Possible involvement of two proteins (phosphoprotein and CD9 (p24)) in regulation of platelet calcium fluxes

J. Enouf, R. Bredoux, C. Boucheix*, M. Mirshahi+, C. Soria and S. Levy-Toledano

*Unité de Recherches sur la Thrombose Expérimentale et l'Hémostase INSERM U 150, Hôpital Lariboisière, 6 rue Guy Patin, F75475 Paris Cedex 10, *INSERM U 253 Hôpital Paul Brousse, Villejuif* and +*Service de Médecine Interne et Oncologie, Hôtel Dieu, Paris, France*

Received 19 February 1985

The monoclonal antibody ALB_6 directed against the leukocyte differentiation antigen CD9 (p24) increases the calcium incorporation into isolated platelet membrane vesicles enriched in internal membranes. The similarities of the effects of both the monoclonal antibody and the catalytic subunit of the cAMP-dependent protein kinase (C. subunit), which phosphorylates a protein of an apparent molecular mass of 23 kDa, led us to investigate the relationship between CD9 (p24) and the 23-kDa phosphoprotein (p23). ALB_6 IgG does not inhibit the C.subunit-induced phosphorylation of p23 and the immunoadsorption by ALB_6 IgG of p24 associated to membrane vesicles does not alter the phosphorylation pattern. Thus, proteins of similar molecular mass appear to be involved in calcium fluxes: one is recognized by the ALB_6 antibody while the other can be phosphorylated by the C-subunit.

Platelet membrane *Ca^{2+} transport* *Monoclonal antibody* *Cyclic AMP* *Phosphorylation*

1. INTRODUCTION

In response to a wide variety of stimuli, an increase in cytosolic calcium ion concentration is supposed to be the trigger for activation of cellular processes. In blood platelets, calcium is thought to be implicated in shape change, secretion and aggregation [1,2]. Elevation of intracellular [Ca^{2+}] may occur by an influx through the surface membrane [3] or the intracellular liberation of calcium associated with phospholipids [4]. It may also occur due to release of Ca^{2+} from intracellular storage sites or dense tubular system [2]. A platelet membrane fraction which sequesters calcium was first described by Käser-Glanzmann et al. [5]. The characterization and pharmacological regulation of a modified fraction have been studied [6,7] and we have been able to correlate this calcium transport with the activity of a (Ca^{2+} + Mg^{2+})-ATPase [8]. Also, regulation of the calcium transport appears to proceed through a cAMP-dependent protein kinase-induced phosphorylation of a 23-kDa polypeptide [9]. Recently, a monoclonal antibody called ALB_6 which recognizes a platelet membrane protein of similar molecular mass has been described [10]. The antibody tested on platelet functions acts as an aggregating agent and is thought to be involved in calcium movements. The work reported here was designed to investigate the effect of this monoclonal antibody on the calcium fluxes in the isolated platelet membranes enriched in internal membranes. The findings suggest the presence of two 23-kDa proteins which regulate calcium fluxes in human platelets.

Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; cAMP, cyclic adenosine 3,5'-monophosphate

2. EXPERIMENTAL

The membrane fraction was isolated as previously described and has been found enriched in internal membranes. Briefly, washed platelets

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

were lysed by ultrasonication followed by differential centrifugation at 19000 and 100000 × *g*. The 100000 × *g* pellet was used in the experiments reported here. Characterization of this fraction has been made essentially using enzymatic activities as markers of the different contaminants [6,8]. The platelet membranes/whole platelet lysate average ratio was 1.44 for the plasma membrane marker enzyme phosphodiesterase. The specific activity of the endoplasmic reticulum marker enzyme, the antimycin insensitive NADH-cytochrome *c* reductase, was 9.0 relative to whole platelets. The calcium uptake into the membrane vesicles was measured on fresh material by incubating 0.2 ml of 100000 × *g* membrane fraction in a final reaction mixture of 2 ml constituted by 20 mM Hepes buffer (pH 8), 100 mM KCl, 10 mM potassium oxalate, 5 mM MgATP, 0.5 mM $^{45}CaCl_2$ (1300–1500 cpm/nmol) and 0.5 mM EDTA. Free final calcium concentration was 1 μM. Calculations were reported by Lotersztajn et al. [11] using apparent association constants of 6.02×10^8, 9.7×10^2, 9.55×10^3 and 4.75×10^4 M^{-1} for Ca EDTA, Mg EDTA, Ca ATP and Mg ATP, respectively.

