Variant Bernard-Soulier Syndrome Associated With a Homozygous Mutation in the Leucine-Rich Domain of Glycoprotein IX

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We describe a new variant of Bernard-Soulier syndrome. The patient (W.K.) showed the classic bleeding symptoms together with absence of platelet agglutination to ristocetin plus von Willebrand factor, whereas aggregation to ADP, collagen, and arachidonic acid was normal. Platelets were markedly larger than normal and the patient had life-long thrombocytopenia. Surface-labeling of the platelets and two-dimensional gel electrophoresis showed reduced but detectable amounts of glycoprotein (GP) Ib-IX-V present; however, there was markedly less GPIb (2% ± 1% of normal) than GPIX, Ibβ, or V (7% ± 2% of normal). This disproportion was confirmed by Western blotting. Sequence analysis was performed after polymerase chain reaction amplification of the coding region of the GPIX and GP Ibα genes from the patient. A point mutation (A → G) was found in GPIX converting "Asn to Ser within the leucine-rich domain. No mutations were found in GPIbα. Both alleles of GPIX contained the same defect, which was confirmed by the appearance of a new cleavage site for the restriction enzyme Fnu4HI. This substitution did not affect glycosylation at the neighboring "Asn as judged by the distribution on two-dimensional gels but did appear to change the conformation of the leucine-rich domain, thus reducing surface expression of the complex. The relationship between GPIb and GPV was not affected, indicating that GPIX does not regulate this. This homozygous mutation in GPIX indicates that, among other possible functions, the leucine-rich domains present on all components of GP Ib-IX-V may play a role in the assembly and surface expression of the complex.

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BERNARD-SOULIER syndrome is a rare autosomal recessive genetic disorder associated with prolonged bleeding time, thrombocytopenia, and morphologically unusually large platelets.1,2 Resting Bernard-Soulier syndrome platelets are incapable of binding von Willebrand factor (vWF) and show a dramatically decreased adhesion to subendothelium of damaged vascular wall. In vitro, they do not aggregate to ristocetin or botrocetin in the presence of vWF. Their response to stimulation by ADP, adrenaline, collagen, and arachidonic acid is normal and elicits shape change and aggregation (and secretion to the latter two agonists). A qualitative or quantitative deficiency in the platelet membrane glycoprotein (GP) Ib-IX-V complex is the cause of the syndrome. In the classic disorder, the complex is totally absent from the platelet surface, but intermediate situations with residual amounts of nonfunctional complex have also been reported.3,4

The GPIb-IX-V complex is composed of four GP chains. GPIb (170 kD) consists of two subunits α (140 kD) and β (27 kD) linked by a disulfide bond. The amino-terminus globular domain of the α subunit has binding sites for vWF and thrombin. GPIb associates with GPIIX (22 kD) by strong noncovalent forces.8 GPV (82 kD) is more loosely attached to the others.9 DNA and amino acid sequence analysis have shown that each chain contains a variable number of leucine-rich repeats.10-13 The genes for GP Ibα,14 GPIIX,15 and GPV16,17 have been cloned. The pattern of their respective 5′ untranslated and coding segments is very similar. Each coding domain is contained within one exon, whereas a small intron cuts the 5′ untranslated sequence upstream from the initiation site. The coordination of complex expression via the individual genes, unlike the situation with GPIIb-IIIa,18 remains unclear. Variable levels of complex have been detected on the platelet surface of patients suffering from variant Bernard-Soulier syndrome. Sometimes the quantity of GPIbα, GPIbβ, and GPIIX chains was reduced in parallel, suggesting that the complex assembly was either regulated by coordinate expression of the components or directed by one of the chains.4 Discrepancies in the expression of the chains have also been noted.5,7 So far, no clear cases have been described in which the genetic defect underlying classic Bernard-Soulier syndrome has been determined. In a few cases of variant Bernard-Soulier syndrome, the molecular basis leading to the disorder has been established. The defects were traced to modifications in the coding sequence of the GPIbα gene, leading to substitutions of 14tryptophan by a nonsense codon,19 "leucine by phenylalanine,"20 and 18alanine by valine in the leucine-rich amino acid domain of the protein.21 Double heterozygote defects have also been described recently within the GPIX gene.22 In these patients, two missense mutations in the coding region of GPIX, one located in codon 21 in one allele and the other in codon 45 in the other allele, were associated with the Bernard-Soulier syndrome. Residual amounts of GPIbα but no GPIX or GPV were reported.

