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Research Article

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Characterization of the Platelet Membrane Glycoprotein Abnormalities in Bernard-Soulier Syndrome and Comparison with Normal by Surface-labeling Techniques and High-resolution Two-dimensional Gel Electrophoresis

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ABSTRACT The platelets from three patients with Bernard-Soulier syndrome have been analyzed by surface-labeling coupled with two-dimensional gel electrophoresis and compared with normals. As well as the previously described absence or deficiency in glycoprotein (GP) Ib(α) it could be shown that GP Ib β and an additional low molecular weight glycoprotein GP₁₇^{5,8-6.5} were not detectable using carbohydrate-labeling methods or deficient to the same extent as the GPIb α subunit. In addition, the thrombin cleavable glycoprotein could not be detected using carbohydrate-labeling methods in two patients and was deficient in a third. This finding was confirmed in a fourth patient by one-dimensional gel electrophoresis. Thus, the changes in the membrane of Bernard-Soulier platelets are more complex than previously thought.

INTRODUCTION

Bernard-Soulier syndrome is an inherited bleeding disorder characterized by reduced adherence of the platelets to subendothelium (1), the presence of "giant" platelets in blood smears (2) and a reduced platelet survival time in circulation (3) resulting in thrombocytopenia. Bernard-Soulier platelets aggregate nor-

mally with collagen, ADP, epinephrine and arachidonic acid (4) but do not aggregate with ristocetin/FVIII:WF (5) or bovine von Willebrand factor (4). Aggregation to, and binding of, thrombin is reduced compared to normal platelets (6, 7).

Biochemical studies on Bernard-Soulier platelets first showed a reduction in sialic acid content (3) and later a deficiency in the membrane glycoprotein Ib was demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with (8) or without (9) surface-labeling of platelets and has been confirmed in several additional studies (10-13) including crossed immunoelectrophoresis (14). Glycocalicin, a readily solubilized platelet glycoprotein derived from glycoprotein Ib was also shown to be decreased in Bernard-Soulier syndrome (10, 11).

Unlike Glanzmann's thrombasthenia, another platelet disorder, involving principally glycoproteins IIb and IIIa, where considerable heterogeneity has been reported (14), little is known about heterogeneity and its relation to clinical symptoms in Bernard-Soulier syndrome, due largely to the rareness of this disorder. It has not yet been established if the genetically determined abnormality of the membrane of Bernard-Soulier platelets is restricted to glycoprotein Ib or if other membrane components are affected. A report has appeared that there are changes in glycosylation in Bernard-Soulier platelets compared with normal (15). Glycoprotein Ib consists of two subunits, α and β , linked by at least one disulphide bond (16). Since previous studies had been carried out on relatively low concentration polyacrylamide gels in order to examine the major glycoproteins, nothing was known about the

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expression of the β -subunit in Bernard-Soulier syndrome.

To examine the question of heterogeneity in Bernard-Soulier syndrome and to see if glycoprotein Ib is the only glycoprotein affected in this disorder, we have surface-labeled Bernard-Soulier platelets and analyzed these by high-resolution two-dimensional polyacrylamide gel electrophoresis.

METHODS

Four patients fulfilling the diagnostic criteria of Bernard-Soulier syndrome were studied. All four had experienced bleeding episodes and had increased bleeding times but have led reasonably normal lives since being under medical control. Patient V.S. (female, 28 yr old) from Switzerland had a bleeding time slightly longer than normal of 8 min (normals 1.5–6 min) and a decreased platelet count of 39,000–72,000/ μ l. The Factor VIII complex components were normal or increased, VIII:C, 100%; VIII:R:WF, 100%; VIII:Ag, 170%. Aggregation of platelets to ADP, collagen and epinephrine were normal. Platelets did not aggregate to ristocetin at 1.5 mg/ml. The peripheral blood smear showed typical giant platelets.

Patient F.S. (female, 14 yr old) from France had a bleeding time in excess of 30 min and had had thrombocytopenic episodes that were eventually treated by splenectomy. Since the platelet counts have been within the normal range. She also had typical giant platelets in the peripheral blood smear (4.51 μ m vs. 2.9–3.5 μ m Diam for normals). Factor VIII complex components were elevated; VIII:C, 248%; VIII:R:WF, 145%; VIII:Ag, 555%. Platelets aggregated normally to ADP, collagen, and epinephrine but failed to aggregate to ristocetin at 1.2, 1.4, and 2.0 mg/ml.