The reaction was started by adding the membrane suspension to the incubation medium and stirred at 37°C for different times. Aliquots of 0.2 ml were withdrawn and filtered through Millipore HAWP filters (0.45 μm pore size) previously soaked in a 2 mg/ml bovine serum albumin solution. Filters were washed with 10 ml of 0.1 M $CaCl_2$ and counted in 10 ml of Unisolve scintillation liquid.

The monoclonal antibody tested here was produced as described previously [12,13]. For this study, we used purified antibody fractions. IgG were obtained by precipitation in ammonium sulfate followed by ion exchange chromatography. After reprecipitation with ammonium sulfate ALB_6 IgG was dissolved and dialysed against 0.1 M phosphate buffer. ALB_1 (CD10) and ALB_9 (CD24) monoclonal antibodies of the same IgG subclass were used as controls. CD_{10} is a common antigen of human acute lymphoblastic leukemia and CD24 is a granulocyte and B cells lineage antigen [14]. The 100000 × *g* treatment for phosphorylation was as follows: platelet membrane vesicles were resuspended and lysed in cold buffer containing: 1% NP40, 1 mM EDTA, 2 mM PMSF, 5 mM ATP, 30 mM NaCl, 30 mM Tris (pH 7.0) and ultracentrifuged in an airfuge (Beckman) for 10 min at 100000 × *g* to eliminate the residual membranes. This extract was mixed gently with immunoadsorbents (glutaraldehyde-activated ultrogels coated with ALB_6 IgG or ALB_1 IgG according to the technique of Kahn et al. [15] in order to retain the antigen defined by the monoclonal antibody ALB_6. After centrifugation at 700 × *g* for 2 min, ultrogel pellets previously washed in 0.1 M phosphate buffer and supernatants were then phosphorylated. Phosphorylations were carried out at 30°C for 10 min in a reaction mixture containing 10 mM $MgSO_4$, 100 mM NaF, 6 mM [^{32}P]ATP (7.4 Ci/nmol), 25 mM sodium phosphate buffer (pH 7.0), in the presence or absence of the catalytic subunit of the cAMP-dependent protein kinase (enzyme/substrate: 0.01). The reactions were initiated by addition of ATP. Supernatant protein phosphorylations were stopped by addition of 0.3 M $HClO_4$. After centrifugation, the precipitated proteins were resuspended in the following buffer: 0.0625 M Tris (pH 6.8) containing 2.5% SDS, 0.001% (v/v) bromophenol blue, 5% (v/v) sucrose, 5% (v/v) β-mercaptoethanol. Ultrogel pellet protein phosphorylations were stopped by addition of the same buffer (v/v). After dissociation for 10 min in boiling water, samples were applied to an SDS-polyacrylamide gradient gel (5–20%) according to Laemmli [16]. Electrophoresis was performed at 30 volts overnight. Gels were stained with Coomassie brilliant blue and dried, then autoradiographed using Kodak X-Omat AR films at −80°C with intensifying screens.

3. RESULTS AND DISCUSSION

Fig.1 shows the dose response relationship of ALB_6 on the time course of calcium uptake by isolated platelet membranes enriched in internal membranes. The monoclonal antibody was added at time 0 of the calcium uptake study. As a control other monoclonal antibodies, ALB_9 IgG (fig.1) and ALB_1 IgG (not shown) obtained under the same conditions, but which were without any effect on platelet function, were used. It is clear that the monoclonal antibody induces an increase in the calcium incorporation into the membrane vesicles either by affecting the rate or the steady state level

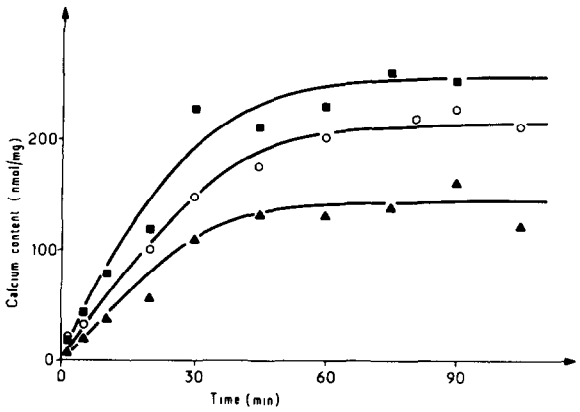

Fig.1. Time course of the calcium uptake into isolated platelet membrane vesicles (100 μg/ml) in the presence of different concentrations of the monoclonal antibody ALB_6 IgG: ○—○, 140 μg/ml; ■—■, 280 μg/ml. The control ALB_9 IgG was added at the final concentration of 280 μg/ml, ▲—▲.