The patient presented here is a rare case of variant Bernard-Soulier syndrome showing a pronounced imbalance in the expression of GPIIX in relation to the GPIbα, GPIbβ, and GPIV chains of the complex.

MATERIALS AND METHODS

Blood samples were collected from the patient, his immediate family members, and normal volunteers. All individuals gave informed consent.

Patient. The patient (W.K.), a 23-year-old man from Vienna has a life-long bleeding problem, including epistaxis. He is thrombocytopenic since childhood, with a platelet count of 30,000 to 60,000/μL, and has a prolonged bleeding time. Peripheral blood smear showed giant platelets. Platelets aggregated normally to ADP, collagen, and epinephrine but failed to aggregate to ristocetin at 1.2, 1.4, and 2.0

mg/mL. The patient’s mother had a mild bleeding tendency since childhood. The father displayed a completely normal hemostasis. The brother had a mild thrombocytopenia since childhood but no bleeding tendency. The sister was normal.

Platelet isolation. Platelet-rich plasma (PRP) from the Bernard-Soulier syndrome patient was prepared from 10 mL of acid-citrate-dextrose (ACD) blood as described previously.3 In the first step, the anticoagulated blood was allowed to sediment (tube tilted at 45°) at room temperature for 4 hours. The PRP was carefully removed and the platelets were isolated by centrifugation; washed twice in 60 mmol/L sodium citrate, pH 6.5, 30 mmol/L glucose, 120 mmol/L NaCl, and 5 mmol/L EDTA in the presence of Iloprost (a kind gift from Schering AG, Zurich, Switzerland) at 1 ng/mL; and washed once in 10 mmol/L Tris-HCl, pH 7.4, 154 mmol/L NaCl, and 5 mmol/L EDTA, before resuspension in the appropriate buffer (2 × 10^7 platelets/mL). Normal platelets were isolated from ACB blood by differential centrifugation; washed twice in 60 mmol/L sodium citrate, pH 6.5, 30 mmol/L glucose, 120 mmol/L NaCl, and 5 mmol/L EDTA in the presence of Iloprost at 1 ng/mL; and washed once in Tris-HCl, pH 7.4, 154 mmol/L NaCl, and 5 mmol/L EDTA. They were resuspended at 10^8 platelets/mL.

Platelet surface-labeling. Washed platelets from a healthy donor and from the patient were surface-labeled in parallel by the metaperiodate/[H]borohydride procedure as optimized for platelets by Steiner et al.25 After labeling, the platelets were solubilized in 1% sodium dodecyl sulfate (SDS) in the presence of 2 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 0.2 mg/mL leupeptin (Fluka AG, Buchs, Switzerland) to inhibit proteases. The protein content of each lysate was established using the Pierce protein assay (Oud-Beijerland, The Netherlands) and for each donor an equal amount of protein was submitted to isoelectric focusing. Two-dimensional isoelectric focusing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described earlier.24 The isoelectric focusing step was run for 18 hours at 260 V. The second dimension was a Laemmli gel with a 7% to 17% acrylamide gradient. The gels were treated for fluorography25 before exposure to Kodak X-Omat films (Eastman Kodak, Rochester, NY). Densitometry of individual spots was performed using a video camera-based, computer-controlled densitometer.

Western blots. Known amounts of platelet lysate protein from normal donors and patient were separated under nonreducing conditions by SDS-PAGE (7% to 17% acrylamide), transferred by the semi-dry technique to nitrocellulose sheets (Schleicher and Schuell, Dassel, Germany), and incubated with appropriate antibodies. Rabbit polyclonal antisera against specific components of the GPIb-IX complex were used. The antibody recognizing GPIbα was raised against the 45-kD fragment obtained by trypsin digestion of GPIbα. The antibodies directed against GPIbβ and GPIX were raised against purified GPIbα subunit and GPIX, respectively.14 Phosphatase-labeled goat antirabbit IgG (Bio-Rad, Glattburg, Switzerland) or phosphatase-labeled rabbit antimouse IgG (Sigma, St Louis, MO) were used in the presence of p-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma) to detect the primary antibodies.

