performed for 50 cycles using a programmable heating and cooling system (Microcycler; Eppendorf Corp, Fremont, CA) under the following conditions: denaturation of strands at 94°C for 1 minute, annealing at 54°C for 1 minute, and extension at 72°C for 2 minutes. Amplification products were labeled with [32P]dCTP by a polymerase chain reaction (PCR) using a programmable heating and cooling system (Microcycler; Eppendorf Corp, Fremont, CA) under the following conditions: denaturation of strands at 94°C for 1 minute, annealing at 54°C for 1 minute, and extension at 72°C for 2 minutes. Amplification products were labeled with [32P]dCTP by a polymerase chain reaction (PCR) using a programmable heating and cooling system (Microcycler; Eppendorf Corp, Fremont, CA) under the following conditions: denaturation of strands at 94°C for 1 minute, annealing at 54°C for 1 minute, and extension at 72°C for 2 minutes.

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**Table 1. Routine Hemostatic Studies**

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<th>II-2</th>
<th>II-3</th>
<th>II-4</th>
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<td>Severe epistaxis</td>
<td>NH</td>
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<td>Platelet count (150-400 k/μL)</td>
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<td>6</td>
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<td>Platelet volume (7.5-11.0 fl)</td>
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<td>38-91</td>
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<td>Platelet diameter (blood film)</td>
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<td>15.6</td>
<td>—</td>
</tr>
<tr>
<td>PRP platelet aggregation</td>
<td>Increased</td>
<td>Increased</td>
<td>N</td>
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**Abbreviations:** PRP, pooled normal plasma; GPIbα, desialylated von Willebrand factor; NH, negative history; N, normal.

**Fig. 1. Platelet membrane glycoproteins labeled with [3H]p-hydroxide and analyzed by SDS-PAGE from a normal control (N) and the affected BSD male sibling patient II-4 (P).** Labeled polypeptides detected by fluorography after exposure for 5 days (A) show an absence of GPIb and GPIIX in the patient. After a 7-week exposure (B), a trace amount of GPIb is detected in the patient. A band at 82 Kd corresponding to GPV can be detected in the normal control, but not in the patient.
Fig 2. Taq I RFLP analysis by Southern blotting of GP Ibα genomic DNA. There is a constant 1.1 kb fragment in all lanes. Individuals are either homozygous for the 0.7 kb band (type A/A, lanes B through E), homozygous for the 4 kb band (type B/B, lane G), or heterozygous for this polymorphism (Type A/B, lanes A and F). Lanes: A through D, normal controls; E, classic BSD-affected male sibling (patient II-4); F, classic BSD-affected female sibling (patient II-1); G, normal control.

for 45 seconds, primer annealing at 49°C for 1 minute, and primer extension at 72°C for 3 minutes. No additional Taq polymerase was added to the reaction during the PCR cycles. After the PCR reaction, both intact PCR product and product digested for 4 hours at 37°C with Hae III restriction endonuclease were analyzed by agarose gel electrophoresis.

RESULTS

Routine hemostatic studies. Routine laboratory studies are shown in Table 1. Patient II-1 and patient II-4 presented with prolonged bleeding times, thrombocytopenia, and giant platelets on peripheral blood films. Platelet aggregation studies showed decreased responsiveness with ristocetin and with asialo-vWF, but normal responses to all other agents. Normal bleeding time, platelet count, platelet size, and platelet aggregation studies were obtained in all other family members available for study, including patient III-5, a newborn male child of patient II-1.

Glycoprotein analysis. Glycoprotein analysis was done using platelets from a normal control or from BSD patient II-4, with 1H-labeled platelet lysates electrophoresed nonreduced on SDS-PAGE. Labeled polypeptides were detected by fluorography. After a 5-day exposure, absence of GP Ibα was demonstrated in the patient as compared with the normal control (Fig 1A). After a 7-week exposure (Fig 1B), GP Ibα could be detected in the patient. While not well visualized in the figure, a trace amount of GP IX could also be seen in the 7-week exposure of the original autoradiograph. However, the 82 Kd band corresponding to GPV remained

Fig 3. Schematic representation of Taq I polymorphism of the GP Ibα gene. The DNA fragments obtained after digestion of genomic DNA with Taq I are represented by the hatched bars. The constant 1.1 kb band present in all persons is between Taq sites 612 bp and 1769 bp. The polymorphic site (Taq I*) is located approximately 76 bp 3' to the end of the known cDNA sequence. Presence of the polymorphic site results in a 0.7 kb band designated Allele A. Lack of the polymorphic site results in a 4 kb band designated Allele B.
Fig 4. Absence of linkage between GPIbα Taq I polymorphism and inheritance of BSD. Patients with a history of thrombocytopenia and giant platelets are shown in hatched symbols; asymptomatic individuals, open symbols. Individuals homozygous for the presence of the polymorphic site are denoted as A/A; heterozygous individuals, A/B. Note that BSD patients II-1 and II-4 have different Taq I polymorphic patterns. Two children of patient II-1, patients III-1 and III-2, also show different Taq I polymorphic patterns, but neither has clinical or laboratory evidence of BSD.

undetectable in the patient. Additionally, when ³H-labeled platelets from patient II-1 or II-4 were immunoprecipitated with the anti-GPIb MoAbs AP-1 or AS-7, bands comigrating with normal GPIb were seen by fluorography; however, by densitometry, the patient GPIb bands showed only 1% to 5% as much intensity as the normals.

