Biochem. J. (1994) 297, 343–350 (Printed in Great Britain)

Correlated expression of the 97 kDa sarcoendoplasmic reticulum Ca^{2+}-ATPase and Rap1B in platelets and various cell lines

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Evidence has accumulated that cyclic AMP (cAMP)-induced phosphorylation of a Ras-related protein (Rap1) regulates platelet Ca^{2+} transport. As this transport was recently found to be controlled by two isoforms of sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA), the 100 kDa SERCA2b and the newly identified 97 kDa SERCA, we attempted to establish which isoform is involved in this regulation. For this purpose, we studied the expression and regulation of both the SERCA and Rap1 isoforms in platelets, haemopoietic cells and various cancer cell lines. SERCA2b was shown to be equally expressed in all the cell lines tested, as determined by detection of its phosphoenzyme formation and by Western blotting using an isoform-specific antibody. In contrast, the expression of the 97 kDa SERCA, studied by the same methods, varied from total absence in the cancer cells to high levels in the megakaryocytic cell lines. With regard to the potential regulatory Rap1 proteins, Western blotting showed different expression of total Rap1 isoforms among the cell lineages, thus ruling out any possible relationship between Rap1 and SERCA2b. However, the expression of Rap1 proteins correlated with that of the 97 kDa SERCA isoform. More refined analysis of the *rap*1A and *rap*1B isoforms by reverse transcription PCR and by determining cAMP-induced phosphorylation of Rap1B, i.e. its functional mechanism, confirmed the correlation between Rap1B and the 97 kDa SERCA expression. This relationship was also established by the concerted up-regulation of these two proteins demonstrated in the pathological model of platelets from hypertensive rats. It is concluded that the expressions of 97 kDa SERCA and Rap1B are related, suggesting that regulation of the platelet Ca^{2+}-ATPase system by cAMP-induced phosphorylation of Rap1B specifically involves the 97 kDa SERCA.

INTRODUCTION

Cytosolic Ca^{2+} concentration plays an important role in platelet function. An increase in this concentration is a necessary event for platelet activation, which involves a shape change and exocytosis processes leading to aggregation [1–4]. This increase in cytosolic Ca^{2+} concentration is brought about by both Ca^{2+} influx from the extracellular medium and inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release from intracellular Ca^{2+} stores [5,6], and is controlled by a specific Ca^{2+}-ATPase system. Thus cells generally express both plasma-membrane Ca^{2+}-ATPases, which extrude Ca^{2+} ions from the cell [7], and sarcoendoplasmic reticulum Ca^{2+}-ATPases (SERCAs), which sequester Ca^{2+} in intracellular Ca^{2+} pools [8]. In platelets, the Ca^{2+}-ATPase system was recently demonstrated to consist of two distinct isoforms of the SERCA family, the 100 kDa SERCA2b isoform and an as yet unidentified 97 kDa SERCA isoform [9–11].

Platelet activation can be reversed by triggering agents which raise the level of cyclic AMP (cAMP) which is known to activate cAMP-dependent protein kinase. As this reversal was found to be associated with a decrease in intracellular Ca^{2+} concentration, it was thought to occur through regulation of a Ca^{2+}-dependent process such as an action on Ca^{2+}-ATPases [12,13]. An interesting early finding was that the addition of cAMP to a cytosolic fraction containing cAMP-dependent protein kinase stimulated platelet Ca^{2+} transport and phosphorylated a 22–24 kDa protein [14]. Various authors subsequently confirmed this finding, using different approaches. First, Fox *et al.* [15,16] treated intact platelets with agents that increased the cAMP level, and demonstrated that a 22 kDa membrane protein was phosphorylated concomitantly with an increase in Ca^{2+} uptake mediated by the Ca^{2+}-ATPases. This effect of cAMP on both the phosphorylation of a 22 kDa protein and Ca^{2+} uptake was further confirmed by treating isolated membrane vesicles with the purified catalytic subunit of the cAMP-dependent protein kinase (C.Sub.) [17–19]. However, this correlation between phosphorylation of the 22 kDa protein and Ca^{2+} transport was recently the subject of some controversy [20–22], based on two observations: first, the C.Sub. did not stimulate Ca^{2+} uptake [20,21], and secondly, the time course of cAMP-induced phosphorylation of the 24 kDa protein was different from that of the regulation of Ca^{2+}-ATPase [22]. The first studies showed that the increased uptake of Ca^{2+} correlated with the phosphate concentration of the commercial C.Sub. solution, and not with its enzyme activity [20,21]. However, this cannot explain the results obtained with membrane fractions isolated from platelets previously treated with cAMP-enhancing agents and without using C.Sub. [15,16]. In the third study, Fischer and White [22] used very short phosphorylation times, and subsequent studies have now clearly demonstrated that the 22 kDa protein cannot be phosphorylated under these conditions [23], highlighting the issue of the identity of the protein involved.

The presence of low-molecular-mass GTP-binding proteins

Abbreviations used: cAMP, cyclic AMP; Rap, Ras-proximate protein; SERCA, sarcoendoplasmic reticulum Ca^{2+}-ATPase; RT-PCR, reverse transcription PCR; IP_3, inositol 1,4,5-trisphosphate; C.Sub., catalytic subunit of cAMP-dependent protein kinase; DTT, dithiothreitol; WKY, Wistar–Kyoto; SHR, spontaneously hypertensive rats; MMLV-RT, murine Moloney leukaemia virus reverse transcriptase; 1 × SSC, 15 mM sodium citrate/150 mM NaCl.

