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Research Article

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Variant Bernard-Soulier Syndrome Type Bolzano

A Congenital Bleeding Disorder Due to a Structural and Functional Abnormality of the Platelet Glycoprotein Ib-IX Complex

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Abstract

We have studied a patient with a congenital bleeding disorder and phenotypic manifestations typical of Bernard-Soulier syndrome, including giant platelets with absent ristocetin-induced von Willebrand factor binding. Two monoclonal antibodies reacting with distinct epitopes in the amino-terminal domain of the α -chain of glycoprotein (GP) Ib were used to estimate the number of GP Ib molecules on the platelet membrane. In the patient, binding of one antibody (LJ-Ib10) was $\sim 50\%$ of normal, while binding of the other (LJ-Ib1) was absent. Binding of both antibodies was reduced to $\sim 50\%$ of normal in the mother and one sister of the propositus, and their platelets exhibited $\sim 70\%$ of normal von Willebrand factor binding. Immunoblotting studies confirmed the presence of GP Ib α , as well as GP IX, in patient platelets. Antibody LJ-Ib10, but not LJ-Ib1, could immunoprecipitate the patient's GP Ib α from surface-labeled proteins. Thus, platelets from the propositus contained a structurally and functionally altered GP Ib-IX complex lacking a specific antibody epitope and the ability to bind von Willebrand factor. In contrast, the binding of human α -thrombin to the patient's platelets was normal, and three classes of binding sites with high, intermediate, and low affinity could be detected. These studies define a distinct variant form of Bernard-Soulier syndrome and provide evidence, based on a naturally occurring mutant molecule, that the amino-terminal region of GP Ib α contains a von Willebrand factor-binding domain distinct from the high affinity thrombin-binding site. Use of different monoclonal antibodies with distinct epitope specificities appears to be essential for a correct identification of variant Bernard-Soulier syndrome. (*J. Clin. Invest.* 1990. 86:25-31.)

Key words: Bernard-Soulier syndrome • platelet glycoproteins • von Willebrand factor • thrombin

Introduction

In the rare congenital bleeding syndrome first described by Bernard and Soulier (1), platelets are characterized by the pronounced decrease or absence of the glycoprotein (GP)¹ Ib-IX complex (2, 3), a major membrane component that serves as one of the two known binding sites for vWf (4) as well as a high affinity binding site for thrombin (5). GP Ib, with an apparent molecular mass of 170 kD, is the major sialylated glycoprotein of the platelet membrane and consists of two disulfide-linked subunits, GP Ib α (145 kD) and GP Ib β (22 kD) (6). GP IX, with an apparent molecular mass of 18 kD, is a single-chain molecule and is linked to GP Ib in a noncovalent complex (7). The primary structures of all three components of the complex have been determined (8-11). Patients with Bernard-Soulier syndrome typically show moderate to severe thrombocytopenia, with the presence of enlarged platelets on peripheral blood smears. These platelets show multiple functional defects, including altered response to thrombin stimulation (12, 13) and inability to bind vWf (14-16). As a consequence, platelets fail to adhere to damaged vascular structures and show abnormal thrombus formation at sites of vascular injury (14).

Bernard-Soulier syndrome has been shown to be heterogeneous in its phenotypic manifestations (17), but structural and functional abnormalities of the GP Ib-IX complex have been poorly characterized to date. In the present communication we describe a patient whose platelets contained a dysfunctional GP Ib molecule that failed to bind vWf but expressed normal interaction with thrombin. In addition to the defective vWf-binding capacity, the platelets of this patient failed to express the epitope of a monoclonal anti-GP Ib antibody that has previously been shown to react with the amino-terminal region of the α -chain (18). These findings, while clearly defining a distinct variant form of Bernard-Soulier syndrome, provide evidence based on a naturally occurring mutant molecule that elements essential for vWf binding reside in the amino-terminal domain of GP Ib α , as previously suggested using other experimental approaches (19, 20), and are distinct from the thrombin-binding site. Moreover, they demonstrate that the correct identification of variant Bernard-Soulier syndrome may require the use of monoclonal anti-GP Ib antibodies of distinct epitope specificity.

