The phosphoprotein that regulates platelet Ca^{2+} transport is located on the plasma membrane, controls membrane-associated Ca^{2+} -ATPase and is not glycoprotein Ib β -subunit

Agnès DARNANVILLE,* Raymonde BREDOUX,* Kenneth J. CLEMETSON,† Nelly KIEFFER,‡
Nathalie BOURDEAU,* Sylviane LEVY-TOLEDANO,* Jacques P. CAEN* and Jocelyne ENOUF*§

*U 150 INSERM, URA 184 CNRS, 6 rue Guy Patin, Hôpital Lariboisière, 75475 Paris Cedex 10, France,

†Theodor Kocher Institute, University of Berne, Berne, Switzerland, and ‡COR Therapeutics, Suite 80, 256E Grand Avenue, South San Francisco, CA 94080, U.S.A.

The localization and identity of the human platelet 24 kDa cyclic AMP (cAMP)-dependent phosphoprotein, previously reported to regulate Ca^{2+} transport, was investigated. It was found to be located on plasma membranes after isolation of these membranes from microsomes. Thus cAMP-dependent regulation of Ca^{2+} transport was associated with the plasma membrane fraction. Time course studies showed that the catalytic subunit of cAMP-dependent protein kinase (c-sub) induced a maximal 2-fold stimulation of Ca^{2+} uptake by the plasma membrane vesicles. This stimulation was dose-dependent up to 15 μg of c-sub/ml. The increase in Ca^{2+} uptake also depended upon the outside Ca^{2+} concentration, and was maximal at 1 μM . As regards the identity of the phosphoprotein, it was clearly distinct from the β -subunit of glycoprotein Ib, as after electrophoresis under reduced conditions it appeared as a 24 kDa protein, but under non-reduced conditions it appeared as a 22 kDa and not as a 170 kDa protein. Nevertheless, glycoprotein Ib was certainly present, because it was detected with two polyclonal antibodies raised against its two subunits. Furthermore, the 24 kDa phosphoprotein was also present in membranes isolated from platelets obtained from patients with Bernard Soulier Syndrome; these membranes contain no glycoprotein Ib.

INTRODUCTION

Human platelet activation is associated with an intracellular increase in Ca^{2+} concentration. Cyclic AMP (cAMP), an inhibitor of platelet activation, lowers this concentration by acting on Ca^{2+} pumps [2–5]. These membrane pumps transport Ca^{2+} on activation of Ca^{2+} -ATPases to lower the cytoplasmic Ca^{2+} concentration [6–14]. The action of cAMP on these pumps has been shown in two ways. The first of these was by raising the cAMP concentration in intact platelets and then isolating membrane vesicles and testing their Ca^{2+} uptake. Two phosphoproteins of 22 and 24 kDa were detected after prostaglandin E_1 (PGE_1)-induced cAMP phosphorylation, and Ca^{2+} uptake by the isolated mixed membranes was increased [15]. These authors concluded that cAMP-induced phosphorylation plays a role in Ca^{2+} transport. In a second approach, different membrane vesicle fractions were isolated from platelets and the effect of cAMP on Ca^{2+} uptake by the vesicles was studied using the catalytic subunit of cAMP-dependent protein kinase (c-sub). In this case, only one phosphoprotein was detected, of 22 or 24 kDa depending upon the authors [7,10,16,17], and an increase in Ca^{2+} uptake was also observed. Ca^{2+} uptake was also inhibited by the protein kinase inhibitor. Lastly, the c-sub was shown to alter the V_{max} of the Ca^{2+} -transport system [17].

These results were interpreted in the literature as showing that regulation of Ca^{2+} transport in platelets resembled that observed in cardiac sarcoplasmic reticulum [18–22]. In the latter case, this transport is also regulated by cAMP, which acts through the phosphorylation of a 23 kDa protein called phospholamban. This implied that the Ca^{2+} -ATPase concerned was the intracellular membrane-associated enzyme, which at the time was the

only one described. However, subsequent studies showed that the platelet phosphoprotein and phospholamban were different. At the same time, two platelet Ca^{2+} -ATPases, respectively associated with plasma membranes and intracellular membranes, were identified [23–26]. Consequently, the phosphoprotein controlling Ca^{2+} transport in platelets has still not been identified or localized.

It has been demonstrated that the 24 kDa phosphoprotein detected after PGE_1 -induced cAMP-dependent phosphorylation was identical with the β -subunit of glycoprotein Ib (GP Ib- β), a major plasma membrane protein which might be the platelet phosphoprotein referred to above [27]. We therefore attempted to establish whether this phosphoprotein was in fact located on the plasma membranes, in which case it would regulate the membrane-associated Ca^{2+} -ATPase, and whether it corresponded to GP Ib- β .