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has been demonstrated in human platelets [24–29]; one of these proteins was phosphorylated *in vivo* in response to an increase in cAMP concentration in intact platelets induced by prostaglandin E_1, and *in vitro* by the cAMP-dependent protein kinase [29]. This GTP-binding protein was also found to be Ras-related [28,30,31] and the most recent studies established its identity precisely as a Rap1 protein [32,33]. As Rap1 proteins themselves consist of two isoforms, Rap1A and Rap1B [34–36], the initially described cAMP-induced 22 kDa platelet phosphoprotein was shown to be the Rap1B isoform [32], i.e. regulation of platelet Ca^{2+}-ATPases in fact occurred via cAMP phosphorylation of Rap1B [33], which is evidence for a new role for Rap1 in platelet function inhibition. In addition, recent investigations have demonstrated other possible roles for Rap1 related to platelet activation. Fischer et al. [37] demonstrated that Rap1B is associated with the cytoskeleton during platelet activation and therefore concluded that Rap1B is a component of the activation-dependent cytoskeleton. Recently, Torti and Lapetina [38] proposed that Rap1B might form a complex with Ras–GTPase-activating protein and serve as a signal to recruit phospholipase Cγ1 to the cell membrane, where it can act on membrane-bound inositol phospholipids.

Thus, the aim of the present study was to advance the understanding of the role of Rap1B in platelet function by further investigating its role in Ca^{2+}-ATPase activity. To do this, we first attempted to establish which of the two newly distinguished platelet Ca^{2+}-ATPases is the target of the Rap1B protein, using an approach based on the hypothesis that, if this protein really regulates the activity of Ca^{2+}-ATPases, the expression and regulation of the interrelated proteins should be concerted.

We thus investigated the possible relationship between the expression of Rap1 and SERCA proteins by testing their relative expression and regulation in platelets and selected cell lines. The presence of the different SERCA isoforms and their levels of expression were studied by the detection of specific Ca^{2+}-ATPase phosphoenzymes and by Western blotting with SERCA isoform-specific antibodies. For Rap1 proteins, we first tested the total expression of Rap1A and Rap1B isoforms by Western blotting, using an anti-Rap1 antibody. Next, we assayed the Rap1B protein by studying its cAMP-induced phosphorylation on isolated membranes, and comparing the mRNA levels corresponding to the *rap*1A and *rap*1B isoforms by PCR followed by Southern blotting with specific cDNA probes. The overall results showed that the 100 and 97 kDa platelet-type Ca^{2+}-ATPases are differentially expressed in platelets and various cell lines. In addition, the expression of the 97 kDa SERCA clearly correlated with that of the regulatory protein, Rap1, and more specifically with that of its Rap1B isoform.

EXPERIMENTAL

Cell culture

This study was conducted on human platelets and the following human cell lines: Dami (megakaryoblastoid [39]), HEL (human erythroleukaemic [40]), HL-60 (promyelocytic [41]), Jurkat (T lymphoblastoid), HeLa (epithelial-like), KATO III (gastric carcinoma), MG-63 (osteosarcoma), DU 4475 (breast carcinoma), SK-MEL-1 (melanoma) and Y79 (retinoblastoma) cells.

All cell lines were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.), except the Dami cell line which was generously given by Professor J. Peries, U107 INSERM, Paris, France. Dami, HEL, HL-60, Jurkat, KATO III, DU 4475 and Y79 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (Boehringer, Mannheim, Germany). HeLa, MG-63 and SK-MEL-1 cells were cultured similarly, but RPMI medium was replaced by Eagle's minimal essential medium with non-essential amino acids and Earle's balanced salt solution. All cell lines were incubated and cultured at 37 °C with 5% CO_2 in a humidified atmosphere.

Membrane preparation

The cells grown in suspension were harvested by centrifugation at 150 *g* for 10 min and lysed in an ice-cold buffer containing 10 mM Hepes, pH 7, 10 mM KCl, 0.05 mM EGTA, 0.05 mM dithiothreitol (DTT) and a mixture of protease inhibitors (0.1 mg/ml soyabean trypsin inhibitor, 0.05 mg/ml aprotinin and 0.01 mg/ml leupeptin; Sigma, St. Louis, MO, U.S.A.). Cell suspensions were homogenized in a Teflon/glass homogenizer at 4 °C, sonicated on ice and centrifuged for 10 min at 4 °C and 3500 *g*. The supernatants were ultracentrifuged for 60 min at 4 °C and 100000 *g* and the resulting pellets were resuspended in 17 mM Hepes, pH 7, buffer containing 160 mM KCl and 0.1 mM DTT. The resulting microsomes were frozen and stored at −80 °C.

Human platelet membranes were prepared by a similar method, as described previously [33,42], and platelet membranes from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) rats were isolated as described by Papp et al. [43].

Formation of the phosphorylated intermediate (E ~ P) of Ca^{2+}-ATPases

Ca^{2+}-ATPases of various cell membranes were autophosphorylated on ice in a reaction buffer containing 17 mM Hepes, pH 7, 160 mM KCl, 1 mM DTT and 0.05 mM $CaCl_2$, in the presence or absence of 0.1 mM EGTA. The reactions were started by adding 0.05 μM [γ-^{32}P]ATP (2×10^5 c.p.m./μl; Amersham International). After 1 min, they were stopped by adding ice-cold 6% trichloroacetic acid solution containing 1 mM ATP and 10 mM phosphoric acid. The resulting precipitates were washed in trichloroacetic acid, dissolved in the electrophoresis sample buffer and submitted to acidic SDS/PAGE (7.5% gels) by the method of Papp et al. [9]. After electrophoresis, proteins were transferred on to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and autoradiographed for 48 h at −80 °C using Kodak X-Omat AR films.

Antibody characteristics

The IID8 and PL/IM 430 anti-Ca^{2+}-ATPase monoclonal antibodies were kindly given by Dr. K. Campbell, Iowa City, IO, U.S.A. and Dr. N. Crawford, London, U.K. respectively. IID8 is a mouse monoclonal antibody raised against dog cardiac muscle SERCA by Grover et al. [44]. This antibody recognized a shared epitope present on cardiac muscle (SERCA2a) as well as on smooth muscle (SERCA2b [45]). PL/IM 430 was elicited against platelet internal membranes, inhibited the active Ca^{2+} transport across these membranes [46] and specifically recognized the 97 kDa SERCA isoform [11].