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1. Abbreviations used in this paper: GP, glycoprotein.

Methods

Blood samples were obtained from patients and normal volunteers with their informed consent and in accordance with the Declaration of Helsinki. They had taken no medication for at least 2 wk. Bleeding time and platelet count were performed as previously described (21). Platelet-rich plasma for binding studies was prepared from blood collected into one-tenth final volume of 0.11 M trisodium citrate anticoagulant containing 50 mM disodium EDTA and 200 U/ml (kallikrein inhibitor units) of aprotinin (Sigma Chemical Co., St. Louis, MO). For selected experiments, the anticoagulant used also contained 600 μ g/ml of soybean trypsin inhibitor (Sigma Chemical Co.). Platelet-rich plasma was prepared by collecting the supernatant at the end of each of two successive centrifugations performed at low speed (70 g for 6 min) to avoid excessive sedimentation of the large platelets in the patient's blood.

Platelets for immunoblotting analysis were washed twice by differential centrifugation in modified calcium-free Tyrode buffer, pH 7.35, containing 200 μ g/ml leupeptin, 10 mM EDTA, and 20 U/ml aprotinin. Solubilization of membrane proteins was obtained by resuspending washed platelets in a buffer composed of 20 mM Tris, 150 mM NaCl, pH 7.4 (Tris buffer), containing 10 mM EDTA, 5 mM *N*-ethylmaleimide, 1 mM PMSF, and 2% SDS. Washed platelets to be used for vWf- and thrombin-binding studies were prepared from blood drawn into one-sixth final volume of acid/citrate/dextrose, pH 4.5, and washed free of plasma constituents by the albumin density gradient technique of Walsh et al. (22), with minor modifications (23). Washed platelets to be used for surface labeling of membrane glycoproteins were prepared from blood drawn in acid/citrate/dextrose containing soybean trypsin inhibitor (250 μ g/ml) and EDTA (10 mM). Platelets were washed three times by differential centrifugation and resuspension in Tris buffer containing 1 mM EDTA, 25 μ g/ml soybean trypsin inhibitor, and 20 nM prostaglandin E_1 (Sigma Chemical Co.); only EDTA was added to the buffer in the final suspension.

Lactoperoxidase-catalyzed iodination of membrane proteins was achieved by mixing the platelet suspension (containing 2×10^9 platelets for the normal control and 0.9×10^9 platelets for the propositus) with 0.5 mg of lactoperoxidase (Worthington Biochemical Corp., Freehold, NJ) and 2.5 mCi of $Na^{125}I$ (Amersham Corp., Arlington Heights, IL) in a final volume of 1 ml. Three successive aliquots of H_2O_2 , each consisting of 25 μ l of a 1:200 dilution freshly prepared from a 35% stock solution, were then added every 3 min to the platelet suspension kept on melting ice. The reaction was stopped by the addition of 5 ml of Tris buffer containing 1 mM EDTA. The labeled platelets were then washed three times using the same buffer. After the final centrifugation the platelet pellet was lysed in a buffer composed of 20 mM Tris, 150 mM NaCl, pH 8, containing 1% Nonidet P-40 (Eastman Kodak Co., Rochester, NY), 1 mM PMSF, 200 U/ml aprotinin, 100 μ g/ml soybean trypsin inhibitor, and 1 mM EDTA.

Immunoprecipitation of radiolabeled membrane proteins was performed after preclearing the platelet lysate by mixing for 2 h at 4°C with one-eighth volume of protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) resuspended in Tris buffer, pH 8. The supernatant of this mixture was then incubated with the anti-GP Ib monoclonal antibodies (see below) added at a final concentration of 1 mg/ml. After mixing for 18 h at 4°C the immunocomplexes were precipitated by adding one-tenth volume of protein A-Sepharose beads and mixing for 2 h at 4°C. The beads were then washed four times using Tris buffer, pH 8, containing 1 mM EDTA and 0.05% Nonidet P-40. The washed beads were then mixed with electrophoresis sample buffer (24) to give a final concentration of 2% SDS and 20 mM dithiothreitol. After incubation in boiling water for 5 min the samples were centrifuged at 12,000 g for 4 min and then applied to a 12% polyacrylamide gel prepared according to Laemmli (24). After electrophoresis the gels were stained with Coomassie blue and dried, and an autoradiograph was obtained.