Patient E.H. (male, 8 yr old) from France had a decreased platelet count and a bleeding time in excess of 30 min. In the peripheral blood smear the platelets were only slightly larger than normal. Platelets from this patient responded normally to ADP and collagen but showed a decreased response to thrombin. The initial slope of aggregation was 40% of the value obtained with normal platelets at the same platelet concentration and the maximum aggregation was 63% of the value obtained with normal platelets. No response was found to ristocetin at 1.2 mg/ml but at 1.4 and 3.0 mg/ml ~20% of the normal response was obtained. The initial slope of aggregation was 20% of the value obtained with normal platelets at the same platelet concentration and the maximum aggregation was 15% of the value obtained with normal platelets. Factor VIII complex was as follows: VIII:C, 140%; VIII:R:Ag, 100%; VIII:R:WF, 77%.

Patient M.S. has been previously described (13).

Human blood platelets. The study was conducted according to the principles embodied in the Declaration of Helsinki. The isolation of a homogeneous preparation of Bernard-Soulier syndrome platelets was performed as previously described by Jamieson and Okumura (7), with minor modifications. Blood (70–80 ml) was drawn from informed, consenting patients or normal donors, collected in 10-ml tubes containing 1.4 ml acid-citrate-dextrose, 10 mM EDTA anticoagulant, and allowed to sediment (tubes tilted at an angle of 45°, at room temperature (20°C), for 4 h. The platelet-rich plasma was then gently removed and diluted with an equal volume of Hanks' buffered saline containing 10 mM EDTA and no calcium or magnesium. The diluted platelet-rich plasma (5 ml) was then layered on top of 4 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden)

in 10 ml plastic or siliconized glass tubes and centrifuged at 800 g for 10 min. Erythrocytes and granulocytes sedimented to the bottom of the tube, while platelets and contaminating lymphocytes were present as a narrow band at the interface.

The interface was removed, mixed with 0.012 M sodium citrate, 0.03 M glucose, 0.12 M NaCl pH 6.5 containing 10 mM EDTA to a volume of 8 ml and centrifuged at 80 g for 5 min. Supernatant and pellet were examined by phase-contrast microscopy or by bright field using a differential Wright stain. The supernatant was then centrifuged repeatedly at 100 g for 5 min, discarding the pellets until a homogeneous platelet population was obtained.

Labeling. Platelets from each Bernard-Soulier syndrome patient and a healthy donor were immediately washed after isolation and were labeled in parallel by lactoperoxidase-catalyzed iodination, periodate plus NaB³H₄ or neuraminidase, galactose oxidase plus NaB³H₄ as previously described (8, 13). Isoelectric focusing and SDS-polyacrylamide gel electrophoresis (5–20% acrylamide linear gradient gels) were carried out as described (17). After fixing, the gels containing ¹²⁵I-labeled proteins were washed under running water for 1 h, dried under vacuum and indirect autoradiography was carried out with Kodak X-Omat R film (Eastman Kodak Co., Rochester, NY) and Cronex Lightning Plus intensifier screens (Du Pont & Co., Inc., Wilmington, DE) as previously described (18). Gels containing ³H-labeled glycoproteins were impregnated with PPO, (Packard Instrument, Co., Zürich), dried under vacuum and fluorography carried out with Kodak X-Omat R film (19).

Incubation of labeled normal platelets in Bernard-Soulier or normal blood. Platelets from two normal donors with the same blood group (Al Rh+) as the Bernard-Soulier patients (E.H. and F.S.) were isolated, washed, and surface-labeled by the neuraminidase, galactose oxidase plus NaB³H₄ or periodate plus NaB³H₄ methods. The pellets of labeled platelets were resuspended in platelet poor plasma (3 ml), to which 7 ml of whole blood was added (total volume 10 ml, platelets adjusted to 250,000/ μ l). The platelet poor plasma and whole blood were from the Bernard-Soulier syndrome patients or from the normal donors. The labeled normal platelets resuspended either in Bernard-Soulier patient blood or in blood from the same donor were incubated for 30 min at 37°C, then isolated and washed twice in 0.01 M Tris, 0.005 M EDTA, 0.154 M NaCl, pH 7.4 buffer before solubilization in SDS for SDS-polyacrylamide gel electrophoresis. Labeled normal platelet samples from the two donors were also directly solubilized for gel electrophoresis without incubation in blood.