Anti-Rap1 antibody was elicited in rabbits against a synthetic peptide encompassing residues 121–137 of Rap1 and common to both Rap1A and Rap1B. The preparation and specificity of this antibody have both been previously described [47], and it has been purified on an agarose column coupled to the immunizing peptide. Briefly, the anti-Rap1 antibody recognized and immuno-

precipitated a single band of 22 kDa in extracts from recombinant bacteria induced to produce Rap1A in Rat-1 fibroblasts and in HL-60 cells, which amply express this isoform [48]. Neither recombinant Rap2 nor Ha-Ras proteins reacted with this antibody.

SDS/PAGE and Western blotting

Membrane fractions were solubilized and reduced for 30 min at room temperature in 50 mM Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 0.01% (v/v) Bromophenol Blue, 25% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol. Samples were analysed by SDS/PAGE (8% gels), electroblotted on to nitrocellulose membranes and blocked overnight at room temperature in quenching solution [10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5% (w/v) fat-free dry milk and 0.1% (v/v) Tween 20]. The membranes were then incubated for 1 h at room temperature with the first antibody (a 1:1000 dilution of either IID8 or anti-Rap1 antibody or a 1:5 dilution of hybridoma supernatant for PL/IM 430) in quenching solution. Nitrocellulose membranes were washed five times with the quenching solution, and further incubated for 1 h with the second antibody–peroxidase conjugates (1:1000 dilution of anti-mouse IgG for PL/IM 430 and IID8, or 1:10000 dilution of anti-rabbit IgG for anti-Rap1 antibody) in quenching solution. After further washing, peroxidase staining was revealed with a chemiluminescence kit (Amersham International) and autoradiography was performed with Kodak X-Omat AR films at room temperature. The bands were quantified by densitometry using an LKB Ultrascan XL laser densitometer.

Membrane protein phosphorylation induced by cAMP

cAMP-induced phosphorylation was conducted on cell membranes. Cell membranes were incubated at 30 °C for 10 min in 10 mM sodium phosphate buffer, pH 7, containing 14 mM $MgSO_4$, 100 mM NaF and 0.1% (v/v) Triton X-100, in the presence of 10 μg/ml C.Sub. (Sigma; enzyme/substrate ratio of 0.02) or without C.Sub. Reactions were started by adding 6 mM [γ-^{32}P]ATP (460 MBq/mmol) and stopped with an equal volume of solubilization buffer [50 mM Tris/HCl, pH 6.8, 1% (w/v) SDS, 0.01% (v/v) Bromophenol Blue and 25% (v/v) glycerol]. cAMP-induced phosphorylated proteins were reduced with 5% 2-mercaptoethanol, separated by SDS/PAGE (11% gels), electroblotted on to nitrocellulose membranes, and autoradiographed for 24–48 h at −80 °C using Kodak X-Omat AR films.

RNA extraction

Total RNA was isolated from various cell lines as described by Chomczynski and Sacchi [49]. Briefly, cells were lysed using guanidinium thiocyanate denaturing solution. RNA was then extracted at 4 °C with phenol/chloroform, precipitated with propan-2-ol, analysed on 1% agarose gel and stored at −80 °C.

PCR

For *rap*1A and *rap*1B amplification, two synthetic 3′ primers (21-mer) were chosen in the non-conserved region (bp 508–528 for both) to ensure that they were specifically complementary to either the *rap*1A or *rap*1B nucleotide sequences. As regards the 5′ primer, a 23-mer oligonucleotide (bp 167–189) that was common to both sequences was designed. For SERCA2b amplification, we used two synthetic primers (bp 3110–3130 for the 3′ primer and bp 2861–2882 for the 5′ primer), the 3′ primer being located in the 3′ region of the mRNA, after the splicing site, because the SERCA2b isoform and the cardiac muscle isoform, SERCA2a, have a common sequence up to the 2980 bp site of the 3′ end region, but thereafter their sequences differ.

Total RNA template (250 ng) was denatured by heating to 75 °C for 3 min and annealed to 21 pmol of the 3′ primers corresponding to *rap*1A, *rap*1B and SERCA2b respectively, by slow cooling to 30 °C. Reverse transcription was carried out by adding to this preparation 200 units of murine Moloney leukaemia virus reverse transcriptase (MMLV-RT) (USB, Cleveland, OH, U.S.A.) and incubating it for 1 h at 42 °C in PCR buffer containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin and 2.5 mM each dATP, dTTP, dCTP and dGTP, in a total volume of 20 μl. Reverse transcription was terminated by heat-inactivating the MMLV-RT for 10 min at 95 °C. The PCR, first described by Saïki et al. [50], was carried out in the same reaction mixture, adjusted to 100 μl with the PCR buffer containing 20 pmol of the 5′ primers (*rap*1A, *rap*1B and SERCA2b) and 2.5 units of *Taq* polymerase (Boehringer). PCR consisted of denaturation at 95 °C for 45 s, annealing at 55 °C for 1 min and extension at 70 °C for 2 min, for 35 cycles, in an automated thermocycler (Hybaid, Teddington, Middx., U.K.). A negative control was run in the absence of reverse transcriptase, in order to check the specificity of the amplification.