The anti-GP Ib monoclonal antibodies used in this study (LJ-Ib1 and LJ-Ib10) were prepared and characterized as published in detail elsewhere (18). Both react with the α -chain of GP Ib and recognize distinct epitopes (8, 18). The murine monoclonal antibody LJ-RA8 (specific for GP IX; see Results) was obtained and selected according to a published procedure (18) after immunization with reduced and alkylated GP Ib-IX complex prepared as reported (18). Purified IgG was obtained from mouse ascitic fluid using staphylococcal protein A coupled to Sepharose 4B-CL (Sigma Chemical Co.) or DEAE-AffiGel blue chromatography (Bio-Rad Laboratories, Richmond, CA) as described previously (25, 26). Immunoblotting studies were performed following published methods (18, 27); the monoclonal antibodies used were LJ-Ib10 and LJ-RA8, while LJ-Ib1 failed to react with transblotted protein.

The method used for the purification of vWf from cryoprecipitate has been published in detail elsewhere (28). Characterization of the purified vWf was performed as previously described (23). Purified human α -thrombin was a generous gift of Dr. John W. Fenton II (Griffin Laboratories, New York State Department of Health, Albany, NY). Proteins were iodinated with ^{125}I (Amersham Corp.) using Iodogen (Pierce Chemical Co., Rockford, IL) according to the procedure of Fraker and Speck (29). The methods used to measure the binding of monoclonal IgG and vWf (ristocetin-dependent binding) to platelets have been previously described in detail (4, 18). The binding of α -thrombin was measured according to the method of Harmon and Jamieson (5). Binding parameters were calculated using the computer-assisted program LIGAND (30) assuming molecular masses for monoclonal antibodies of 170 kD and for vWf of 275 kD. Nonspecific (nonsaturable) binding was calculated as the ratio of bound over free at infinite free ligand concentration (30). For all the curves analyzed in the course of these studies, the best fit of experimental data was obtained without a nonsaturable component in the total binding isotherm; thus, nonspecific binding was assumed to be 0 for all curves.

Flow cytometric analysis was performed on platelet-rich plasma diluted with homologous platelet-poor plasma to obtain a platelet count of 5×10^7 /ml. 100 μ l of the platelet suspension was then incubated with increasing concentrations of monoclonal antibody LJ-Ib10 for 45 min at 37°C. Goat anti-mouse IgG conjugated to FITC (Ortho Pharmaceutical, Raritan, NJ) was then added and incubated at 37°C for 45 min. Fluorescent cells were analyzed using a Spectrum III flow cytometer (Ortho Pharmaceutical) after further dilution of the sample (1:10) in a buffer composed of 20 mM sodium phosphate and 150 mM sodium chloride, pH 7.3. In selected experiments the blood used for flow cytometric analysis was drawn into anticoagulant containing soybean trypsin inhibitor (see above).

Results

Case report. The propositus (D.V.G.) had been previously described as having classical Bernard-Soulier syndrome, a conclusion based on the results of platelet-binding studies performed with monoclonal antibody AP-1. According to these previous findings, antibody binding was absent in the propositus and decreased to $\sim 50\%$ of normal in the parents and four sisters, who were considered to be heterozygotes (21).