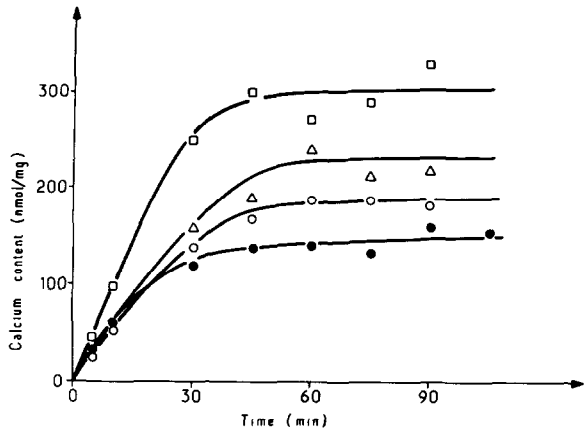

Fig.2. Time course of calcium uptake into isolated platelet membrane vesicles (380 μg/ml) in the presence of different concentrations of C. subunit (Sigma) dissolved in water immediately prior to use: ○—○, 5 μg/ml; △—△, 7.5 μg/ml; □—□, 10 μg/ml, or ●—●, in the absence of C. subunit.

which reaches a factor 1.8 when ALB_6 IgG was used at 280 μg/ml. This effect is not detectable when using 100 μM calcium concentration instead of 1 μM. The stimulation depends on the ratio between the immunoglobulin and the membrane vesicle protein concentration. A stimulation factor of 1.5 was obtained in 5 different experiments when using a mean value of 1.2 for this ratio. Fig.1 represents a typical experiment but similar results were obtained with different successive antibody preparations.

To investigate further the effect of the monoclonal antibody, we compared the effect of cAMP, which phosphorylates a 23-kDa protein, on the calcium transport [9]. The experiments were conducted using the catalytic subunit of the cAMP dependent protein kinase (C. subunit) because of the very small amounts of endogenous cAMP-dependent protein kinase holoenzyme [6]. Fig.2 shows that 10 μg/ml C. subunit induces an increase in the calcium uptake reaching a factor 2 at the steady state level. The response is dose dependent at enzyme concentrations of 5–10 μg/ml. These results differ from those obtained with high calcium concentrations but they allow us to explain the transient stimulation by C. subunit on calcium uptake we observed [6] in the first 2–5 min and the more pronounced stimulation from 0.5–5 min noted by Käser-Glanzmann et al. [5]. Such a modulation of the effect of cAMP as a function of the calcium concentration has been previously described in the cardiac system [13]. Moreover, the C. subunit-induced increase of calcium uptake is obtained with very low Ca^{2+} concentration (1 μM) which is consistent with the inhibitory role of cAMP on platelet activation.

The similarities of the effects of both the monoclonal antibody and the C. subunit, added to the fact that they act on proteins of the same molecular mass, led us to investigate the relationship between these two effectors. This was carried out by experiments on calcium transport and phosphorylation in the presence of ALB_6 IgG together with C. subunit.

Fig.3 shows the results obtained on calcium transport: both effectors are incubated with the membrane fraction under conditions where each effector involved a stimulation by itself. We can see that in the presence of an almost saturating amount of ALB_6 IgG the steady state level does not increase by adding suboptimal concentrations of C. subunit. To determine if both effectors are directed to the same target, we then continued the investigations through the phosphorylation experiments induced by the C. subunit. The monoclonal antibody ALB_6 added to the same incubating medium never modified the C. subunit-induced phosphorylation of the 23-kDa protein

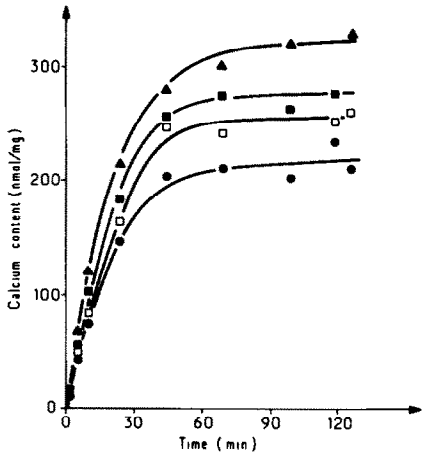

Fig.3. Effect of ALB_6 IgG and C. subunit on calcium uptake into membrane vesicles. Membrane proteins (194 μg/ml) were incubated with buffer (●—●) or with ALB_6 IgG (▲—▲) 240 μg/ml, or C. subunit (□—□) 5 μg/ml, or both proteins (■—■).