Southern blotting

The PCR products (one-tenth of the reaction volume) were submitted to electrophoresis on 1.5% ethidium bromide-stained agarose gel, and transferred on to Biohylon Z+ membranes (Bioprobe, Montreuil-sous-Bois, France) as described by Sambrook et al. [51]. Membranes were prehybridized for 2 h at 65 °C in a hybridization oven, in the presence of the hybridization solution (5 × SSC, 5 × Denhardt's solution, 0.1% SDS and 100 μg/ml denatured herring sperm DNA). Hybridization was performed overnight at 65 °C in the same solution in the presence of 2×10^6 c.p.m./ml of the labelled cDNA probes. The following three probes were labelled with [α-^{32}P]dCTP by random priming (Random Priming DNA Labelling Kit, Boehringer): the *rap*1A probe was a 760 bp *Hind*III–*Eco*RI cDNA corresponding to total human *rap*1A [34], the *rap*1B probe, a 730 bp *Hind*III–*Eco*RI cDNA totally covering human *rap*1B [36], and the SERCA2b probe, an 848 bp cDNA fragment of SERCA2b, obtained by screening of a human platelet cDNA library [10]. Membranes were washed twice at high stringency in 1 × SSC/0.1% SDS for 20 min at 55 °C, and twice in 0.1 × SSC/0.1% SDS for 20 min at 65 °C (55 °C for the SERCA2b probe). Southern blots were autoradiographed with Kodak X-Omat AR films for 2 h.

RESULTS

Formation of Ca^{2+}-ATPase phosphorylated intermediates in various cell lines

To establish whether the 100 kDa SERCA2b or the 97 kDa SERCA platelet Ca^{2+}-ATPase is the target of the Rap1 protein regulating Ca^{2+} transport, we looked for cells expressing one or both of these SERCAs, preferably in different amounts. For this purpose, our choice of cell lines was based on the results of our preliminary investigation, which showed that the 97 kDa SERCA was present in several haemopoietic cell lines and absent from a cancer cell line [11]. Consequently, we tested a panel of haemopoietic and cancer cell lines, and investigated isolated membranes for the presence of the different SERCA isoforms by following the formation of the phosphorylated intermediate (E∼P) complex corresponding to the transient step in the catalytic cycle of these isoforms (Figure 1).

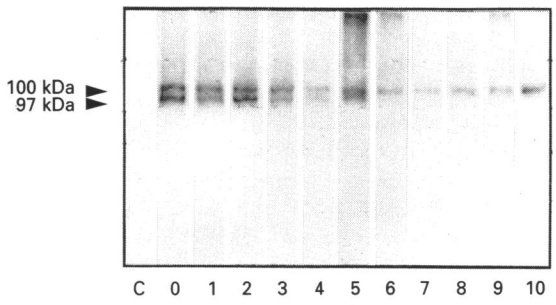

Figure 1 Demonstration of the presence of different SERCA isoforms in human platelets and various cell lines

Mixed membrane fractions (100000 *g*) were isolated from either human platelets or different cell lines. They were phosphorylated on ice by 0.05 μM [γ-^{32}P]ATP in the presence of 0.05 mM $CaCl_2$, with or without 0.1 mM EGTA as described in the Experimental section. After trichloroacetic acid precipitation, the phosphorylated proteins were processed by SDS/PAGE (7.5% gels), electroblotted on to nitrocellulose, and autoradiographed at −80 °C for 48 h. Lane C, phosphorylation of 100 μg of mixed human platelet membranes in the presence of 0.1 mM EGTA; lanes 0–10, phosphorylation, in the presence of 0.05 mM $CaCl_2$, of 100 μg of membranes from platelets and the following cells: Dami, HEL, HL-60, Jurkat, HeLa, KATO III, MG-63, DU 4475, SK-MEL-1 and Y79 respectively. The molecular masses of the Ca^{2+}-ATPases, estimated with standard markers, are indicated. This Figure is typical of four experiments.

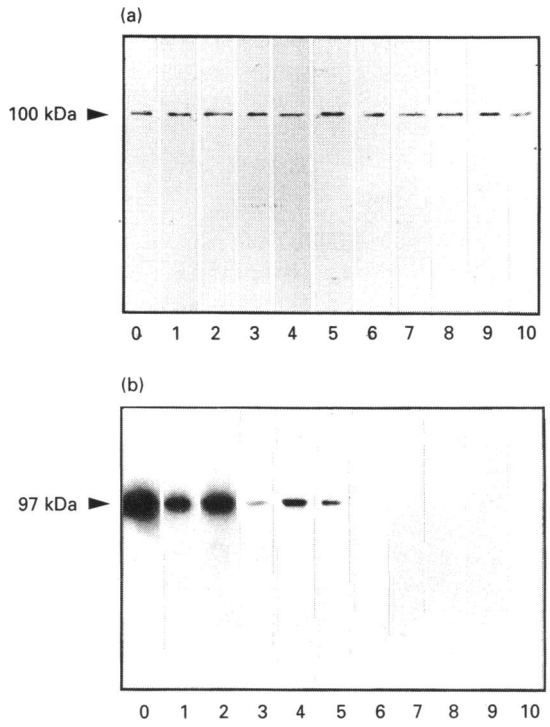

Figure 2 Expression of SERCA2b and 97 kDa SERCA in the different cells tested

Various cell membranes (100 μg of each) were reduced, analysed by SDS/PAGE (8% gels) and electroblotted on to nitrocellulose sheets. Immunodetection of SERCA2b (**a**) was performed by Western blotting using IID8 antibody, and that of the 97 kDa SERCA proteins by using PL/IM 430 antibody (**b**), as described in the Experimental section. Lanes 0–10 were respectively loaded with membranes isolated from platelets, and from Dami, HEL, HL-60, Jurkat, HeLa, KATO III, MG-63, DU 4475, SK-MEL-1 and Y79 cells. The molecular masses of the proteins detected, estimated with standard markers, are indicated on the left. (**a**) and (**b**) are each typical of six experiments.

When human platelets were phosphorylated in the presence of 0.1 mM EGTA, no phosphorylation was detected (Figure 1, lane C) thus showing the Ca^{2+} specificity of the E∼P formation. The presence of the 100 and 97 kDa Ca^{2+}-ATPases was checked by phosphorylating human platelet membranes in the presence of 0.05 mM Ca^{2+} (lane 0). Lanes 1–5 show the phosphorylation of the Ca^{2+}-ATPases of the haemopoietic cell lines Dami, HEL, HL-60 and Jurkat (lanes 1–4 respectively) and the epithelial-like HeLa cells (lane 5). These results demonstrate the presence of both 100 and 97 kDa phosphorylated bands in all the above cell lines. In contrast, only the 100 kDa isoform was present in the cancerous cell lines, i.e. KATO III, MG-63, DU 4475, SK-MEL-1 and Y79 (lanes 6–10 respectively).