Abnormal expression of a GP Iba epitope in the propositus. Two monoclonal antibodies, LJ-Ib1 and LJ-Ib10, which recognize distinct epitopes in the α -chain of GP Ib (18), were used for these experiments. In particular, LJ-Ib1 reacts with a conformational epitope present only in native GP Ib and located within the first 293 amino-terminal residues (8, 18); it completely inhibits vWf binding to GP Ib (18), but has no effect on thrombin binding (unpublished observation). LJ-Ib10 reacts with an epitope present in native as well as denatured, reduced,

and S-carboxymethylated α -chain, and located between residues Ala²³⁸ and Arg²⁹³ (8, 18); it has no effect on ristocetin-mediated vWf binding to platelets (18), but inhibits thrombin binding (unpublished observation). The interaction of both antibodies with patient platelets was decreased, but the degree of abnormality varied considerably depending on the antibody used. There was no detectable binding of LJ-Ib1, whereas binding of LJ-Ib10 corresponded to $\sim 50\%$ of normal (Table I). Similar results were also observed when studies were performed immediately after collection of blood in anticoagulant containing aprotinin, EDTA, and soybean trypsin inhibitor (Table I). Without these precautions, a decrease of 15–20% in the binding of monoclonal anti-GP Ib antibodies to normal platelets may occur within 2–3 h from blood collection. In contrast to the findings in the propositus, both antibodies bound concordantly to platelets obtained from the mother and one sister of the patient, and the number of molecules bound corresponded to $\sim 50\%$ of normal (Table I). With both antibodies, the dissociation constants calculated for binding to platelets from the propositus or his relatives were in close agreement with those calculated for normal platelets (Table I).

Monoclonal antibody LJ-Ib10 reacted with normal GP Ib α -chain blotted onto nitrocellulose after polyacrylamide gel electrophoresis in the presence of SDS (Fig. 1), and was thus used to study the electrophoretic mobility of the patient GP Ib α -chain. Two bands could be detected. One, with an apparent molecular mass of 145 kD, was similar to normal GP-Ib α ; the other, with a molecular mass of 105 kD, corresponded to a band seen in normal platelets only when a high concentration of membrane proteins was applied to the gels (Fig. 1). The pattern seen in platelets from the patient's mother and sister

Table I. Binding of Anti-GP Ib Monoclonal Antibodies to Platelets

	LJ-Ib1	LJ-Ib10
D.V.G. (propositus)		
B_{\max}	0 (0)	11,283 (7,625)
K_d	0 (0)	2.7 (3.93)
L.E. (mother)		
B_{\max}	8,424	7,850
K_d	2.5	2.5
D.V.C. (sister)		
B_{\max}	11,965	10,631
K_d	3.3	2.0
Normal		
B_{\max}	19,564 (26,200)	20,041 (22,044)
K_d	2.2 (6.1)	1.5 (3.4)

Citrated platelet-rich plasma (final platelet count, $1 \times 10^8/\text{ml}$) was incubated for 30 min at room temperature with increasing concentrations of radiolabeled monoclonal IgG (2.9–46 $\mu\text{g}/\text{ml}$ final concentration). Separation of bound from free ligand was obtained by centrifuging the platelets through 20% sucrose. Binding parameters were calculated with the computer-assisted program LIGAND (30). B_{\max} indicates the number of molecules per platelet bound at saturation; K_d is the dissociation constant expressed in moles per liter, and the values reported must be multiplied by 10^{-8} . The values reported in parentheses for D.V.G. and normal represent the results of one experiment performed in the presence of soybean trypsin inhibitor (see Methods).