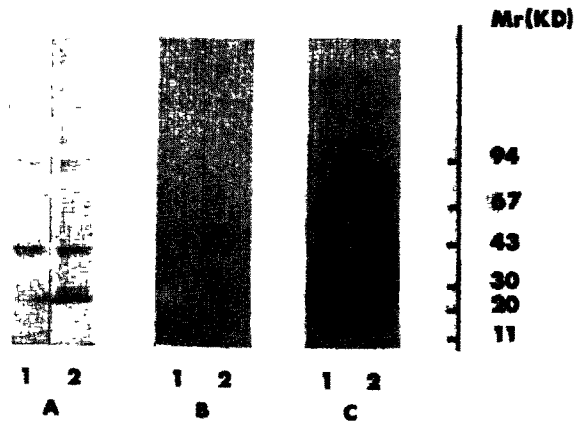

Fig.4. SDS–PAGE autoradiograms of platelet membrane proteins phosphorylated by C. subunit after immunoadsorption treatment described in section 2. The membrane vesicles were treated with the immunoadsorbents linked to monoclonal antibodies ALB_6 (2) and ALB_1 (1) as a control. Membrane vesicles (1500 μg/ml) were incubated for phosphorylations with the C. subunit (25 μg/ml). (A) and (B) refer to ultrogel adsorbed proteins: (A) SDS–PAGE, (B) autoradiograms, (C) autoradiograms of treated membrane vesicles. The figure represents the molecular mass of the following markers: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lactalbumin.

(not shown). This result was obtained either by increasing ALB_6 IgG concentrations and keeping a constant C. subunit concentration or by varying C. subunit concentrations in the presence of a fixed ALB_6 IgG concentration. Hence, it is likely that the monoclonal antibody and the enzyme recognize two different sites.

To define whether these two sites belong to one or two proteins, immunoadsorption experiments were then carried out. Platelet membranes were treated with immunoadsorbents to eliminate the protein recognized by the antibody and then submitted to phosphorylation procedures in the presence of the C. subunit.

Results are given in fig.4: on the left side (A), one can see the efficiency of the immunoadsorption treatment because an ultrogel adsorbed protein of 23 kDa appears after incubation with ALB_6 but not with ALB_1 (lanes 2 and 1). This result was confirmed in a quantitative manner as assessed by the enzyme-linked immunoadsorbent assay (not shown). On the right side of the figure (C), we can see that the phosphorylation still occurs when the membrane vesicles are depleted in the protein recognized by the monoclonal antibody: phosphorylation intensities are the same in lanes 1 and 2. This result is confirmed by the absence of phosphorylation of the protein recognized by ALB_6 as shown on (B) of the same figure. Thus the immunoadsorption treatment allows us to isolate a 23-kDa protein which is not phosphorylated by the C. subunit. It seems, then, different from the phosphoprotein.

In summary, previous work has shown that the antibody recognized a 24-kDa protein in the external membranes which has been shown to be involved in platelet aggregation [10]; it now seems that it also recognizes a protein of the same molecular mass in a 100000 × *g* fraction enriched in internal membranes which actively sequesters calcium. These results raise different hypotheses:

(i) ALB_6 IgG recognizes a 24-kDa protein of the external membranes and we cannot exclude a contamination of the 100000 × *g* fraction by these membranes; the binding of the monoclonal antibody to this antigen could interfere with the ATPase associated with the external membranes and could account for the observed stimulation of calcium uptake. Boucheix et al. [10] proposed that the ALB_6 platelet interaction could involve a

calcium channel, which suggests that a relationship between the different systems would exist in calcium transport, i.e., ATPase pump and calcium channel. However, we cannot exclude that either the increase of calcium uptake or the activation of whole platelets induced by this antibody is directly triggered by the interaction of the antigen: this calcium effect could be the secondary effect of an activation process.