The results show that the protein pp^{22–24} phosphorylated by cAMP was associated with the plasma membrane fraction, and that it regulates Ca^{2+} uptake, thus providing new insight into the regulation by cAMP of Ca^{2+} transport. The possibility that GP Ib- β regulates this transport can also be ruled out.

MATERIALS AND METHODS

Materials

BSA, Hepes, ATP (sodium salt) and c-sub from bovine heart were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The following products for electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.): glycine, Tris, SDS, acrylamide, bis-acrylamide, $(\text{NH}_4)\text{HSO}_4$, β -mercaptoethanol, $\text{NNN}'\text{N}'$ -tetra-

Abbreviations used: Ca^{2+} -ATPase, Ca^{2+} -activated Mg^{2+} -dependent ATPase; PGE_1 , prostaglandin E_1 ; cAMP, cyclic AMP; TEMED, $\text{NNN}'\text{N}'$ -tetramethylethylenediamine; DTT, dithiothreitol; GP Ib, platelet glycoprotein Ib; GP Ib- β , β -subunit of glycoprotein Ib; c-sub, catalytic subunit of cAMP-dependent protein kinase.

§ To whom correspondence and reprint requests should be addressed.

methylethylenediamine (TEMED) and Coomassie Blue R-250. All other chemicals were of reagent grade. $^{45}\text{CaCl}_2$ and ^{125}I -Protein A were purchased from the Amersham International. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was from Du Pont-NEN. The antibody used to detect GP Ib- α was prepared against a major water-soluble proteolytic fragment called glycofalicin. The antibody used to detect the β -subunit was prepared against free reduced GP Ib- β [28].

Patient

The case history of patient NV, with Bernard Soulier Syndrome, including haematological data and the demonstration that platelets were deficient in GP Ib, has already been reported [29–31]. The present investigation was performed according to the Declaration of Helsinki and informed consent was obtained.

Preparation of the crude platelet membrane fraction

Ca^{2+} -accumulating membrane vesicles were prepared as previously described [10,32]. Briefly, fresh platelets were isolated and washed in Tyrode's modified buffer. The cells were then disrupted by controlled ultrasonication in a lysis buffer containing 25 mM-Hepes, pH 7.5, 100 mM-KCl, 2 mM-MgSO₄, 25 mM-NaCl, 12 mM-sodium citrate, 10 mM-glucose, 5 mM-ATP, 0.35% (w/v) BSA, 0.8 mg of benzamidine/ml, 0.1 unit of aprotinin/ml, 4.8 μg of leupeptin/ml, 1 mM-phenylmethanesulphonyl fluoride and 1 mM-EGTA with or without 1 mM-dithiothreitol (DTT). Lysed platelets were centrifuged at 19000 *g* to eliminate unlysed platelets, mitochondria and granules. The supernatant was centrifuged at 100000 *g* and the pellet obtained contained a crude membrane fraction consisting of both plasma and intracellular membranes as previously characterized [10,32].

Subfractionation of membrane vesicles

The 100000 *g* membrane fraction was resuspended in an incubation medium containing 10 mM-potassium oxalate, 100 mM-KCl, 10 mM-MgCl₂, 5 mM-ATP and 20 mM-Hepes, pH 7.4, and was layered on a 40% (w/v) sucrose cushion and centrifuged at 100000 *g* for 120 min. After washing, the two membrane subfractions obtained (interface and pellet) were characterized by the assessment of immunological and biochemical parameters [33].

Briefly, the pellet membrane fraction exhibited weak [^3H]concanavalin A labelling, low phosphodiesterase activity and a lower cholesterol/phospholipid ratio [33]. These first values gave between 4 and 12% of plasma membranes parameters in the pellet membrane fraction compared with 96–88% in the interface membrane fraction. These indicative criteria were then confirmed by two studies. Electrophoresis of the two membrane subfractions revealed that they had different profiles, with different proportions of high- and low-molecular-mass proteins. One significant difference between the two subfractions was the enrichment of the pellet membrane fractions in protein(s) of about 55 kDa [24]. The protein profile of the intracellular membranes isolated by free-flow electrophoresis using Coomassie Blue staining showed a similar enrichment [34]. The IgG L alloantibody, which is specific for the platelet surface-oriented glycoprotein GP IIb–IIIa, gave 10% cross-reactivity with the pellet membrane fraction using a quantitative immunofluorescence technique [33], whereas the anti-Lek^a antibody, which recognizes GP IIb, gave 0% cross-reactivity. Similar results were obtained by immunoblotting [35]. Finally, 90% of the cytochrome *c*:NADH reductase activity (EC 1.6.99.3) (intracellular membrane marker) was found associated with the pellet membrane fraction [36].

Based on these different characteristics, we concluded that the interface and pellet membrane subfractions were respectively plasma membranes and intracellular membranes. However,

mainly plasma membranes were used in the experiments described in this study.