Polymerase chain reaction (PCR). Genomic DNA samples were isolated from peripheral blood leukocytes. After cell lysis, the nuclei were collected by centrifugation and treated with protease K (0.075 mg/mL; Boehringer, Mannheim, Germany) in 1% sodium lauroyl sarcosinate at 42°C followed by RNase. The protein was removed by phenol extraction and the DNA was recovered by precipitation with ethanol. The coding regions of the GPIbα gene from bp 2796 to bp 5309 and GPIX gene from bp 1471 to bp 2172 were amplified in segments using the PCR. In both cases, the 5' untranslated sequence and the intron located a few base pairs upstream of the start codon were included in the analysis. Specific primers were designed from the published sequences of the GPIbα and GPIX genes (EMBL sequence database, Heidelberg, Germany) and are listed in Table I. Amplification of the GPIbα fragments was performed in a volume of 50 μL containing 250 ng genomic DNA, 0.200 mmol/L of each dNTP, 100 ng of each primer in PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, and 1.5 mmol/L MgCl2). The primer pairs were Iba1 + Iba3, Iba2 + Iba3, Iba4 + Iba5, and Iba6 + Iba7. The samples were heated for 7 minutes at 94°C before adding 1 U Taq DNA polymerase (Perkin-Elmer Cetus, Rotkreuz, Switzerland). Thirty cycles were performed with denaturation for 1 minute and 15 seconds at 94°C, annealing for 2 minutes at 52°C, and extension for 2 minutes at 72°C. The conditions were modified slightly for the amplification of GPIX.

Two hundred fifty nanograms of genomic DNA was mixed in PCR buffer (10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 0.01% gelatine, 1.75 mmol/L MgCl2), with 0.200 mmol/L of each dNTP and 150 ng of each primer. The primer pairs were IX1 + IX2, IX3 + IX2, and IX3 + IX4. The DNA was denatured for 7 minutes at 94°C before adding 2 U of Taq polymerase. Thirty cycles were used with 1 minutes and 15 seconds of denaturation at 94°C, 2 minutes of annealing at 52°C, and 3 minutes of elongation at 72°C.

Subcloning. The PCR fragments were cleaned on agarose gel (SeaKem; FMCG BioProducts, Vallensbaek Strand, Denmark) recovered by electroelution and introduced in the vector BlueScript KS+ (Stratagene, La Jolla, CA) by blunt-end ligation or using restriction sites when these were available.

Sequencing. Sequencing was performed with Sequase II (US Biochemical Corp, Cleveland, OH) on double- or single-stranded DNA with primers complementary to vector or insert sequences.

Restriction analysis of fragments amplified by PCR. Ten microliters (0.5 vol) of PCR reaction was diluted twofold and incubated with various restriction endonucleases following the manufacturer's instructions. The products of digestion were analyzed by agarose gel electrophoresis using a 3% agarose gel (NuSieve; FMCG BioProducts).

RESULTS

Platelet surface-labeling. To examine the expression of the constituents of the GPIb-IX complex on platelets of the