(ii) ALB_6 IgG might recognize a protein on internal membranes which would be different from the phosphoprotein and which would play a role on calcium uptake. Then the use of the ALB_6 monoclonal antibody and the C. subunit allowed us to distinguish between two proteins of the same molecular mass, both involved in calcium fluxes. Interestingly, both sarcolemmal and sarcoplasmic reticulum membranes in the cardiac system have been described as bearing a 23-kDa protein which modulates calcium fluxes. The activator of the $(Ca^{2+} + Mg^{2+})$-ATPase is the phospholamban which binds the enzyme of the sarcoplasmic reticulum [18] and the calciductin is suggested to constitute the gate responsible for opening the surface membrane Ca^{2+} channel [19]. These latter proteins would differ by their isoelectric point and their capacity to be phoshorylated [19]. The question of the similarities between the two proteins is still open [20–22]. A salient feature of the results presented here is that there exists a family of proteins of similar molecular mass which regulate calcium movements directly or indirectly. A more profound study of the structure of the platelet proteins and their involvement in calcium fluxes is being further investigated on purified external and internal platelet membranes [23].

ACKNOWLEDGEMENT

Rhone Poulenc Laboratories (Paris) are fully acknowledged for their grant.

REFERENCES

[1] Detwiler, T.C., Charo, I.F. and Feinman, R.D. (1978) Thrombos. Haemostas. (Stuttg.) 40, 207–211.
[2] Massini, P., Käser-Glanzmann, R. and Lüscher, E.F. (1978) Thrombos. Haemostas. (Stuttg.) 40, 212–217.
[3] Rink, T.J., Smith, S.W. and Tsien, R.Y. (1982) FEBS Lett. 148, 21–26.
[4] Vickers, J.D., Kinlough-Rathbone, R.L. and Mustard, J.F. (1982) Blood 60, 1247–1250.
[5] Käser-Glanzmann, R., Jakabova, M., George, J.N. and Lüscher, E.F. (1977) Biochim. Biophys. Acta 446, 429–440.
[6] Le Peuch, C., Le Peuch, D., Katz, S., Demaille, J., Hinckle, M., Bredoux, R., Enouf, J., Levy-Toledano, S. and Caen, J. (1983) Biochim. Biophys. Acta 731, 456–464.
[7] Enouf, J. and Levy-Toledano, S. (1984) Br. J. Pharmacol. 81, 509–518.
[8] De Metz, M., Lebret, M., Enouf, J. and Levy-Toledano, S. (1984) Biochim. Biophys. Acta 770, 159–165.
[9] Käser-Glanzmann, R., Gerber, E. and Lüscher, E.F. (1979) Biochim. Biophys. Acta 558, 344–347.
[10] Boucheix, C., Soria, C., Mirshahi, M., Soria, J., Perrot, J.Y., Fournier, N., Billard, M. and Rosenfeld, C. (1983) FEBS Lett. 161, 289–295.
[11] Lotersztajn, S., Hanoune, J. and Pecker, F. (1981) J. Biol. Chem. 256, 11209–11215.
[12] Boucheix, C., Perrot, J.Y., Mirshahi, M., Bernadou, M. and Rosenfeld, C.J. (1983) J. Immunol. Meth. 57, 145–150.
[13] Boucheix, C., Perrot, J.Y., Mirshahi, M., Giannoni, F., Billard, M., Bernadou, A. and Rosenfeld, C. (1985) Leukemia Res., in press.
[14] IUIS-WHO. Nomenclature Subcommittee on Human Leukocyte Differentiation Antigens (1984) Immunol. Today 5, 158–159.
[15] Kahn, A., Cottreau, D., Daegelen, D. and Dreyfus, J.C. (1981) Eur. J. Biochem. 116, 7–12.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Enouf, J., Bredoux, R. and Levy-Toledano, S. (1984) Biochim. Biophys. Acta 772, 251–258.
[18] Kirchberger, M.A. and Tada, M.L. (1976) J. Biol. Chem. 251, 725–729.
[19] Rinaldi, M.L., Le Peuch, C.J. and Demaille, J.G. (1981) FEBS Lett. 127, 139–143.
[20] Will, H., Levchenko, T.S., Levitsky, D.O., Smirnov, V.N. and Wollenberger, A. (1978) Biochim. Biophys. Acta 543, 175–193.
[21] Lamers, I.M.J. and Stinis, J.T. (1980) Biochim. Biophys. Acta 624, 443–469.
[22] Manalan, A.S. and Jones, L.R. (1982) J. Biol. Chem. 257, 10052–10062.
[23] Enouf, J., Bredoux, R., Boizard, B., Wautier, J.L., Chap, H., Thomas, J., De Metz, M. and Levy-Toledano, S. (1984) Biochem. Biophys. Res. Commun. 123, 50–58.