Two groups of cells can therefore be distinguished in terms of their Ca^{2+}-ATPase systems. The first includes the haemopoietic cells, which express both the 100 kDa and 97 kDa SERCAs, and the second, the cancerous cells, which express only the 100 kDa Ca^{2+}-ATPase.

Expression of SERCA2b and 97 kDa SERCA

To check the exact identity of the Ca^{2+}-ATPases corresponding to these two SERCAs, and to estimate their relative expression, we performed Western blotting of cell membranes. We used the IID8 and PL/IM 430 monoclonal antibodies previously shown to react specifically in human platelets with the 100 kDa SERCA2b and 97 kDa SERCA respectively [11]. The IID8 antibody recognized the 100 kDa band in all the cell lines tested (Figure 2a, lanes 0–10); the corresponding SERCA2b protein was expressed in similar amounts.

When the same cell membranes were then immunoblotted with the anti-(97 kDa SERCA) antibody PL/IM 430, the results established that the 97 kDa SERCA expressed in the haemopoietic cells (Figure 2b, lanes 0–4) and epithelial cells (lane 5) is the same as the SERCA in platelets, and that it is not expressed in the cancer cell lines tested (Figure 2b, lanes 6–10). In addition, as expected with this fairly quantitative technique, large differences were found in the relative expression of this 97 kDa SERCA. It was interesting to note that its most abundant expression was in the haemopoietic cells, especially the megakaryocytic cells (platelets, Dami and HEL cells, lanes 0–2), but it was also detectable in the HeLa epithelial cells (lane 5). Thus, there were striking differences between the relative expression of SERCA2b and 97 kDa SERCA. Whereas SERCA2b seemed to be expressed equally in the different cell lines, the 97 kDa SERCA was expressed in a specific manner in the haemopoietic cells, even though its distribution in these cell types was heterogeneous.

Expression of Rap1 proteins

To explore the possible relationship between the expression of Rap1 and SERCA proteins, we first performed Western blots of cell membranes using the anti-Rap1 antibody, which recognized a common epitope on both Rap1A and Rap1B [47], as described in the Experimental section. Rap1 proteins are known to display an electrophoretic-mobility shift which varies depending on whether reducing (24 kDa) or non-reducing conditions (22 kDa [33]) are used. Total expression of Rap1A and Rap1B (Figure 3) showed that the membranes isolated from all the cell types contained Rap1 proteins (lanes 0–10), as indicated by the recognition of the 24 kDa band by the antibody under reducing conditions. However, there were large differences in the relative

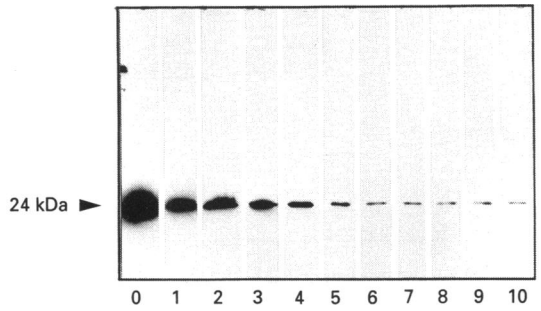

Figure 3 Western blotting of Rap1 proteins in the different cells tested

Membranes were isolated from the different cells, analysed by SDS/PAGE (8% gels) and electroblotted on to nitrocellulose. Immunodetection of Rap1 proteins was conducted using the anti-Rap1 antibody common to the Rap1A and Rap1B isoforms. Lanes 0–10 were respectively loaded with 100 μg of membranes from platelets and the following cells: Dami, HEL, HL-60, Jurkat, HeLa, KATO III, MG-63, DU 4475, SK-MEL-1 and Y79. The molecular mass on the left indicates the size of the Rap1 proteins, estimated with standard markers. This Figure is typical of six experiments.

expression of these proteins. As for the expression of 97 kDa SERCA, two groups of cell lines were distinguished: in the first, Rap1 expression was strong and heterogeneously distributed, and in the second, it was weak but equally distributed. The first group of cell lines comprised exactly the same cells as those that expressed the 97 kDa SERCA, i.e platelets, Dami and HEL cells (Figure 3, lanes 0, 1 and 2 respectively) in which the largest amounts of Rap1 proteins were present, HL-60 and Jurkat cells (lanes 3 and 4) in which intermediate amounts were present, and HeLa cells (lane 5) in which a small amount was present. The second group consisted of the five other cancer cell lines (lanes 6–10). This study of total Rap1 protein expression shows that it is not related to expression of the 100 kDa SERCA2b, but that it is related to that of the 97 kDa SERCA. Thus a clear relationship emerged between (1) the cells that expressed the largest amounts of 97 kDa SERCA and Rap1 proteins and (2) the cells that did not express the 97 kDa SERCA and only expressed Rap1 proteins to a low level. Exceptionally, for the HL-60 cells, which belonged to the first group, the relationship between the expression of 97 kDa SERCA and Rap1 was weaker.