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<th>Table 1. Sequences and Locations of Nucleotide Primers Used in the PCR Amplification of GPIbα and GPIX</th>
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patient with Bernard-Soulier syndrome, isolated intact platelets were surface-labeled by the periodate-borohydride method. Platelets from a healthy donor labeled in parallel were used as references. Fluorograms of surface-labeled GPs after separation by two-dimensional isoelectric-focusing SDS-PAGE are presented in Fig 1. The patterns derived from a normal donor (Fig 1A) compared with the patient (Fig 1B) showed significant differences. GPIIbα and GPIlbβ were only present in trace amounts on the patient’s platelets, whereas GPV and GPIX were not detectable after exposure times sufficient to show them in the fluorogram of healthy patients. GPIIbα with its high O-glycosylation content is al-
ways intensively surface-labeled by the periodate\[1^H\]-sodium borohydride method, whereas GPV gives a fainter reaction. Longer exposure times were necessary to show this protein on the fluorogram of the patient. When the weaker signals were enhanced, the presence of GPV was established easily and in amounts that were in the normal relationship to those of GPlb\alpha and GPlb\beta (Fig 1C). GPIX, which is hardly detectable in Fig 1B, was easily identified in Fig 1C as a characteristic group of spots located between pI 5.8 and 6.5 with an electrophoretic mobility of 22 kD. However, the relative intensities of GPlb\beta and GPIX were not equivalent because GPlb\beta was much denser than GPIX. This disparity tended to indicate that the natural ratio between GPlb\beta and GPIX was modified in the Bernard-Soulier syndrome platelets. Therefore, a quantitative evaluation of the patient GPlb-IX complex was performed by densitometry. The relative amount of each constituent, including GPV, was compared with that of healthy donors. GPlIb (GPIV, CD36) and GPl-Ilb\beta (\alpha_{\text{llb}} light chain), both GPs that were found in normal amounts on the defective platelets, were chosen as internal standards. GPlba, GPlb\beta, and GPV were present in much reduced quantities on the patient’s platelets and the level of their expression seemed to be affected in a comparable manner. Each was present at 7% \pm 2% of the value measured on normal platelets. GPIX was found in even lower concentration and did not exceed 2% \pm 1% of the value of GPIX in normals.

**Western blot experiments.** The labeling results were supported by immunoblot experiments of platelet lysates with antiserum against GPIX. In Fig 2, a band at 22 kD corresponding to GPIX is visible on nonreduced blots of platelet lysates from both normal and patient separated by gel electrophoresis and incubated with a polyclonal antibody raised against purified GPIX. However, the band from the patient is very weak in comparison with that of the control. One hundred micrograms of protein, equivalent to 6 \times 10^8 normal platelets, was necessary to get a positive answer with the patient lysate, whereas the protein corresponding to 5 \times 10^7 platelets from an healthy donor gave an equivalent signal. The additional bands seen on the blot come from the reaction of the GPlb\beta subunit with the antiserum used here that was not absolutely specific for GPIX but also recognized GPlb\beta weakly. The major band at 170 kD corresponded to the integral GPlb protein, whereas the others represented proteolytic fragments of GPlba associated with GPlb\beta. The cleavage affected the N-terminal part of the GPlba chain leaving intact the macroglycopeptide domain and GPlb\beta, because no binding was observed with an antibody raised against the 45-kD fragment of GPlba. Autoradiograms of gels run on the patient’s labeled platelets under nonreduced conditions showed the expected normal band for GPlb at 170 kD (data not shown).

**Analysis of the patient DNA.** To try to detect whether an anomaly in the amino acid sequence of one of the GPs composing the GPlb-IX complex could lead to the defect observed on the platelets of the patient, an analysis was performed at the DNA level. Based on the results of the surface-labeling, the gene for GPIX was analyzed first. The DNA sequence of the genes for GPlba\alpha and GPIX\alpha have been described and both coding regions are only interrupted by a small intron upstream from the initiation codon. This particularity allows an amplification of the entire coding region from genomic material. Fragments covering the entire coding region of GPIX and extending 174 bp upstream of the initiation codon were amplified from the patient genomic DNA with different primers. The fragments were subcloned and sequenced. A unique mutation was found in all the clones analyzed. The base 1826 from the GPIX gene was changed from A to G, causing an alteration in codon 45 from AAC to AGC (Fig 3). This mutation introduced a modification in the codon for \textit{asparagine} of GPIX that was replaced by a serine. The possibility of a mistake introduced by the PCR technique could be eliminated by comparing the results of several different amplification reactions. The mutation introduced a new cleavage site for the restriction enzyme Fnu4HI. In the modified sequence, a shorter fragment of 105 bp is substituted for the 151 bp encountered in normals after cleavage of GPIX DNA by this restriction enzyme. Forty healthy donors were screened for the polymorphism of GPIX in that location. The DNA from the patient and his closer relatives were digested with several restriction enzymes. Taq I and Smal I were used as controls.