Immunoprecipitation was carried out by the method of Kessler (20) with some modifications. Platelets (10⁹), surface-labeled by the periodate plus NaB³H₄ method were solubilized in 250 μ l of 0.15 M NaCl, 0.005 M EDTA, 0.5% Nonidet P-40, 0.05 M Tris/HCl pH 7.4, (NET) buffer, followed by centrifugation at 100,000 g for 30 min. The supernatant was divided into two aliquots of 100 μ l that were mixed with 100 μ l of antiglycocalicin IgG (1 mg/ml) (21), or 100 μ l of anti-glycoprotein IIb/IIIa IgG (1.3 mg/ml) (21, 22), respectively, and left for 8 h at 4°C. Two aliquots (2 ml) of a 10% suspension of *Staphylococcus aureus* Cowan I (IgG Sorb, The Enzyme Center, Boston, MA) were centrifuged for 10 min at 6,000 g and the supernatants discarded. The antigen-antibody mixtures were added to the pellets, mixed well, and left for 1 h at 4°C. After centrifugation for 10 min at 6,000 g the supernatants were discarded and the pellets were washed three times with NET buffer. To each of the washed sediments was added 200 μ l of 4% SDS, 4% dithiothreitol in

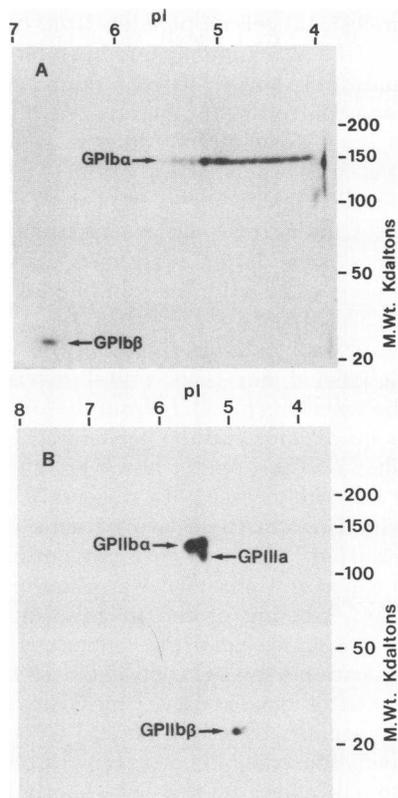


FIGURE 2 Fluorograms of two-dimensional separations under reducing conditions, of immunoprecipitates from normal platelets labeled with the periodate/ NaB^3H_4 technique, solubilized in Nonidet P-40 detergent, after precipitation with: (a) Rabbit anti-glycocalicin IgG. (b) Rabbit anti-glycoproteins IIb/IIIa IgG. The anti-glycocalicin antibodies precipitate GPIIb from platelets that have been washed and labeled in the presence of EDTA to prevent degradation by endogenous calcium-activated proteases (21).

body against GPIIb/IIIa (Fig. 2b) and is absent when Glanzmann's thrombasthenia platelets were analyzed by two-dimensional polyacrylamide gel electrophoresis in the reduced state. Traces of a labeled glycoprotein were found in this position with unreduced normal platelet samples after long exposure times.

All of the glycoproteins not detected with patients V.S. and F.S. could be seen with the platelets from patient E.H. (Fig. 1d). However, they were clearly present in lower amounts than normal. Because the x-ray films used were flashed to increase their sensitivity, the response to light is approximately linear (19), and densitometry of the spots on the film can be used to estimate the quantity of the component present on the platelet surface. Although GPIIb α cannot be easily assessed in this manner because of the proximity of other glycoproteins and also the heterogeneity of GPIIb α in the isoelectric focusing dimension, it was possible to

show that E.H. had $\sim 40 \pm 5\%$ of the normal amounts of GPIIb β and of GP $_{17}^{5.8-6.5}$, using several other glycoproteins, including GPIIb β , as standards. Based on densitometry of one-dimensional gels (not shown) E.H. had $\sim 30 \pm 10\%$ of the normal amount of GPIIb α . The differences in GPV were confirmed on one-dimensional gels run on platelets from patient M.S. (not