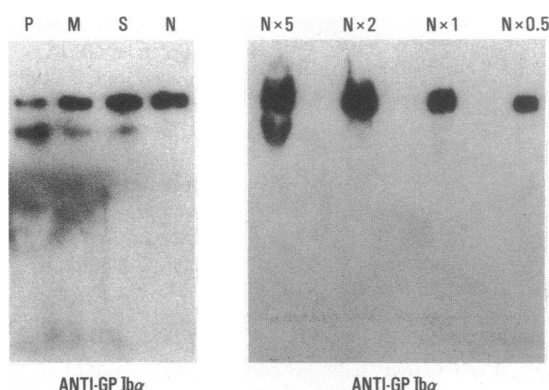


Figure 1. Immunoblotting of platelet GP Ib. Membrane glycoproteins were solubilized by treating washed platelets with 2% SDS in Tris buffer, pH 7.4, containing 10 mM EDTA, 5 mM N-ethylmaleimide, and 1 mM PMSF. After reduction of disulfide bonds with 20 mM dithiothreitol, reduced lysates (corresponding to 7×10^7 platelets/lane) were analyzed by 7.5% SDS-PAGE using the discontinuous buffer system of Laemmli (24), followed by electrophoretic transfer onto nitrocellulose paper and immunoblot analysis using monoclonal antibody LJ-Ib10. Positive bands were visualized by incubation with rabbit anti-mouse IgG labeled with ^{125}I followed by autoradiography. Left, Results observed in the propositus (P), his mother (M) and sister (S), and a normal control (N). Right, Results observed when normal platelets were analyzed at different concentrations: $N \times 1$, the lane containing the same concentration used for the samples in the left panel (7×10^7 platelets/lane); the other lanes contained half, double, or five times that amount, as indicated. The band seen in all normal samples corresponds to intact GP Ib α (molecular mass 145 kD). Note that a band of faster mobility, corresponding to a molecular mass of 105 kD, is visible in normal only when the sample is overloaded ($N \times 5$). The propositus shows a relative increase of the smaller band relative to intact GP Ib α . The smaller band is also evident in the mother and sister of the propositus.

was similar, although relatively smaller amounts of the 105-kD bands were seen. Immunoblotting was also performed with monoclonal antibody LJ-RA8, which recognizes an epitope located on GP IX (Fig. 2). One band was seen with platelets from the propositus and his mother, showing a mobility similar to that of normal GP IX (Fig. 2).

Immunoprecipitation of surface-labeled glycoproteins demonstrated abnormalities in agreement with those seen in immunoblotting studies. With patient platelets, antibody LJ-Ib10 precipitated two bands of similar intensity with apparent molecular masses of 145 and 105 kD, respectively; with normal platelets, only the band at 145 kD was distinctly evident (Fig. 3). An additional band with an apparent molecular mass of 38 kD was seen in the sample from the propositus but not in normal, while another minor band with an apparent molecular mass of 70 kD in normal showed decreased intensity and faster mobility (apparent molecular mass of 66 kD) in the propositus (Fig. 3). The exact nature of these two polypeptides, as well as of two bands larger than reduced GP Ib α (Fig. 3), remains to be identified. Immunoprecipitation with antibody LJ-Ib1 gave results identical to LJ-Ib10 in normal platelets, whereas no detectable immunoprecipitates were observed with platelets from the propositus (not shown).

Flow cytometric analysis was performed using saturating concentrations of monoclonal antibody LJ-Ib1 and LJ-Ib10.

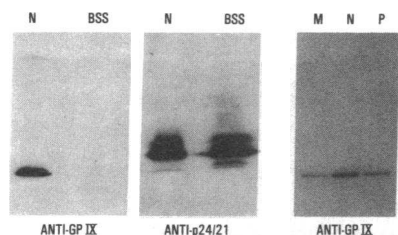


Figure 2. Immunoblotting of platelet GP IX. The procedure followed for this experiment was similar to that described in the legend to Fig. 1, except that the samples were analyzed without reduction of disulfide bonds, and the mono-

clonal antibody used (LJ-RA8) was specific for GP IX. Evidence for this specificity is shown in the left panel, where platelets from a patient (B.L., reference 21) with typical Bernard-Soulier syndrome (BSS) exhibit lack of reactivity with this antibody, whereas normal platelets exhibit a reactive band with an apparent molecular mass of 18 kD. An antibody against a group of related platelet membrane proteins similar in size to GP IX (anti-p24/21; this antigen is also known as CD9) was used to demonstrate the efficacy of electrophoretic transfer of membrane proteins of Bernard-Soulier platelets. The right panel shows that platelets from the propositus described in this study (P), as well as his mother (M) and a normal control (N), contain GP IX since they react with antibody LJ-RA8.