Measurement of Ca^{2+} uptake by membrane vesicles

Ca^{2+} uptake by plasma membrane vesicles was measured with fresh material by incubating 0.1 ml of the membrane fraction (200 μg of membrane protein) in 1 ml of a final reaction mixture containing 20 mM-Hepes buffer, pH 7.4, 100 mM-KCl, 5 mM-MgATP, and different $^{45}\text{CaCl}_2$ and EGTA concentrations giving different final free Ca^{2+} concentrations calculated according to Fabiato & Fabiato [37] using a computer program kindly provided by Dr. L. Brass (Hematology–Oncology Section, Hospital of the University of Pennsylvania, Philadelphia, PA, U.S.A.). The reaction was started by adding the membrane suspension to the incubation medium and stirring at 37 °C. At different time intervals, 100 μl portions were withdrawn and filtered through Millipore HAWP filters (0.45 μm pore size) previously soaked in 2 mg of BSA/ml. The filters were washed three times with 5 ml of 0.1 M- CaCl_2 and counted for radioactivity in 10 ml of Unisolve scintillation liquid.

SDS/polyacrylamide gel electrophoresis

Membranes were solubilized in 0.05 M-Tris buffer, pH 6.8, containing 2.3% (v/v) SDS, 0.01% (v/v) Bromophenol Blue and 25% (v/v) glycerol for 10 min at 100 °C. Reduced samples were treated for 5 min at 100 °C with 5% (w/v) β -mercaptoethanol. The samples were then applied to an SDS/7%- or 11%-polyacrylamide slab gel according to Laemmli [38]. Electrophoresis was performed at 40 V overnight.

Membrane phosphorylation

Phosphorylations were carried out at 30 °C for 10 min in a reaction mixture containing 10 mM-sodium phosphate buffer, pH 7, 15 mM-MgSO₄, 100 mM-NaF, 6 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (7.4 Ci/nmol) and 0.1% Triton X-100, in the presence or absence of the cAMP-dependent protein kinase (enzyme/substrate ratio = 0.01–0.02) [16]. The reactions were initiated by adding $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and stopped by adding an equal volume of solubilization buffer. Membrane proteins were then separated by SDS/PAGE as described above. In some experiments, the proteins were electrotransferred on to a nitrocellulose membrane for 2 h at 60 V and 4 °C, before autoradiography using Kodak X-Omat AR films at –80 °C with intensifying screens.

Western blot procedure

Proteins were transferred electrophoretically from unstained polyacrylamide gels to nitrocellulose membranes [39]. Membranes were incubated with either anti-glycocalicin or anti-(GP Ib- β) antibody, as previously described [25]. Briefly, they were first incubated at 42 °C for 1 h with TBS (10 mM-Tris, pH 7.5, 140 mM-NaCl and 0.2% Tween) and then with the antibodies at room temperature for 90 min using a 1:250 dilution of the anti-glycocalicin antibody or a 1:200 dilution of the anti-(GP Ib- β) antibody, then washed five times with TBS. Antibody binding was revealed by incubating the nitrocellulose membranes for 1 h at 37 °C with 1200 MBq of ^{125}I -labelled Protein A/mg (30000 c.p.m./strip) (Amersham International) and washing five more times with TBS. Autoradiography of the dried nitrocellulose membranes was done with Kodak X-AR films using intensifying screens.

RESULTS

Membrane location of the phosphoprotein

Previous studies using the same mixed 100000 *g* membrane fraction showed that c-sub induced phosphorylation of a 24 kDa

Fig. 1. Effect of c-sub on 100000 g, plasma and intracellular membranes

The different membrane fractions (23 μg of membrane protein) were incubated with [$\gamma\text{-}^{32}\text{P}$]ATP as described in the Materials and methods section, in the absence (–) or in the presence (+) of c-sub (10 $\mu\text{g}/\text{ml}$). The membrane proteins were submitted to SDS/PAGE and the dried gels were autoradiographed. A, 100000 g membrane fraction; B, intracellular membranes; C, plasma membranes.

protein (reducing conditions). To locate the phosphoprotein, we isolated the plasma membranes and intracellular membranes from this mixed fraction as previously described [33]. Fig. 1 shows the effect of c-sub on the 100000 g (A), plasma membrane (C) and intracellular membrane (B) fractions. The amount of endogenous protein kinase in all three fractions was negligible because only minor protein phosphorylation could be detected when c-sub was not added. When it was added to the membrane preparations, a phosphorylated 24 kDa protein appeared under reduced conditions as a single band which was mainly associated with the plasma membrane fraction (compare lanes C+ and B+). Consequently, the presence of the phosphoprotein detected in the 100000 g membrane fraction (lane A+) was essentially due to the presence of plasma membranes.