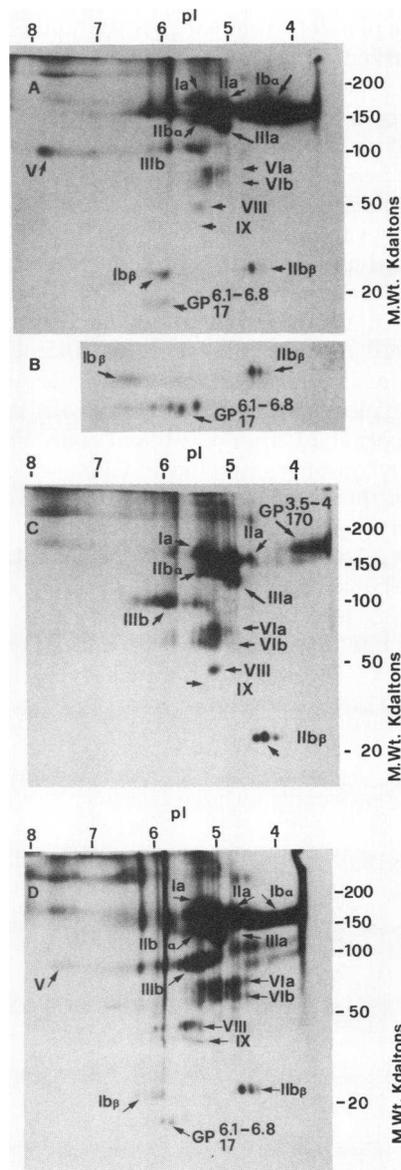


FIGURE 3 Fluorograms of two-dimensional separations, under reducing conditions, of platelets surface-labeled by the neuraminidase/galactose oxidase/ NaB^3H_4 technique. (a) Normal platelets. (b) Lower part of gel with platelets from one normal donor. (c) Platelets from Bernard-Soulier syndrome patient, F.S. (d) Platelets from Bernard-Soulier syndrome patient, E.H.

shown). Differences in the GPIb region have already been described with this patient (13).

One of the normal controls showed a different pattern for GP₁₇^{5.8-6.5} (in the reduced state) compared with other single normals or to normal pool platelets. Instead of the usual two major spots and one minor, four spots were seen with the additional spots more acidic than normal (Fig. 1b).

Neuraminidase + galactose oxidase + NaB³H₄ labeling. The platelets from patients V.S. and F.S. again showed marked differences from normal (Fig. 3). GPIb α was absent or present only in trace amounts. The same two low molecular weight glycoproteins were absent as with periodate/NaB³H₄ labeling. In normals the position of GPIb β was not greatly affected by neuraminidase but GP₁₇^{5.8-6.5} was shifted to a more basic pI, now running nearer to GPIb β at pI 6.1-6.8. The thrombin-cleavable glycoprotein termed GPV or SP85 (23-25) was not detectable. This glycoprotein was identified by thrombin treatment of surface-labeled normal platelets. Fig. 4 shows fluorograms of two-dimensional gels of surface-labeled normal platelets, normal platelets after treatment with thrombin and the supernatant after treatment with thrombin. The only glycoprotein affected is GPV, which is removed from the platelet surface. At the same time a smaller glycopeptide, which is a fragment of GPV termed GPV_{f1} (26), appears in the supernatant. Patient E.H. again showed an intermediate situation with levels of GPIb α less than normal but clearly visible (Fig. 3d), while densitometry gave values for GPIb β , GP₁₇^{6.1-6.8} and GPV (the thrombin-cleavable glycoprotein) of $\sim 40 \pm 7\%$ of normal.

One-dimensional gels on platelets from patient M.S. showed that GPV was virtually undetectable compared with normals (not shown). As with periodate/NaB³H₄ labeling, one control (the same one) gave a different pattern of spots for GP₁₇^{6.1-6.8} in the reduced state showing four major spots instead of the two major, one minor generally obtained. The extra spots were on the acidic side of the normal and are apparently not due to differences in sialylation as all four persist although they are more basic after neuraminidase treatment (Fig. 3b).