With platelets from the propositus, antibody LJ-Ib1 demonstrated a negative GP Ib phenotype similar to that seen in another patient previously diagnosed as a case of classical Bernard-Soulier syndrome (patient B.L., reference 21). In contrast, antibody LJ-Ib10 demonstrated one population of platelets with the GP Ib positive phenotype in the propositus, but gave a negative phenotype in the patient with classical Bernard-Soulier syndrome.

Binding of vWf and α -thrombin to platelets. Binding of 125 I-vWf to washed platelets in the presence of ristocetin (0.8 mg/ml) was undetectable in the propositus, while it was decreased to $\sim 70\%$ of normal in his mother and sister (Table II). In contrast, binding isotherms obtained with 125 I-labeled α -thrombin demonstrated that platelets from the propositus, like normal platelets, expressed three classes of binding sites with high, moderate, and low affinity (Table III). This was in contrast to the results obtained with platelets from the patient with classical Bernard-Soulier syndrome (patient B.L., reference 21), which failed to express high affinity binding sites (Table III).

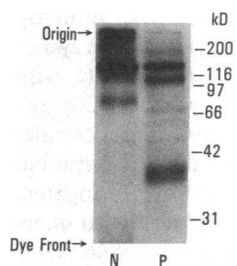


Figure 3. Immunoprecipitation of surface-labeled platelet glycoproteins with monoclonal anti-GP Ib antibody. Membrane glycoproteins labeled with 125 I were immunoprecipitated from either normal platelets (N) or platelets from the propositus (P) using the anti-GP Ib monoclonal antibody LJ-Ib10. Reduced samples were electrophoresed in a 12% polyacrylamide gel in the presence of SDS using the discontinuous buffer system of

Laemmli (24), and radioactive bands were visualized by autoradiography. The band at the top of the gel (Origin) is located at the interface between stacking and running gel, while the arrow at the bottom of the gel indicates the dye front. The cathode is at the top. Normal GP Ib α corresponds to the prominent band seen in the control (left lane) above the 116-kD mark. The abnormal 105-kD species can be seen in the propositus (right lane) just below this mark.

Table II. Binding of 125 I-labeled vWf to Platelets

	B_{max}	K_d
D.V.G. (propositus)	0	0
L.E. (mother)	22,231	0.57
D.V.C. (sister)	24,289	0.41
Normal	33,761	0.42

Washed platelets were incubated at room temperature with increasing concentrations of 125 I-vWf (1.6–25.6 μ g/ml final concentration) in the presence of ristocetin (0.8 mg/ml final concentration). The assay was performed as previously described in detail (4). Binding parameters were calculated with the computer-assisted program LIGAND (30). B_{max} indicates the number of molecules per platelet bound at saturation; K_d is the dissociation constant expressed in moles per liter, and the values reported must be multiplied by 10^{-8} .

Discussion

The propositus described in the present report was previously identified as a case of Bernard-Soulier syndrome with typical phenotypic expression (21). The new results introduced here nevertheless demonstrate that he is clearly distinct from any other patient reported in the literature. In fact, GP Ib could be detected on the membrane of his platelets, albeit abnormal in its structure and function, whereas this glycoprotein is absent in individuals with typical Bernard-Soulier syndrome. The distinct defects of this mutant GP Ib molecule include failure to express vWf-binding function, as well as loss of the epitope of a monoclonal antibody (LJ-Ib1) that is known to react with the amino-terminal region of GP Ib α and that inhibits the vWf-platelet interaction when bound to normal platelets (18). In contrast, platelets from this propositus exhibited normal interaction with α -thrombin and with another anti-GP Ib an-

Table III. Binding of 125 I-labeled α -Thrombin to Platelets

	Normal	B.L.	D.V.G.
K_d			
H ($\times 10^{-10}$)	2.5–13	0	7.5
M ($\times 10^{-8}$)	3.0–7.7	0.34	1.1
L ($\times 10^{-7}$)	4.0–24	12	4.9
B_{max}			
H	103–344	0	448
M	3,422–8,200	1,818	1,656
L	30,327–225,863	95,239	85,000