Fig. 2. Effect of c-sub on the time course of Ca^{2+} uptake by plasma membrane vesicles

Membrane vesicles (200 $\mu\text{g}/\text{ml}$) were incubated with 1.6 μM - Ca^{2+} (0.50 mM- $^{45}\text{CaCl}_2$ and 0.50 mM-EGTA) in the reaction mixture as described in the Materials and methods section. Vesicles were sonicated for 5 s before incubation. Ca^{2+} uptake was initiated by adding membrane vesicles and stopped after the incubation times indicated. Vesicles were then treated as described in the Materials and methods section. Results are expressed as nmol of Ca^{2+} incorporated into the vesicles/mg of protein as a function of incubation time, and are the means $\pm$ S.E.M. of 12 experiments. ●, Ca^{2+} uptake in the absence of c-sub; ▲, Ca^{2+} uptake in the presence of 15 μg of c-sub/ml. The arrows indicate the addition time of 2 μM -ionophore A23187.

Effect of c-sub on Ca^{2+} uptake by plasma membranes

If the phosphoprotein regulating Ca^{2+} uptake is in fact associated with plasma membranes, it must be established that it regulates the Ca^{2+} transport recently shown to be associated with these membranes. We therefore tested the characteristics of the cAMP-dependent regulation of Ca^{2+} transport of the plasma membrane fraction.

Effect of c-sub on the time course of Ca^{2+} uptake by membrane vesicles. Fig. 2 shows the time course of Ca^{2+} uptake by the membrane vesicles. In the absence of c-sub, the Ca^{2+} uptake was 2.5 nmol/min per mg, which corresponded to the uptake previously shown [24]. This was due to the trapping of residual oxalate inside the vesicles, which occurred during membrane isolation. The addition of c-sub (15 $\mu\text{g}/\text{ml}$; maximum conditions) raised Ca^{2+} uptake. The maximum stimulation was 2.5-fold, giving a Ca^{2+} uptake of 6.25 nmol/min per mg. As a control of Ca^{2+} uptake by the membrane vesicles, 2 μM -ionophore A23187 was added after 15 min of treatment. In both cases, 75 % of the incorporated Ca^{2+} was released, thus eliminating the possibility of Ca^{2+} binding to the membranes.

Effect of c-sub as a function of different Ca^{2+} concentrations outside the membrane vesicles. As Fig. 3 shows, the stimulatory effect of c-sub (15 $\mu\text{g}/\text{ml}$) on Ca^{2+} uptake by membrane vesicles was regulated by the Ca^{2+} concentration. Interestingly, the maximum stimulation of Ca^{2+} uptake by c-sub was observed within the range of Ca^{2+} concentrations found in platelets during their activation (approx 1 μM). This range of concentrations resulted in a doubling of Ca^{2+} uptake by membrane vesicles, as shown in the experiments illustrated in Fig. 2. At low Ca^{2+} concentrations corresponding to those found in resting platelets (10 nM), or at high Ca^{2+} concentrations (> 1 μM), the effect of c-sub was negligible.

These results explain why, when we studied the effect of c-sub on the mixed 100000 g membrane fraction, an external Ca^{2+} concentration of 100 μM had no significant effect, but at 1 μM , the effect of c-sub was maximal. They also explain the different

Fig. 3. Effect of c-sub on Ca^{2+} uptake as a function of different Ca^{2+} concentrations

Ca^{2+} uptake by membrane vesicles (200 $\mu\text{g}/\text{ml}$) was measured as described in the Materials and methods section for 10 min, in the absence (●) or in the presence (▲) of c-sub (15 $\mu\text{g}/\text{ml}$) using different Ca^{2+} concentrations (0.5 mM-EGTA, variable $^{45}\text{CaCl}_2$ concentration). Free Ca^{2+} concentration were calculated and expressed as pCa. Ca^{2+} uptake is expressed as a percentage of the maximum Ca^{2+} incorporation obtained in the presence or the absence of c-sub as a function of pCa; 100 % Ca^{2+} uptake corresponded to the Ca^{2+} uptake obtained at pCa 6. Results are the means of 4 experiments.

Fig. 4. Comparative effects of c-sub on Ca²⁺ uptake by plasma membranes (○) and intracellular membranes (□)

Plasma membrane and intracellular membrane vesicles (100 µg/ml) were incubated with 1.6 µM-Ca²⁺ (0.5 mM-⁴⁵CaCl₂ and 0.5 mM-EGTA) in the reaction mixture described in the Materials and methods section. Ca²⁺ uptake was measured in the absence or in the presence of c-sub (15 µg/ml). Vesicles were then treated as described in the Materials and methods section. Ca²⁺ uptake in the presence of c-sub is expressed as a percentage of the maximum Ca²⁺ incorporation, obtained in the absence of c-sub. Results are the means of three experiments.

degrees of stimulation reported by authors working on different membrane fractions, because these studies were generally carried out at a single Ca²⁺ concentration.