Determination of *rap*1A and *rap*1B mRNA expression using RT-PCR

To determine whether the Rap1A or Rap1B protein was related to the 97 kDa SERCA, we studied the RNAs of the cell lines tested, because no isoform-specific antibodies were available. We determined specific regions in the *rap*1A and *rap*1B nucleotide sequences, and designed primers and probes corresponding to each mRNA, as described in the Experimental section. We then performed reverse transcription followed by PCR amplification of the *rap*1A and *rap*1B cDNAs obtained from the RNAs of the different cell lines. Negative controls of the amplifications were run in the absence of reverse transcriptase, to assess the specificity of the amplification (results not shown). To obtain additional controls, we took advantage of the results in Figure 2(a), showing ubiquitous distribution of SERCA2b in all the cells tested. Thus, parallel amplification of SERCA2b cDNA allowed us to obtain an original control of the RT-PCR amplification. As expected,

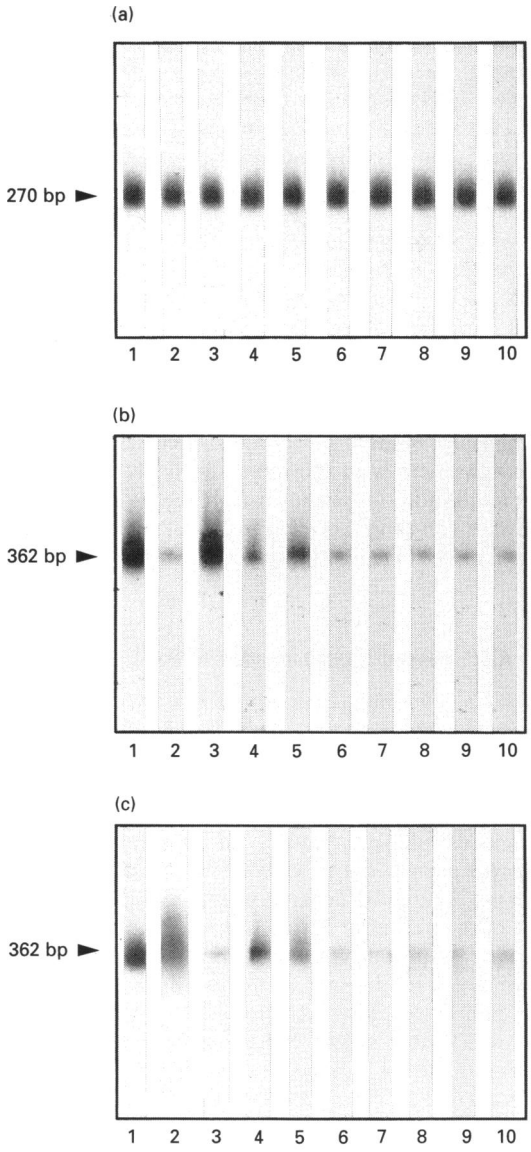

Figure 4 Comparative mRNA expression of the *rap*1A and *rap*1B isoforms, shown by RT-PCR

Total RNAs were extracted from platelets and the various cell lines as described in the Experimental section. Each RNA (250 ng) underwent reverse transcription using the specific 3′ primers and MMLV-RT under the conditions described in the Experimental section. PCR was then performed and one-tenth of the amplification products were analysed by Southern blotting using, as probes, ^{32}P-labelled cDNA of SERCA2b (**a**), *rap*1A (**b**) and *rap*1B (**c**). After different washing steps at high stringency, the blots were autoradiographed for 2 h using Kodak X-Omat AR films. Lanes 1–10 respectively show the amplification products from the RNAs of the following cells: Dami, HEL, HL-60, Jurkat, HeLa, KATO III, MG-63, DU 4475, SK-MEL-1 and Y79. Numbers on the left give the approximate sizes of the amplified SERCA2b, *rap*1A, and *rap*1B fragments. Each part is typical of six experiments.

the results obtained in the absence of reverse transcriptase did not allow the detection of any amplified products in any of the amplification systems (*rap*1A, *rap*1B and SERCA2b; results not shown).

With regard to the amplification performed with SERCA2b primers (Figure 4a), Southern blotting of the amplified fragments (270 bp) after hybridization with the labelled SERCA2b probe showed that, again as expected, identical amounts of SERCA2b mRNA were present in all cell lines tested (lanes 1–10). Figures

4(b) and 4(c) respectively show the Southern blots resulting from hybridization at high stringency of the amplified fragments of *rap*1A and *rap*1B (362 bp) with the respective labelled probes. The haemopoietic cell lines exhibited different amounts of mRNA for *rap*1A and *rap*1B: Dami and HL-60 cells (Figure 4b, lanes 1 and 3) contained the most *rap*1A, and HEL cells, the most *rap*1B (Figure 4c, lane 2). In contrast, the tumour cell lines KATO III, MG-63, DU 4475, Y79 and SK-MEL-1 (Figures 4b and 4c, lanes 6–10 respectively) seemed to possess identical and low mRNA levels for both *rap*1A and *rap*1B.

The mRNA levels of both *rap*1A and *rap*1B totally corroborated the results for total Rap1 proteins and confirmed, at the RNA level, the relationship established between the 97 kDa SERCA and Rap1 proteins. These results also explain why the HL-60 cell line constitutes an exception to the Rap1/97 kDa SERCA relationship, because it exhibits particularly high levels of Rap1A, in agreement with previous reports [47,48]. Finally, these results provide additional information about the identity of the Rap isoform involved in this relationship, as it seems that the correlation with the 97 kDa SERCA involved Rap1B rather than Rap1A.