Lactoperoxidase catalyzed iodination. The patterns of the labeled membrane proteins were virtually identical between the platelets from the Bernard-Soulier syndrome patients and the normals and are therefore not shown. There were, however, minor differences that were noticeable with patients V.S. and F.S. but not with E.H. In these two patients GP Ib was absent or shifted. This was only perceptible at relatively low exposure times as it was otherwise masked by the heavy labeling of the neighboring proteins. With patient E.H. no clear differences could be seen. In all these patients several spots could be seen in the GPV region, after long exposure times. However, there were no differences from normal and GPV could not be definitively identified in normals by thrombin treatment presumably because it is very poorly labeled by iodination. The lower molecular mass (<40,000 daltons) regions also showed no difference from normal.

Incubation of labeled normal platelets in Bernard-Soulier or normal blood. Normal platelets were labeled by either the periodate/NaB³H₄ or the neuraminidase/galactose oxidase/Na³H₄ method and then

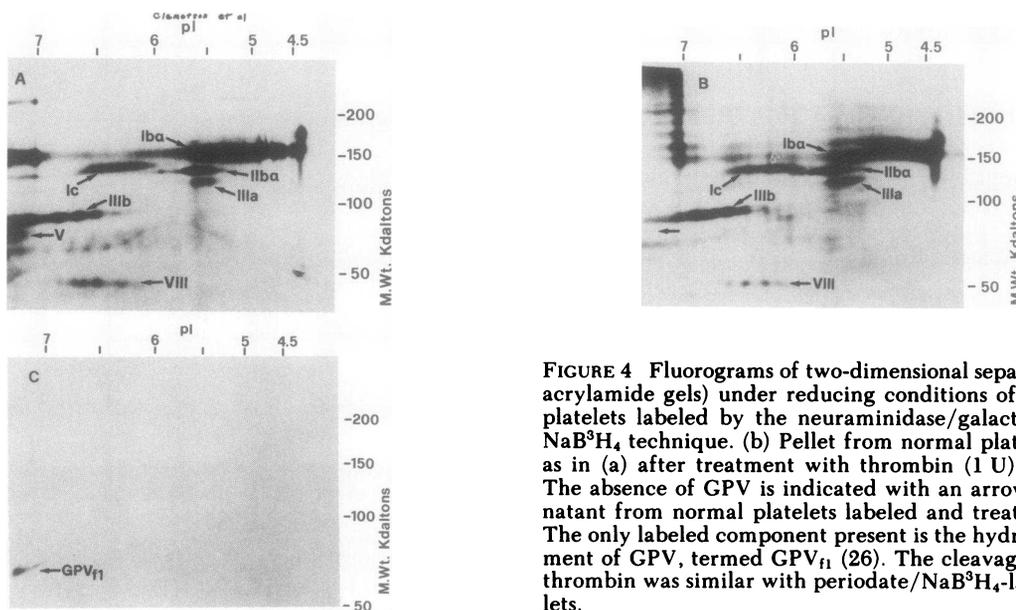


FIGURE 4 Fluorograms of two-dimensional separations (7.5% acrylamide gels) under reducing conditions of: (a) Normal platelets labeled by the neuraminidase/galactose oxidase/NaB³H₄ technique. (b) Pellet from normal platelets labeled as in (a) after treatment with thrombin (1 U) for 10 min. The absence of GPV is indicated with an arrow. (c) Supernatant from normal platelets labeled and treated as in (b). The only labeled component present is the hydrophilic fragment of GPV, termed GPV_{f1} (26). The cleavage of GPV by thrombin was similar with periodate/NaB³H₄-labeled platelets.

incubated in either normal blood or Bernard-Soulier blood. After reisolation and gel electrophoresis followed by fluorography the results of the different treatments were compared. Fig. 5 shows two-dimensional separations of periodate platelets + NaB³H₄ labeled in the reduced state. As can be clearly seen there are no obvious differences between the labeled normal platelets (Fig. 5a), the labeled normal platelets incubated in normal blood (Fig. 5b), and the labeled normal platelets incubated in Bernard-Soulier blood (Fig. 5c). In particular there were no changes in GPV or in GP₁₇^{5,8-6.5}. Densitometry of one-dimensional gels (not shown) also showed no difference between the variously treated platelets.