Comparative effects of c-sub on Ca²⁺ uptake by plasma membranes and intracellular membranes. The characteristics of the cAMP regulation of Ca²⁺ transport previously observed with the 100000 g membrane fraction also appear to be associated with the plasma membrane fraction. To ensure that the c-sub-sensitive Ca²⁺-accumulating activity in this membrane fraction is not due to contamination by intracellular membranes, we tested the comparative effects of c-sub on Ca²⁺ uptake by the plasma membrane and intracellular membrane vesicles. Fig. 4 shows that the stimulatory effect of c-sub (15 µg/ml) is mainly associated with the plasma membrane fraction, thus agreeing with the location of the cAMP phosphoprotein shown in Fig. 1.

Consequently, the platelet phosphoprotein investigated here is located on the plasma membrane and does regulate membrane-associated Ca²⁺ transport. This observation made it essential to investigate the possibility that GP Ib-β is involved in regulating platelet Ca²⁺ transport in our membrane preparation.

Immunological detection of GP Ib

For this investigation, we first performed experiments to confirm the presence of GP Ib. As GP Ib consists of a 145 kDa α-chain, disulphide-linked to a 24 kDa β-chain [40], it was necessary to reduce the disulphide bridge in order to demonstrate the presence of the GP Ib β-subunit. In addition, to preserve Ca²⁺-ATPase activity, platelet lysis was performed in the presence of 1 mM-DTT, which might also have reduced some of the GP Ib. We therefore used membrane preparations with or without DTT in the lysis step.

Fig. 5(a) indicates the results of immunoblotting experiments obtained with an anti-glycocalicin antibody, which clearly show that GP Ib was present in our membrane preparation. In the absence of DTT, a 170 kDa protein was detected (A, lane NR). The addition of 5% β-mercaptoethanol (conditions described in the legend to Fig. 5) caused only a partial reduction of GP Ib, as two protein bands were still visible at 170 and 140 kDa (A, lane R). When 10% β-mercaptoethanol was added, GP Ib was completely reduced (results not shown). The results obtained when the membrane preparations were treated with 1 mM-DTT during platelet lysis showed that 5% β-mercaptoethanol totally reduced GP Ib (B, lane R; A, lane NR).

These experiments with an anti-glycocalicin antibody were

Fig. 5. Detection of GP Ib in platelet plasma membranes using anti-glycocalicin polyclonal antibody (a) and anti-(GP Ib-β) polyclonal antibody (b)

Membrane fractions were prepared in the absence (A) or in the presence (B) of 1 mM-DTT during platelet lysis. Fractions of 25 µg of non-reduced proteins (NR) or of proteins reduced by 5% β-mercaptoethanol (R) underwent electrophoresis on an SDS/7% (a) or 11% (b) polyacrylamide gel and subsequent transfer on to nitrocellulose membranes. Membrane fractions were then treated for immunodetection of the GP Ib, using a 1:250 (a) or 1:200 (b) antibody dilution (see the Materials and methods section).

complemented by similar studies using an anti-(GP Ib-β) antibody (Fig. 5b). Again, GP Ib was partly reduced when membranes were prepared in the presence of DTT, as GP Ib-β could be detected (B, lane NR). The GP Ib-β band was more intense after 5% β-mercaptoethanol treatment (B, lane R) and was also present in the absence of DTT after 10% β-mercaptoethanol treatment (A, lane R). No band was seen in the absence of both DTT and β-mercaptoethanol (A, lane NR).

Relationship between the phosphoprotein and GP Ib-β

The results of the above experiments indicated that if the phosphoprotein is indeed GP Ib-β, the difference between the two proteins should be clearly visible in experiments with and without membrane protein reduction. Consequently, we isolated plasma membranes with or without DTT in the lysis step, and performed c-sub-induced phosphorylation of these membranes, followed or not by membrane protein reduction. Fig. 6, which illustrates the results obtained without DTT, shows that addition of c-sub (15 µg/ml) to the incubation medium in the absence of β-mercaptoethanol before gel electrophoresis (lane 1) led to ³²P incorporation into a protein band of 22 kDa. After treatment with 5% β-mercaptoethanol, the main incorporation of ³²P was in the 24 kDa protein band (lane 2). When DTT was added for the lysis step there was some reduction, and two protein bands of 24 and 22 kDa were visible, the latter being more intense under non-reduced conditions whereas the 24 kDa protein was observed under reduced conditions (results not shown). Thus under non-reducing conditions, c-sub did not induce phosphorylation of the high-molecular-mass protein (170 kDa).