Comparative cAMP-induced phosphorylation of Rap1 proteins

As the mechanism governing the action of Rap1 proteins on the SERCAs is their cAMP-induced phosphorylation, it seemed essential to first study this functional property of Rap1B in the relationship with the 97 kDa SERCA, as only this isoform was found to be phosphorylated in platelets [32]. For this, we carried out cAMP-induced phosphorylation on membranes isolated from the selected representative cells, using C.Sub. The same experiments were conducted in the absence of C.Sub. to check the specificity of the phosphorylation by cAMP. Figure 5 shows the comparative results of a typical experiment. No phosphorylated Rap1 proteins were detected in the membranes of any cell type in the absence of C.Sub., as expected for these negative controls. Figure 5 further shows that the cAMP-induced major phosphorylation of the 24 kDa band occurred in platelets (lane 1, +C.Sub.) and HEL cells (lane 3, +C.Sub.). In Dami cells (lane 2, +C.Sub.), the amount of Rap1 protein phosphorylated was smaller, and the same applied to Jurkat cells (lane 5, +C.Sub.) but to a lesser extent. HL-60, KATO III and DU 4475 cells (lanes 4, 6, and 7, +C.Sub. respectively) exhibited very weak Rap1 phosphorylation. These results totally corroborate the data obtained from Rap1B expression at the RNA level, because the different amounts of Rap1B mRNA shown by RT-PCR fit the amounts of protein phosphorylated by cAMP, and most of the Rap1B in platelet membranes was found to be phosphorylated by cAMP. Thus, the amount of 97 kDa SERCA is related not only to the amount of Rap1B protein, but also to this protein's capacity for cAMP-induced phosphorylation, i.e. to its mode of regulating Ca^{2+}-ATPases.

Concerted up-regulation of the 97 kDa SERCA and Rap1 proteins

Finally, to establish the relationship between the expression of the 97 kDa SERCA and Rap1 proteins, we used the results of our recent investigations, which showed overexpression of the 97 kDa SERCA protein in platelets isolated from SHR compared with normotensive WKY rat platelets [43]. If the expression of 97 kDa SERCA and Rap1 proteins is correlated, they should be regulated in the same way. Consequently, we attempted to establish how the expression of Rap1 proteins is regulated in SHR platelets, which exhibit the same Ca^{2+}-ATPase system as human platelets, consisting of the 100 kDa SERCA2b and

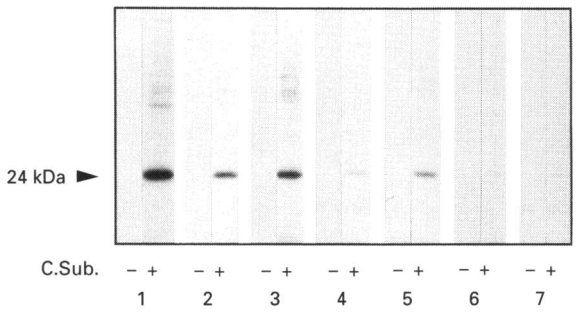

Figure 5 cAMP-induced phosphorylation of Rap1 protein in different cell lines

Membrane proteins (100 μg) were isolated from platelets and from Dami, HEL, HL-60, Jurkat, KATO III and DU 4475 cells. They were phosphorylated for 10 min at 30 °C by cAMP-dependent protein kinase, using 10 μg/ml C.Sub, reduced with 2-mercaptoethanol, separated by SDS/PAGE (11% gels), transferred on to nitrocellulose and autoradiographed for 48 h. To check the specificity of the experiment, cAMP-induced phosphorylation was conducted in either the absence (−) or presence (+) of C.Sub. Lanes 1–7 respectively show the cAMP-induced phosphorylation of cell membranes from platelets, and Dami, HEL, HL-60, Jurkat, KATO III and DU 4475 cells. The approximate molecular mass of Rap1 protein, estimated with standard markers, is given on the left. This Figure is representative of three experiments.

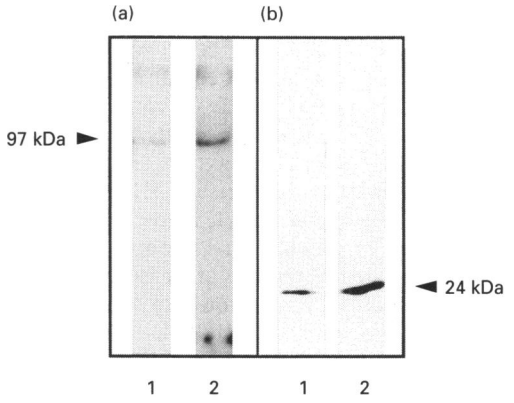

Figure 6 Comparative expression of the 97 kDa SERCA and Rap1 proteins in platelets of normal WKY rats and SHR

Platelet membranes (obtained by 100000 **g** centrifugation) were isolated from 16-week-old WKY rats and SHR. These membranes (100 μg) were then submitted to electrophoresis, electrotransferred on to nitrocellulose and immunoblotted with either an anti-(rat 97 kDa SERCA) antibody (**a**) or the anti-Rap1 antibody (**b**), as described in the Experimental section. Lane 1, membranes isolated from WKY rat platelets; lane 2, membranes isolated from SHR platelets. The approximate molecular masses of the 97 kDa SERCA and Rap1 proteins, estimated with standard markers, are indicated. This Figure is representative of three experiments.

97 kDa SERCA [43]. Figure 6 shows the expression of 97 kDa SERCA and Rap1 proteins in SHR and WKY rat platelet membranes, as revealed by Western blotting. Figure 6(a) shows that the amount of 97 kDa SERCA was larger in SHR platelets (lane 2) than in normal platelets (lane 1), as previously observed [43]. Figure 6(b) shows that there was also a larger amount of Rap1 proteins in SHR platelets (lane 2) than in WKY rat platelets (lane 1). Densitometric measurements allowed estimation of the parallel increase in 97 kDa SERCA and Rap1 expression, which reached a factor of 3. Consequently, this finding confirms and completes the results showing a correlated expression of Rap1 and 97 kDa SERCA in the various cell lines

tested, as they were both overexpressed in the platelets of the SHR essential hypertension model.

DISCUSSION

This study demonstrates a clear relationship between the expression and regulation of the 97 kDa SERCA and Rap1B protein in human platelets and various cell lines. This was shown, first of all, by the identical expression of 100 kDa SERCA2b protein in all the cell lines studied, both at the qualitative and quantitative levels. In addition, the study of the expression of the SERCA2b isoform in several cancerous cell lines, which was not yet known, showed that it was expressed equally in each line. These results are consistent with each other, and greatly strengthen the idea that SERCA2b is the product of a housekeeping gene, as already suggested. In contrast, the parallel study of Rap1 proteins eliminated any possible relationship between the expression of Rap1 and SERCA2b, as their total expression in the same cells was different.