Binding parameters for the thrombin-platelet interaction were calculated with the computer-assisted program LIGAND (30) and expressed as dissociation constant (K_d , in moles per liter) and number of molecules bound per platelet at saturation (B_{max}) for three classes of binding sites with high (H), moderate (M), and low (L) affinity. The results shown for the normal control represent the range of eight separate experiments. Patient B.L. was previously diagnosed as a typical case of Bernard-Soulier syndrome (21). Note the normal number of high affinity binding sites in the propositus, in contrast to their absence in patient B.L. Note also that both Bernard-Soulier patients show a decrease in the number of moderate affinity binding sites. The reasons for this finding are presently unexplained.

tibody, LJ-Ib10, which has no inhibitory effect on ristocetin-mediated vWf binding (18) but is known to inhibit the high affinity thrombin-binding sites on normal platelets (De Marco, L., M. Mazzucato, and Z. M. Ruggeri, manuscript in preparation). The present results, therefore, support with the example of a naturally occurring mutant the concept that the amino-terminal region of GP Ib α contains two domains essential for vWf binding (19, 20) and high affinity thrombin binding (5), each distinctly related to the epitopes of antibodies LJ-Ib1 and LJ-Ib10, respectively. Only the vWf-binding domain is structurally and functionally altered in the patient described here, who exhibits a clear dissociation of two typical abnormalities of patients with Bernard-Soulier syndrome, namely, the defective interaction with vWf and thrombin (12–16).

The availability of specific anti-GP Ib monoclonal antibodies has led to the recommendation of their use for the diagnosis of Bernard-Soulier syndrome (31). The present results clearly show that, depending on the epitope specificity of the antibodies used, there is the possibility of reaching significantly different conclusions as to the presence and functionality of platelet GP Ib. For example, this same propositus was previously identified as a typical Bernard-Soulier patient with absent platelet GP Ib (21) as judged by lack of platelet reactivity with antibody AP-1, a well-characterized anti-GP Ib antibody that inhibits vWf interaction with platelets (31). In this regard, the results previously observed with AP-1 are identical to those obtained in the course of these studies with LJ-Ib1. Nevertheless, the presence of GP Ib in the platelets of this patient has now been demonstrated using another antibody that reacts with an epitope not directly related to the vWf binding site. These findings provide an important example that, when using immunological methods of detection, it is necessary to perform studies with antibodies reacting with different epitopes before reaching conclusions about the presence or absence of a certain antigen. In fact, the results obtained here with regard to platelet GP Ib are likely to apply to any other membrane glycoprotein. This may be particularly important in the case of platelets deficient in a certain function, like vWf binding in the case presented here, when the antibodies used inhibit the same function in normal platelets. In fact, these antibodies may fail to detect abnormal molecules when the corresponding epitope is closely related to the altered functional domain.

The exact nature of the structural abnormality and underlying genetic defect of the mutant GP Ib molecule described here remain unknown at present. As a matter of fact, even though the defective functional domain and corresponding LJ-Ib1 epitope have both been located in the α -chain of GP Ib (18–20), it remains possible, although unlikely, that other components of the complex, like the β -chain of GP Ib or GP IX, may themselves be abnormal and responsible for the defect in GP Ib α through conformational effects. Nevertheless, the presence in the patient's platelets of immunoreactive GP Ib α of heterogeneous molecular mass, with 145- and 105-kD species, supports the concept of an intrinsic defect in this glycoprotein. The origin of the smaller GP Ib α molecule remains undefined at present, but the findings presented here are consistent with the concept that it is proteolytically derived from the normal-sized species. In fact, both molecules must share the same structural abnormality since neither of them reacts