Autoradiography and immunoblotting were performed with the same membrane to allow precise localization. Fig. 6 (lane 2) shows that the 24 kDa form of the phosphoprotein had exactly the same molecular mass as the protein band corresponding to GP Ib-β (Fig. 6, lane 3). Consequently, since the molecular mass of the plasma membrane phosphoprotein investigated was 22 kDa under non-reducing conditions and 24 kDa under reducing conditions, we propose calling this phosphoprotein pp²²⁻²⁴. This variation in molecular mass, reported here for the first time to our knowledge, explains why the phosphoprotein previously described in the literature in different mixed or purified

Fig. 6. Comparative molecular masses of the phosphoprotein and of GP Ib- β

Membrane proteins were prepared without DTT during platelet lysis and were first phosphorylated as described in the Materials and methods section. They were then either reduced using 5% β -mercaptoethanol and submitted to electrophoresis on an SDS/11% polyacrylamide gel (lane 2), or underwent electrophoresis without reduction (lane 1). After transfer on to nitrocellulose membranes, autoradiography was performed to detect the phosphorylated bands. Next, membrane proteins were treated for immunodetection of GP Ib- β as described in the legend to Fig. 5. Lane 3, immunoblot of reduced proteins.

intracellular membranes [10,17,21,22] exhibited a molecular mass ranging between 22 and 24 kDa.

Phosphorylation of plasma membranes from platelets of a patient with Bernard Soulier Syndrome

To establish definitely the difference between GP Ib- β and the 24 kDa form of pp^{22-24} , we isolated membranes from the platelets of a patient with Bernard Soulier Syndrome. Such platelets were previously shown by different methods to be completely devoid of GP Ib. These methods included ^{125}I -labelling of the membrane glycoproteins followed by their electrophoresis [31], crossed immunoelectrophoresis using a polyspecific rabbit anti-(human platelet) antibody preparation [41] or a monoclonal antibody specific for GP Ib [42], and binding studies with AN 51, another specific monoclonal antibody [43]. Fig. 7 shows the comparative effects of c-sub (15 $\mu\text{g}/\text{ml}$) on the membrane proteins from normal and Bernard Soulier platelets. These experiments were only conducted under reducing conditions after phosphorylation, because of the limited quantities of membrane available. No differences between the two kinds of membranes were detected (compare lanes C+ and BSS), as the 24 kDa form of pp^{22-24} was present in both cases. Moreover, on the basis of the phosphorylation intensities, it could be assumed that the phosphoprotein was present in similar amounts.

Thus, taken together, the above results led us to conclude that pp^{22-24} and GP Ib- β are totally distinct proteins, despite their identical molecular masses.

DISCUSSION

Although it was shown many years ago that platelet Ca^{2+} transport is regulated by cAMP-dependent phosphorylation, the mechanism of action of this regulation is still unknown. Its

Fig. 7. Phosphorylation of membrane proteins isolated from normal platelets and from platelets of a patient with Bernard Soulier Syndrome

Plasma membrane proteins (70 μg) were phosphorylated as described in the legend to Fig. 6, in the absence or in the presence of c-sub (15 $\mu\text{g}/\text{ml}$). After sample reduction using 5% β -mercaptoethanol, membrane proteins were separated on an SDS/11% polyacrylamide gel which was then autoradiographed. Lane C-, basal phosphorylation of membranes isolated from normal platelets; lane C+, c-sub-induced phosphorylation of membranes isolated from normal platelets; lane BSS+, c-sub-induced phosphorylation of membranes isolated from Bernard Soulier platelets.

definition requires (1) identification of the phosphoprotein concerned, (2) location of the regulation of Ca^{2+} transport, since the existence of intracellular membrane and plasma membrane Ca^{2+} -ATPases was recently reported, and (3) establishment of the relationship between cAMP-dependent phosphorylation and Ca^{2+} transport.

As an approach to the third requirement, a platelet Ca^{2+} -ATPase and a 22 kDa phosphoprotein (pp^{22-24}) were partially purified and their relationship was tested by reconstitution experiments [44]. Although the results suggested that the Ca^{2+} -transport system is not associated with the phosphoprotein, the experimental procedures involved may have degraded the proteins, as suggested by the fact that the partially purified phosphoprotein migrated at 22 kDa under both reduced and non-reduced conditions. This was not the case in the present work when the phosphorylated protein was not partially purified. In addition, the relationship between the Ca^{2+} -ATPase and the phosphoprotein may be direct or indirect, and in the latter case it cannot be shown by reconstitution methods.

Another approach, which we used in this study, was to start by locating and trying to identify the phosphoprotein, because its identification would limit possible mechanisms of action involved. The previously described platelet pp^{22-24} was located on the plasma membranes and shown to be involved in regulating the associated Ca^{2+} transport. This is established by the following: (1) the phosphoprotein is enriched in the plasma membrane fraction; (2) the stimulatory effect observed in the mixed membrane fraction was also found in the plasma membrane fraction, and (3) the minor residual contamination by intracellular membranes cannot account for the degree of stimulation (up to 2-fold). This new insight into platelet Ca^{2+} transport requires some comment, because the results of the studies initially conducted on mixed or enriched intracellular membranes [11-14] suggested that cAMP regulates intracellular membrane Ca^{2+} transport. The present finding that pp^{22-24} is in fact associated with the plasma membranes therefore agrees with the previous observation that the Ca^{2+} transport associated with purified intracellular membranes is not regulated by cAMP [45]. However, it disagrees with other studies [17,21,22] conducted using different platelet sources and different methods of membrane separation. One possible explanation for this disagreement might be that in these latter studies some plasma membranes were recovered with the in-