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**Fig 2.** Western blot of platelet lysate from both patient W.K. (P, 100 \(\mu\)g of protein) and normal (N, 12 \(\mu\)g of protein) incubated with a polyclonal antibody against GPIX. Platelet proteins were separated by gel electrophoresis under nonreducing conditions and transferred onto nitrocellulose. The antiserum against GPIX was not absolutely monospecific and also recognized the GPlb\beta subunit weakly. In the patient, a faint band corresponding to GPIX is visible at 22 kD. The major band at 170 kD is the integral GPlb protein.
and generated fragments of the anticipated lengths (190 bp and 289 bp, respectively) in all the subjects studied (data not shown). When the DNA amplified from the patient was digested with Fnu4HI, a unique fragment of 105 bp in length was found on agarose gel electrophoresis, indicating that both alleles carried the mutation (Fig 4). This result is in good agreement with the data from the sequencing experiments in which all the clones studied showed a modified sequence for codon 45. The fact that the patient was homozygous for the defect indicated that the mutation in each allele probably arose from a common source. The family history was more thoroughly investigated and the parents of the patient were found to be second cousins (Fig 5). The GPIX gene of the closer relatives was studied in more detail. Digestion of the GPIX gene from the patient’s parents and from his brother with Fnu4HI yielded two bands, one at 151 bp corresponding to native GPIX allele and another band at 105 bp representing the mutant allele. The patient’s sister was normal (Fig 4).

In addition to GPIX, the GPlba gene was also investigated. The entire coding region of GPlba as well as 273 bp upstream of the first ATG were amplified by PCR, subcloned, and sequenced. No mistakes were found in any of the clones analyzed.

DISCUSSION

In the Bernard-Soulier syndrome case presented here, despite all the classic symptoms linked with the disorder,
thrombocytopenia, giant platelets, and lack of aggregation to ristocetin with vWF, residual amounts of GPIbα, GPIbβ, and GPV as well as traces of GPIX were detected on the platelet surface of the patient. The defect could be linked to a unique missense mutation in both alleles of the GPIX gene, leading to a modification in the amino acid sequence of the GP.

The mechanism of platelet adhesion to the subendothelium is not yet completely understood. The binding site for vWF is on the GPIbα chain of the GPIb-IX-V complex, at least partially between the leucine-rich domain and the highly glycosylated macroglycopeptide. Inhibition studies with peptides\(^{26,27}\) have defined it more precisely to the double-loop region formed between cysteines 209-248 and 211-264.\(^{28}\) However, little is known about the involvement of the other chains of the GPIb-IX-V complex in the process or their contribution to its spatial organisation.

Glanzmann’s thrombasthenia has been an useful model to study the mechanism of platelet aggregation. Various cases have been described in which the absence of platelet aggregation could be linked to molecular defects in either chain of the GPIIb-IIIa complex.\(^{29}\) Similarly, the analysis of variant Bernard-Soulier syndrome in which platelets display residual amounts of GPIb-IX-V complex may provide useful information on the role of the different complex constituents in platelet adhesion to the subendothelium. Establishing the consequences of defects in one of the four chains on the expression of the GPIb-IX-V complex can help to explain the mechanism of assembly of the GPIb-IX-V complex and its modulation and expression at the platelet surface. In normal platelets, GPIb and GPIX occur in a ratio of 1:1 in the GPIb-IX-V complex,\(^{30}\) whereas GPV is estimated to be present in only half that amount. This difference in stoichiometry has still not been explained. A few cases of variant Bernard-Soulier syndrome have been described in which platelets were unable to adhere to the subendothelium but retained various amounts of GPIb complex. The quantity can vary from normal levels of nonfunctional complex to rare cases in which only one chain is present. In the patient presented here, surface-labeling of intact platelets showed the presence of GPIbα, GPIbβ, and GPV in low amounts. The stoichiometric ratio encountered in the native complex for these three chains was retained. On the other hand, there was clearly less GPIX than the other three. Western blot analysis confirmed these observations and showed the presence of only traces of GPIX in the platelet lysate. The apparently normal relationship of GPIbα, GPIbβ, and GPV indicated that a defect in the GPIX chain was associated with the poor expression of the complex at the platelet surface. Indeed, analysis of the coding region of the GPIX gene showed a unique point mutation that altered codon 45 from AAC to AGC resulting in the conversion of an asparagine residue to a serine. The presence of this unique mutation in all the clones sequenced indicated that both alleles in the patient were affected in the same way. This was checked by restriction fragment length analysis, because the modification introduced an additional restriction site for the enzyme Fnu4HI and allowed a rapid method of identification of the point mutation. All the GPIX DNA fragments amplified from the patient’s genomic DNA carried the additional cleavage site for the enzyme and confirmed that the patient was homozygous. The close relatives were screened and, as expected from the recessive autosomal character of the disorder, the parents were found to be carriers of the defective gene for GPIX. The brother was also heterozygous, whereas both alleles were completely normal in the sister.