with antibody LJ-Ib1. Moreover, both species are immunoprecipitated by antibody LJ-Ib10 from surface-labeled platelets; thus, they are both membrane expressed. It is conceivable, therefore, that the structural abnormality responsible for the altered vWf-binding function and the loss of the LJ-Ib1 epitope determines an increased susceptibility to proteolysis at a specific site in the amino-terminal domain of GP Ib α , with cleavage of a fragment of ~ 40 kD. The existence of a protease-sensitive region in GP Ib α is, indeed, suggested by the observation that small amounts of a cleaved 100-kD species have been found in purified preparations of normal GP Ib-IX complex (32), in agreement with findings reported here (Fig. 1). Interestingly, a fragment of ~ 40 kD could be immunoprecipitated by LJ-Ib10 from surface-labeled proteins of patient platelets. This fragment may represent the amino-terminal portion of GP Ib α , which may remain linked to the rest of the molecule after cleavage because of the existence of intrachain disulfide bonds in that region of the α -chain (8, 19, 20). This hypothesis will have to be tested with future experiments. While proteolysis *in vitro* is difficult to rule out in spite of the use of a mixture of protease inhibitors, it is likely that generation of the smaller GP Ib α species is an *in vivo* proteolytic event on a structurally abnormal mutant molecule. Other possible explanations for the presence of the smaller GP Ib α species, like incomplete glycosylation or synthesis directed by an abnormal mRNA, cannot be ruled out at present, but appear less likely in view of the fact that both GP Ib α species detected in the patient's platelets share the same antigenic defect.

The modality of genetic transmission of this new variant form of Bernard-Soulier syndrome remains unresolved. Typical patients show multiple defects of platelet membrane glycoproteins (2, 11) and lack, among others, three proteins (GP Ib α , GP Ib β , and GP IX) that are the product of distinct genes (9–11) and form a complex in normal platelets (7). This may be the consequence of a single genetic lesion if one postulates that absence of one component of the complex may result in rapid degradation of the others, a situation similar to that proposed as an explanation for the dual glycoprotein defect in Glanzmann thrombasthenia (33–36). The situation in this family appears uniquely different, since the propositus exhibited decreased levels of structurally altered GP Ib α , while two relatives available for study had decreased levels of an apparently normal molecule. A simple explanation for this phenotypic pattern is that the patient may have inherited two different altered alleles from his parents. The phenotype in the mother is compatible with a heterozygous state characterized by the presence of one normal and one null allele, leading to decreased expression of an otherwise normal molecule. The father could be heterozygous with one normal allele and one abnormal allele responsible for the structural defect. This would predict that the father has normal GP Ib α expression as measured by LJ-Ib10, but only 50% as measured by LJ-Ib1, a fact that could not be verified experimentally since the father was not available for additional studies. In the offspring, then, the daughter would have inherited the null maternal allele and the normal paternal allele, thus exhibiting the same phenotype as the mother. The propositus, on the other hand, would have inherited the null maternal allele and the abnormal paternal allele, thus exhibiting decreased expression of a structurally and functionally abnormal molecule.

In contrast with this hypothesis is the finding that the smaller GP Ib α species (the marker of structural abnormality in the patient) could be detected in both mother and sister of the propositus, as shown in Fig. 1, although in lower relative concentration than in the propositus himself. If this is taken as evidence for the presence of abnormal GP Ib α , then both mother and sister may be heterozygous for the same genetic defect existing in the patient. The presence of functionally abnormal GP Ib α was not predictable on the basis of the results of binding studies with monoclonal antibodies and vWf, but it is difficult to anticipate how much of the abnormal molecule would be incorporated into the GP Ib-IX complex in the presence of normal GP Ib α synthesized by the normal allele. Thus, the propositus may have inherited two alleles with the same genetic lesion from his parents, who, although not apparently consanguineous, belong to a community originating from Sicily and now living in northeastern Italy. A definitive clarification of these points cannot be obtained until the nature of the molecular abnormality present in this family becomes known.

In conclusion, we have identified a variant form of Bernard-Soulier syndrome characterized by the presence in platelets of a structurally and functionally abnormal GP Ib molecule. The results observed in this case provide information that may aid in the use of monoclonal antibodies for diagnosing patients with congenital platelet defects. Moreover, ongoing studies aimed at defining in detail the molecular and genetic defects present in this patient should prove important for understanding function and structure of the GP Ib-IX complex. This variant form of Bernard-Soulier syndrome has been identified as type Bolzano, from the patient's city of origin in Italy.

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