DISCUSSION

The Bernard-Soulier syndrome platelets examined in the present study showed the classic membrane glycoprotein defect of this disorder, a deficiency in GP Ib, which has been described by several authors using various techniques (8-14). In the majority of cases described in the literature GPIb appears to be totally absent; however in some cases it appears to be present in reduced amounts. It was, therefore, not entirely unexpected that cases exist such as patient E.H. who

shows an intermediate situation. Despite the fact that this patient does not have "giant" platelets, one of the classic symptoms of this disorder, the poor response to ristocetin and the biochemical differences from normal clearly indicate that he should be considered as a less extreme case of Bernard-Soulier syndrome.

The β -subunit of glycoprotein Ib was either not detectable in Bernard-Soulier syndrome or present in amounts that correlated well with the α -subunit, implying that the synthesis or incorporation of both subunits into the membrane is closely linked. Because little is known about disulphide-linked membrane glycoproteins this conclusion was not necessarily apparent.

The unexpected findings of this study were the inability to detect, by carbohydrate-labeling techniques, a further low molecular weight glycoprotein, GP₁₇^{5,8-6.5}, in the platelets of both patients with fully developed Bernard-Soulier syndrome, which was present in the same relative amounts as GPIb β in E.H., the intermediate case, and the deficiency or change in glycosylation in the thrombin-cleavable glycoprotein [GPV (23) or SP 85 (24)]. Again, E.H. appears to represent an intermediate situation as the GPV was detectable, but not as strongly labeled as in normals.

Previous reports have appeared claiming that changes in glycosylation could be observed in Bernard-

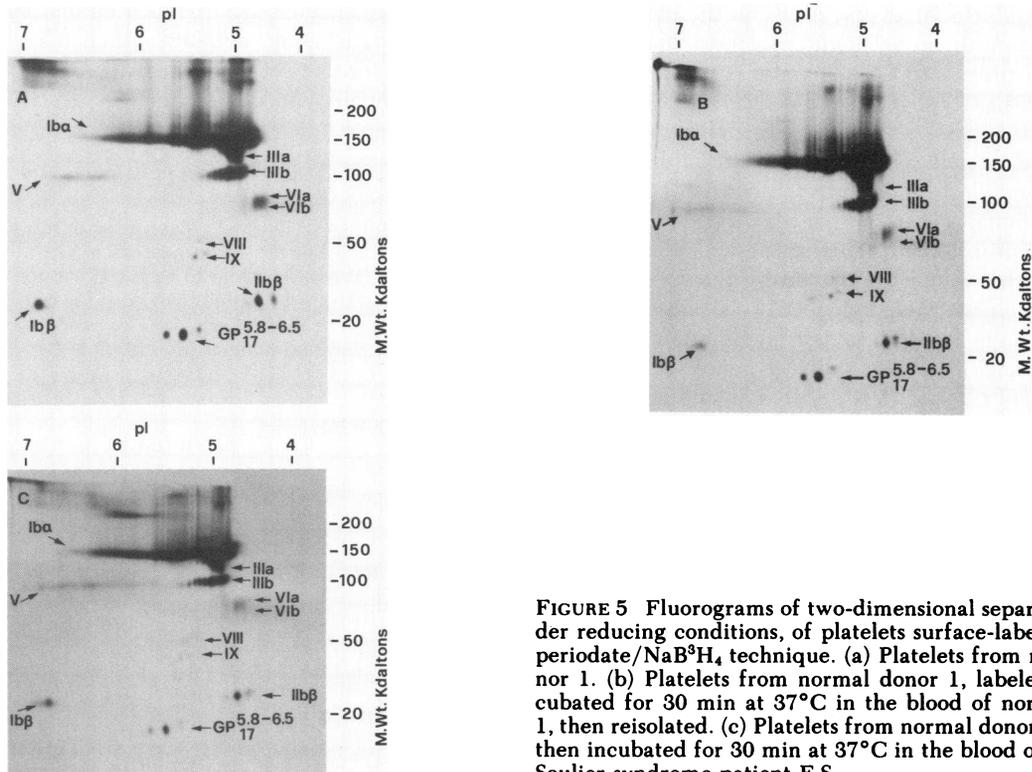


FIGURE 5 Fluorograms of two-dimensional separations, under reducing conditions, of platelets surface-labeled by the periodate/NaB³H₄ technique. (a) Platelets from normal donor 1. (b) Platelets from normal donor 1, labeled then incubated for 30 min at 37°C in the blood of normal donor 1, then reisolated. (c) Platelets from normal donor 1, labeled then incubated for 30 min at 37°C in the blood of Bernard-Soulier syndrome patient F.S.