The mutation discovered here is the same as one of the two single basepair substitutions recently reported for the GPIX gene in double heterozygous patients with Bernard-Soulier syndrome.\(^{22}\) This unusual frequency would suggest that codon 45 in GPIX is rather vulnerable to mutation because the two families are geographically widely separated. A combination of different defects in GPIX is not essential because one homozygous mutation is also associated with the phenotype. It may therefore be expected that the homozygous form of the other mutation (\(^{45}\)Asp to Gly) will also be associated with Bernard-Soulier syndrome, but there may be quantitative differences in expression levels. No missense mutation was detected in the coding region of the patient GPIbα gene. Even if additional mutations in the GPIbβ and GPV genes cannot be totally excluded, they seem unlikely. Despite a poor expression at the platelet surface, the relative amounts of GPIbα, GPIbβ, and GPV are equivalent to those found in the native complex, suggesting a normal relationship between the three chains.

The modification lies in the unique leucine-rich domain of GPIX and is adjacent to the potential N-glycosylation site of the molecule. GPIX contains a single biantennary lactosamine type N-glycosylation chain with no O-glycosylation.\(^{31}\) Although it was not possible to measure accurately the glycosylation in GPIX of the patient, the isoelectric pattern of GPIX was identical of that of normal controls, indicating that the glycosylation at this site was not affected by the mutation. The situation of heterozygotes in Bernard-Soulier syndrome remains controversial. Although some cases have been described in which the platelets from heterozygotes had slight abnormalities, they have generally been reported to be normal. In those that we examined from this family, the brother had life-long thrombocytopenia but no bleeding problems, whereas the mother had life-long mild bleeding problems. The father was completely normal. Thus, mild clinical manifestations in Bernard-Soulier heterozygotes may be related to the nature of the defect but may also possibly be caused by additional, normally subclinical, defects, eg, in coagulation, leading to an additive effect.

Transfection of DNA for GPIbα, Ibβ, and IX into non-megakaryocytic cell lines has helped to define some of the parameters required for surface expression of GPIb-IX. Although these expression systems were useful models for the study of complex formation, they gave some apparently conflicting results. In one case, an adequate expression of GPIb-IX was obtained only after transfection of the three DNA for GPIbα, Ibβ, and IX.\(^{32}\) However, other studies have shown that the transfection of GPIbα alone, despite considerable degradation, could give respectable levels of surface expression of this chain.\(^{33}\) In the case presented here, the substitution of \(^{45}\)Asn by \(^{45}\)Ser in the leucine-rich domain of GPIX, presumably by changing the conformation of the protein, is sufficient to disturb the association of the GPIb-IX-V complex. However, GPIbα, GPIbβ, and GPV are still
coordinately expressed even in the presence of a strongly decreased and nonfunctional GPIX. This probably implies that GPIX is not essential for complex formation of GPIbα and GPIbβ with GPV, but is nevertheless important for the efficient expression of the total complex at the platelet surface. The role of GPV remains unclear, but in a recent study,24 transfection of GPV into HEL cells that express low levels of GPIb-IX but no GPV did not affect levels of GPIb-IX expression. This might suggest that levels of GPV expression are normally controlled by the levels of GPIb rather than the reverse. Although the data presented here together with those of Wright et al22 clearly associate the Asn to Ser mutation with the Bernard-Soulier syndrome phenotype, it will require the transfection of this mutant form of GPIX into cultured cells already expressing GPIb (α and β) to determine whether this is indeed the case. Because, unlike GPIbα, there is so far no function directly linked to GPIX itself, only the effects of the mutant versus normal GPIX on levels of expression of GPIb can be used as an assay. The establishment of controls in such an experiment will not be trivial.

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Variant Bernard-Soulier syndrome associated with a homozygous mutation in the leucine-rich domain of glycoprotein IX

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