tracellular membranes. This could easily happen if the plasma-membrane Ca^{2+} -transport system is located in the open canaliculic system [46], as the latter system can fuse with internal structures upon platelet activation, which may occur during membrane preparation. Another alternative is that the initial localization of intracellular Ca^{2+} -ATPase in fact referred to open canaliculic system Ca^{2+} -ATPase. This was suggested by Herbener & Dean [47] on the basis of the immunolocalization that they observed using an antibody directed against purified intracellular Ca^{2+} -ATPase. In other words, the initially described intracellular Ca^{2+} -ATPase may in fact correspond to the plasma-membrane Ca^{2+} -ATPase studied here.

Secondly, the present work establishes without doubt that the phosphoprotein that we designate as pp^{22-24} is not GP Ib- β . The phosphoprotein concerned was initially identified as a 22, 23 or 24 kDa protein after microsome phosphorylation by cAMP and a cytosolic fraction [7] or by cAMP-dependent protein kinase [10–17,21,22]. These molecular masses were obtained by electrophoresis under non-reduced conditions, and in the present work we demonstrated that pp^{22-24} in fact appears as a 24 kDa phosphoprotein under reducing conditions, and as a 22 kDa protein under non-reducing conditions. This unusual change in electrophoretic migration has not been previously described, and can be explained by the presence of an intracellular disulphide bond. Consequently, this further confirms that the 22–24 kDa platelet phosphoprotein is not 23 kDa phospholamban, as previously deduced from three characteristics: (1) its non-recognition by anti-phospholamban antibody; (2) the inability of Triton X-100 to dissociate it into the same subunits as those of phospholamban; and (3) this phosphoprotein cannot be purified by the same methodology used to purify phospholamban [10–22].

In addition, the electrophoretic behaviour of the pp^{22-24} platelet phosphoprotein shows that it could not be distinguished from GP Ib- β solely on the basis of its molecular mass of 22 kDa. It was also necessary to show that pp^{22-24} does not comigrate with GP Ib on one-dimensional non-reduced gels. This absence of comigration was not due to the absence of GP Ib from our membrane preparation, because GP Ib was detected by antibodies raised both against glycosialin and GP Ib- β . Secondly, we showed that the phosphoprotein was present in membranes isolated from Bernard Soulier Syndrome platelets, which have been shown by various techniques to be completely deficient in GP Ib [41–43]. Consequently, GP Ib- β is certainly not identical with pp^{22-24} , and further investigations into the identity of the phosphoprotein are required.

We are particularly grateful to Dr. J. George for his interest and critical review of the manuscript. We also thank A. Chasme for typing the manuscript.