Further, the 97 kDa SERCA was not expressed in the cancerous cell lines tested, but was expressed in the haemopoietic lineages, i.e. the megakaryocytic Dami cells, the erythroleukaemic HEL cells, the promyelocytic HL-60 cells and the T lymphoid Jurkat cells, as well as in the epithelial HeLa cells, in different amounts. In addition, comparison of total Rap1 expression and 97 kDa SERCA expression in the different cell lines allowed us to conclude that the correlation between them was relatively close, considering the identical expression levels in each lineage.

To discriminate between Rap1A and Rap1B and to ascertain if one of them is preferentially co-expressed with the 97 kDa SERCA, we studied the expression of each isoform at the RNA level by RT-PCR, and, for the Rap1B isoform, by testing the extent of its phosphorylation by cAMP [32]. The results of the RT-PCR experiments were indeed consistent with differential expression of the two isoforms in the various cell lines, although they were both present in all the cells studied. This fits with the results of Culine et al. [52] who reported that both *rap*1A and *rap*1B mRNAs were expressed in all the human tumours and cell lines they tested. Also a more recent study showed the presence of immunoreactivity against the Rap1A and Rap1B isoforms in the membranes of several cell lines [48]. In addition, the present differential expression of *rap*1A and *rap*1B was consistent with the fact that only the expression of *rap*1B correlated with that of the 97 kDa SERCA. The results for cAMP-induced phosphorylation of the various cell membranes are in total agreement with this finding, as the amounts of phosphoproteins, i.e. of functional Rap1B, corresponded to the level of expression of this protein and not that of Rap1A.

Finally, the correlation between the expression of Rap1B and 97 kDa SERCA was confirmed by the demonstration of their concerted up-regulation in the experimental model of platelets from hypertensive rats, because, not only did we observe parallel positive regulation of the proteins concerned, but this regulation also agreed with the stoichiometry of their increased expression, as expected for interrelated proteins.

The present evidence of a clear relationship between the expression of the 97 kDa SERCA and Rap1B proteins requires several comments. First, it means that the initial description of the regulation of platelet Ca^{2+}-ATPases by cAMP-induced phosphorylation of a 22–24 kDa protein (i.e. Rap1B) may not be specific for platelets, but might be a more general process, representative of at least haemopoietic cell lineages.

Secondly, our present findings might also fit the idea that the different SERCA-type Ca^{2+}-ATPases are regulated by specific regulatory proteins: Rap1B for the 97 kDa SERCA, and a 24 kDa protein, phosphorylated by cAMP and termed phospholamban [53–56], for the SERCA2a isoform. This isoform is produced by alternative splicing of the SERCA2 gene and is found in cardiac muscle. With regard to this regulation of SERCA2 by phospholamban, Eggermont et al. [57] also wondered whether the expression of SERCA2 and phospholamban is coupled. They failed to demonstrate co-expression in smooth muscle, but it should be kept in mind that SERCA2b is the predominant SERCA isoform in smooth muscle, and that regulation by phospholamban has only been demonstrated for SERCA2a and not for SERCA2b. Further, the parallel changes reported in the levels of SERCA2a and phospholamban mRNA strongly co-ordinated expression of the SERCA2a protein and its regulatory protein phospholamban [58,59].

Thirdly, the present study provides a basis for further exploration of the action mechanism of this regulation, as the two protein entities involved have now been identified. Our findings define the specific nature of the tools that will be required for this exploration, such as isoform-specific antibodies to both the 97 kDa SERCA and Rap1B proteins. The present identification of the proteins concerned is also particularly important with regard to the recent controversy about the potential regulation of platelet Ca^{2+}-ATPase (see the Introduction). Indeed, Fischer and White [22] failed to detect a 100 kDa platelet SERCA in a complex formed by cross-linking the 24 kDa phosphoprotein with platelet microsomes. This result can be explained by the use of an anti-SERCA1 antibody, and we demonstrate in this study that the protein involved is the 97 kDa SERCA and not SERCA1.

In addition, the present work has established beyond a doubt that the two platelet SERCAs are quite distinct. Previously, the 97 kDa SERCA was found to differ from the 100 kDa SERCA on the basis of its specific recognition by a platelet antibody, its particular trypsin fragmentation and its higher sensitivity to the newly described Ca^{2+}-ATPase inhibitors [9], but it was difficult to rule out the possibility that the 97 kDa isoform might constitute a proteolytic fragment of the 100 kDa isoform. Here, however, we have shown its specific up-regulation in SHR platelets and its absence from cell lines that express the 100 kDa SERCA2b isoform equally, so the distinction between the two Ca^{2+}-ATPases can no longer be questioned.

Finally, these data agree with the idea that the 97 kDa SERCA might be the as yet unidentified SERCA3 protein. We have clearly demonstrated that epithelial cells express the 97 kDa SERCA protein, and it has recently been shown by *in situ* hybridization that SERCA3 is mainly present in endothelial cells (A. M. Lompré, personal communication), which have the same clonal origin as haemopoietic and epithelial cells. Thus it has strengthened the potential identity of the 97 kDa SERCA as being SERCA3, the cDNA of which was recently described [60], and its definitive identification in platelets is in progress.

In conclusion, the relationship established here between the 97 kDa SERCA isoform and the Rap1B protein constitutes a step forward in the understanding of the respective roles of the Rap1 isoforms, especially in megakaryocytic cells which exhibit particularly high levels of these proteins.

This work was supported by grants from the Association Française contre les Myopathies, from the Association pour la Recherche sur le Cancer and from the Institut des Vaisseaux et du Sang. We thank Elisabeth Savariau for her helpful technical assistance which was greatly appreciated.

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Received 4 May 1993/6 August 1993; accepted 19 August 1993