Southern blot analysis. For study of the genomic structure of the GPIbα gene in the affected patients, DNA was isolated from leukocytes, digested with restriction endonucleases, and hybridized with ¹³P-labeled full-length GPIbα cDNA probes. Genomic DNA digested with restriction enzyme BanII revealed a single 6.4 kb band both in normal controls and in the affected patients. When genomic DNA was digested with HindIII, a single 12.9 kb band was seen, both in patients and in normal controls. These findings suggested that there was no gross deletion of the GPIbα gene in the affected patients.

When genomic DNA was digested with Taq I, a constant 1.1 kb band was always seen in all persons studied. However, a Taq I polymorphism was also seen in the normal population, resulting in individuals homozygous for a 0.7 kb band, individuals heterozygous for both a 0.7 and a 4.0 kb band, and individuals homozygous for a 4.0 kb band (Fig 2). Double digestion of genomic DNA with Bgl II and Taq I, followed by probing with labeled cDNA fragments representing only the 3' end or the 5' end of the cDNA confirmed localization of the Taq I polymorphic site approximately 75 bp downstream from the 3' end (bp 2420) of the published¹⁹ cDNA sequence. The presence of the polymorphic site resulted in the 0.7 kb band (designated Allele A), shown schematically in Fig 3. Lack of the polymorphic site resulted in the 4.0 kb band (designated Allele B).

Study of the Taq polymorphism in this family indicated non-linkage with the GPIbα gene. The affected brother, patient II-4 (Fig 2, lane E), was homozygous for the 0.7 kb band (A/A), while his affected sister, patient II-1 (lane F), was heterozygous, showing both the 0.7 and the 4.0 kb bands (A/B). Thus, the brother and sister, both of whom have autosomal recessive BSD, did not inherit the same pair of alleles for GPIbα from their parents (Fig 4). Furthermore, DNA analysis of two of the children of patient II-1 revealed

- Hae III
+ Hae III

314 bp -
230 bp -

Fig 5. Demonstration by PCR of GPIbα mRNA transcript in BSD platelets. After reverse transcription of platelet mRNA and subsequent amplification by PCR, a 314 bp DNA band, corresponding to the region from bases 737 to 1051 of the coding sequence for GPIbα, is seen both in the patient (P) and the normal control (N). After digestion of the PCR products with restriction enzyme HaeIII, the major 230 bp restriction fragment is seen in both the patient and the normal control.
that one (patient III-2) was homozygous for the 0.7 kb band (A/A), while the other (patient III-2) was heterozygous for the polymorphic site (A/B), but neither had any clinical or laboratory evidence of BSD.

**Platelet RNA studies.** To establish whether specific mRNA coding for GPIbα was indeed present in the thrombocytopenic BSD patients, and to facilitate its analysis, the PCR technique was used to amplify the GPIbα RNA sequence. RNA samples were treated with RNase-free DNase to remove any possible contaminating genomic DNA. Two oligonucleotide primers, J9 and J10, were used to synthesize a specific GPIbα cDNA from platelet mRNA. A 314 bp fragment (from bases 737 to 1051) of the RNA coding sequence for GPIbα was reverse transcribed and subsequently amplified, both for patient II-4 and for a normal control (Fig 5). For further confirmation that the 314 bp cDNA PCR product was an amplified copy of the corresponding region in the platelet GPIbα mRNA, the amplified DNA was digested with restriction endonuclease HaellI. After ethidium bromide staining, the predicted major band corresponding to a 230 bp DNA restriction fragment was shown in both the patient and the normal control (Fig 5). Accordingly, these studies provide evidence at the RNA level for the expression of GPIbα in the platelets of these patients with BSD.

**DISCUSSION**

Using DNA and RNA analysis, we have studied possible abnormalities of the GPIbα gene in a family with BSD. Based upon clinical and laboratory evidence, this BSD family demonstrates a classic autosomal recessive pattern of inheritance, with the implication that a pair of defective alleles is needed in order to express disease. DNA analysis after digestion with BamHI or HindIII showed no rearrangements or deletions of the GPIbα gene in these BSD patients. Using restriction fragment length polymorphism (RFLP) analysis, we were able to show that these patients have different Taq I polymorphic patterns, indicating that they each inherited a different pair of GPIbα alleles from their parents. Patient II-4, who is homozygous (A/A), has BSD; patient II-1, who is heterozygous (A/B), is also affected. Furthermore, DNA analysis of two of the children of patient II-1 revealed that one (III-1) is homozygous (A/A), while the other (III-2) is heterozygous (A/B), and neither has evidence of BSD. No construct of heterozygosity or homozygosity for GPIbα alleles in this family is consistent with a model in which one or more defective GPIbα alleles could produce BSD. Thus, the BSD phenotype in this family is not linked to the RFLP that marks the GPIbα gene.

We have also shown the presence of mRNA encoding for GPIbα in the platelets of the BSD patients. This finding is not unexpected, since by the use of sensitive detection procedures, we have also been able to demonstrate GPIbα on the surface of their platelets. Detection of small amounts of platelet GPIbα in other patients with BSD has also been demonstrated recently.31

This report documents the usefulness of RFLP linkage analysis to identify which of several potential genes might be abnormal in a disorder characterized by the absence of a multisubunit membrane complex. Recent studies of other membrane complexes, such as platelet GPIbα-IIb3 and the T-cell receptor/CD3 complex,3 show that normal receptor assembly may require coordinate expression of multiple subunits. Thus, in BSD a defect in the separate genes encoding any one of several membrane glycoproteins, including GPIbα, GPIbβ, GPIX, and, possibly, GPV, could prevent the assembly and cell surface expression of the von Willebrand factor receptor complex.

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Evidence that an abnormality in the glycoprotein Ib alpha gene is not the cause of abnormal platelet function in a family with classic Bernard-Soulier disease

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