REFERENCES

- Rink, T. J. & Hallam, T. J. (1984) *Trends Biochem. Sci.* **376**, 215–219
- Imai, A., Hattori, H., Takahashi, M. & Nozawa, Y. (1983) *Biochem. Biophys. Res. Commun.* **112**, 693–700
- Feinstein, M. B., Egan, J. J., Sha'afi, R. I. & White, J. (1983) *Biochem. Biophys. Res. Commun.* **113**, 598–604
- Yamanishi, J., Kawahara, Y. & Fukuzaki, H. (1983) *Thromb. Res.* **32**, 183–188
- Zavoico, G. B. & Feinstein, M. B. (1984) *Biochem. Biophys. Res. Commun.* **120**, 579–585
- Robblee, L. S., Shepro, D. & Belamarich, F. A. (1973) *J. Gen. Physiol.* **61**, 462–481
- Käser-Glanzmann, R., Jakabova, M., George, J. N. & Lüscher, E. F. (1977) *Biochim. Biophys. Acta* **466**, 429–440
- Käser-Glanzmann, R., Jakabova, M., George, J. N. & Lüscher, E. F. (1978) *Biochim. Biophys. Acta* **512**, 1–12
- Javors, M. A., Bowden, C. L. & Ross, D. H. (1982) *Biochim. Biophys. Acta* **691**, 220–226
- Le Peuch, C. J., Le Peuch, D. A. M., Katz, S., Demaille, J. G., Hinckle, M. T., Bredoux, R., Enouf, J., Levy-Toledano, S. & Caen, J. (1983) *Biochim. Biophys. Acta* **731**, 456–464
- Menashi, S., Authi, K. S., Caret, F. & Crawford, N. (1984) *Biochem. J.* **222**, 413–417
- Fischer, T. H., Campbell, K. P. & White, G. C. (1987) *Biochemistry* **26**, 8024–8030
- Levitsky, D. O., Loginov, V. A., Lebedev, T. S., Levchenko, T. S. & Leytin, V. L. (1984) *FEBS Lett.* **171**, 89–93
- Dean, W. L. & Sullivan, D. M. (1982) *J. Biol. Chem.* **257**, 14390–14394
- Fox, J. E. B., Say, A. K. & Hallam, R. J. (1979) *Biochem. J.* **184**, 651–661
- Enouf, J., Bredoux, R., Boucheix, C., Mirshahi, M., Soria, C. & Levy-Toledano, S. (1986) *FEBS Lett.* **183**, 398–402
- Adunyah, S. E. & Dean, W. L. (1987) *Biochim. Biophys. Acta* **930**, 401–409
- Le Peuch, C. J., Haiech, J. & Demaille, J. G. (1979) *Biochemistry* **18**, 5150–5157
- Tada, M., Inui, M., Kadoma, M., Kuzuya, T., Abe, H. & Kakiuchi, S. (1983) *J. Mol. Cell. Biol.* **15**, 335–346
- Suzuki, T. & Wang, J. H. (1986) *J. Biol. Chem.* **261**, 7018–7023
- Fischer, T. H. & White, G. C. (1987) *Biochem. Biophys. Res. Commun.* **149**, 700–706
- Adunyah, S. E., Jones, L. R. & Dean, W. L. (1988) *Biochim. Biophys. Acta* **941**, 63–70
- Enyedi, A., Sarkadi, B., Foldes-Papp, Z., Monostory, S. & Gardos, G. (1986) *J. Biol. Chem.* **261**, 9558–9563
- Enouf, J., Bredoux, R., Bourdeau, N. & Levy-Toledano, S. (1987) *J. Biol. Chem.* **262**, 9293–9297
- Enouf, J., Lompré, A. M., Bredoux, R., Bourdeau, N., de la Bastie D. & Levy-Toledano, S. (1988) *J. Biol. Chem.* **263**, 13922–13929
- Enouf, J., Bredoux, R., Bourdeau, N., Sarkadi, B. & Levy-Toledano, S. (1989) *Biochem. J.* **263**, 547–552
- Fox, J. E. B., Reynolds, C. C. & Johnson, M. M. (1987) *J. Biol. Chem.* **262**, 12627–12631
- Wicki, A. N., Schnittering, W. & Clemetson, K. (1990) *Methods Enzymol.*, in the press
- Nurden, A. T. & Caen, J. P. (1975) *Nature (London)* **255**, 720–722
- Caen, J. P., Nurden, A. T., Jeanneau, C., Michel, H., Tobelem, G., Levy-Toledano, S., Sultan, Y., Valensi, F. & Bernard, J. (1976) *J. Lab. Clin. Med.* **87**, 586–596
- Nurden, A. T., Dupuis, D., Kunicki, T. J. & Caen, J. P. (1981) *J. Clin. Invest.* **67**, 1431–1440
- Enouf, J. & Levy-Toledano, S. (1984) *Br. J. Pharmacol.* **81**, 509–518
- Enouf, J., Bredoux, R., Boizard, B., Wautier, J. L., Chap, H., Thomas, J., de Metz, M. & Levy-Toledano, S. (1984) *Biochem. Biophys. Res. Commun.* **123**, 50–58
- Menashi, S., Weintraub, H. & Crawford, N. (1981) *J. Biol. Chem.* **256**, 4095–4101
- Fujimoto, T., Fujimura, K. & Kuramoto, A. (1989) *Thromb. Res.* **53**, 99–108
- Peleg, I., Kahane, I., Eldor, A., Groschel-Stewart, U., Levy-Toledano, S. & Muhlrad, A. (1989) *Thromb. Res.* **56**, 1–12
- Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Wicki, A. N. & Clemetson, K. J. (1987) *Eur. J. Biochem.* **163**, 43–50
- Kunicki, T. J., Nurden, A. T., Pidard, D., Russel, N. R. & Caen, J. P. (1981) *Blood* **58**, 1190–1197
- Montgomery, R. R., Kunicki, T. J., Taves, C., Pidard, D. & Corcoran, M. (1983) *J. Clin. Invest.* **71**, 385–389
- Bryckaert, M. C., Pietu, G., Ruan, C., Tobelem, G., Girma, J. P., Meyer, D., Larrieu, M. J. & Caen, J. (1985) *J. Lab. Clin. Med.* **106**, 393–400
- Fischer, T. H. & White, G. J. (1989) *Biochem. Biophys. Res. Commun.* **159**, 644–650
- Hack, N., Crosset, M. & Crawford, N. (1986) *Biochem. J.* **233**, 661–668
- Cutler, L. S., Feinstein, M. B., Rodan, G. A. & Christian, C. P. (1981) *Histochem. J.* **13**, 547–554
- Herbener, G. H. & Dean, W. L. (1988) *Biochem. Biophys. Res. Commun.* **153**, 848–854