Soulier syndrome platelet glycoproteins compared with normal using metabolic labeling with sugars (15). The changes reported here do not appear so general but are nevertheless significant. Bernard-Soulier syndrome has been used as a model for lack of response to von Willebrand Factor/ristocetin and first indications of the role of GPIb as the von Willebrand factor receptor were based on the absence of this glycoprotein in Bernard-Soulier syndrome platelets (8, 9). Shulman and Karpatkin (27) found extensive differences between Bernard-Soulier and normal platelets using crossed-immunoelectrophoresis techniques and suggested that endogenous membrane proteolysis might account for these. To see if proteases or glycosidases present in Bernard-Soulier blood might be responsible for the loss or degradation of the various glycoproteins absent in this disorder, labeled normal platelets were incubated with Bernard-Soulier or normal blood. No obvious differences were found (Fig. 5), which would exclude the presence of soluble enzymes but possibly not that of membrane-bound enzymes.

Peculiarities in the response of Bernard-Soulier platelets to thrombin have been reported (6, 7) that were ascribed to the absence of GPIb, which is known to bind thrombin and has been claimed to be the high-affinity thrombin binding site on platelets (6, 28). In one of these reports both the high affinity and the low affinity binding sites were reduced (7).

Considerable controversy exists on the nature of the platelet thrombin receptor and two possible candidates have been proposed. One of these is GPIb/glycocalicin, which has been reported to have a high affinity for thrombin (28). However, α -thrombin inactivated with reagents such as diisopropyl fluorophosphate or phenyl methyl sulphonyl fluoride does not activate platelets though it binds with the same affinity as untreated thrombin (29). This result led others to seek for a receptor that was cleaved by thrombin and the evidence points to this being GPV (SP 85) (23, 24).

The changes in GPV in Bernard-Soulier syndrome observed here might be a direct consequence of the deficiency of GPIb if this indeed acts as a high-affinity receptor for thrombin. In circulation the Bernard-Soulier platelets would be exposed to low amounts of thrombin and in the absence of the high-affinity receptor this would bind to and cleave GPV. If this took place at a sufficiently low concentration it would probably not activate the platelets but their ability to respond to larger amounts of thrombin would gradually be reduced. It is, of course, not yet possible to distinguish whether GPV is affected in its entirety or only its carbohydrate moiety. In the latter case the changes in thrombin-induced aggregation in Bernard-Soulier platelets might be caused by changes in conformation in GPV as a result of the defective glycosylation.

Since it is known that GPV is a peripheral platelet glycoprotein that can be removed by changes in ionic strength (26) and in view of the transition that occurs in Bernard-Soulier syndrome platelets when they become "giant-sized" the most likely possibility is that GPV is lost from Bernard-Soulier syndrome platelets during the extensive washing that is part of the surface-labeling methods. This would also imply that GPIb and GPV are associated on the platelet surface and that GPIb stabilizes the association of GPV with the membrane, which would fit in well with the idea of coupling of binding of thrombin to signal generation via cleavage of a receptor (30).

One of the diagnostic characteristics of Bernard-Soulier syndrome is the tendency of the platelets to become giant on undergoing "shape-change." These platelets are apparently normal-sized in circulation unlike those in May-Hegglin's disease (31). The role that the membrane glycoproteins play in this phenomenon is still unclear but the deficiency or absence of GPIb with its two-chain structure and of GP₁₇^{5.8-6.5}, which is probably also an integral membrane glycoprotein, must have a profound influence on the structure of the membrane.

Thus, although the absence or strong depletion of both subunits of GP Ib remains the main biochemical characteristic of Bernard-Soulier syndrome platelets there are other changes in the surface glycoproteins, particularly the absence or strong depletion of GP₁₇^{5.8-6.5} with carbohydrate-labeling methods, which may be equally responsible for some of the peculiarities that characterize Bernard-Soulier syndrome platelets.

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