Human platelets express the SERCA_{2-b} isoform of Ca²⁺-transport ATPase

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Previous biochemical studies suggested that the human platelet Ca²⁺ATPase system may be cell-specific. To test this hypothesis, we first undertook the molecular cloning of Ca²⁺ATPase from human erythroleukaemia (HEL) cells, because this human cell line exhibits megakaryocytic features and expresses a Ca²⁺ATPase that cross-reacts with platelet Ca²⁺-ATPase. For this cloning, an HEL-cell cDNA library was screened with a rat cardiac Ca²⁺ATPase cDNA probe. The insert of the longest clone isolated was 3.9 kb and its sequence displayed a 100 % identity with that of the non-muscle human Ca²⁺ATPase 2-b isoform, termed SERCA_{2-b} (sarco-endoplasmic-reticulum Ca²⁺ATPase). The 3.9 kb cDNA covered a subtotal coding region and part of the 3' non-coding end of the SERCA_{2-b} mRNA. It cross-hybridized with the 4 kb transcript species of cardiac SERCA_{2-a} and with non-muscle SERCA_{2-b} mRNAs, but not with fast-skeletal-muscle SERCA₁ mRNA. We next confirmed that SERCA_{2-b} was a component of the platelet Ca²⁺ATPase system because (1) the platelet clones isolated from a platelet cDNA library exhibited a 100 % homology with HEL-cell cDNA; (2) SERCA_{2-b} mRNA was amplified by PCR on total platelet RNA and (3) platelet Ca²⁺ATPase cross-reacted with a polyclonal SERCA_{2-b}-specific antiserum. Platelets therefore contain a Ca²⁺ATPase definitely identified as the SERCA_{2-b} isoform of Ca²⁺ATPase, thus eliminating the possibility that they only contain a single specific Ca²⁺ATPase.

INTRODUCTION

The regulation of the Ca²⁺ concentration in human platelets has been extensively studied. The cytoplasmic Ca²⁺ concentration increases when platelets are activated [1] and declines when platelet activation is inhibited [2]. In several other cell types, this decline is brought about, at least partially, by Ca²⁺-transport ATPases located in both the intracellular organelles and plasma membranes [3–6].

Such Ca²⁺-transport ATPase activity, presumed to be located in intracellular organelles, has been characterized in platelets [7–16]. The Ca²⁺ATPase concerned has a molecular mass of about 100 kDa, as determined by the detection of its phosphorylated intermediate [11,14]. It is related to the fast-skeletal-muscle sarcoplasmic-reticulum Ca²⁺ATPase, because the two enzymes have similar molecular masses and cross-react on immunoblotting [16]. However, structural differences exist between sarcoplasmic-reticulum and platelet Ca²⁺ATPase, since they exhibit distinct trypsin-proteolysis patterns [15]: thus the platelet enzyme gives rise to an 80 kDa fragment absent from intracellular Ca²⁺ATPases in other cell types, whereas the concomitant 55, 35 and 24 kDa fragments are common to all intracellular Ca²⁺-ATPases. Lastly, more recent studies suggest that platelets in fact contain two related but distinct Ca²⁺ATPases [17–19]. These different observations constitute the basis of the concept of platelet Ca²⁺ATPase specificity [20].

Different intracellular Ca²⁺ATPases were recently identified as the products of three distinct genes termed SERCA₁, SERCA₂ and SERCA₃. The SERCA₁ gene codes for the fast-skeletal-muscle Ca²⁺ATPase [21], whereas the SERCA₂ gene [22] codes

for both the slow-twitch cardiac Ca²⁺ATPase (SERCA_{2-a}) and the smooth-muscle/non-muscle-type Ca²⁺ATPase (SERCA_{2-b}) through alternative splicing. SERCA₃ [23] was recently identified after isolation of a cDNA distinct from SERCA₁ and SERCA₂, and its presence has been demonstrated in several muscle and non-muscle tissues.

To determine from which gene(s) the platelet Ca²⁺ATPases are derived, we opted for the use of molecular-cloning technology. As human platelets are anucleated cells, their RNA content is limited and isolation of the remaining RNA is difficult; no platelet cDNA library was available at the beginning of our investigation. Megakaryocytes, which are platelet precursors that contain Ca²⁺ATPase [24], are only present in bone marrow in small amounts, and culture of megakaryocyte colonies *in vitro* yields relatively few cells. For these reasons, we decided to begin this study by using the HEL-cell line to clone Ca²⁺ATPase, as this line exhibits megakaryocytic features [25–29], and cDNAs coding for several platelet proteins have recently been cloned from it [30–32].

In this work, we obtained biochemical evidence that HEL cells express a platelet-type Ca²⁺ATPase. We also isolated cDNA clones from a λgt10 HEL-cell library and showed that they correspond to the mRNA of the SERCA_{2-b} isoform. Lastly, we demonstrated the identical nature of HEL-cell Ca²⁺ATPase and platelet Ca²⁺ATPase by three independent approaches: (1) the isolation of a platelet cDNA clone from an expression library which became available in the course of our investigations, (2) PCR on platelet RNA and (3) immunoblotting of platelet membranes by using an antiserum raised against a synthetic peptide derived from the SERCA_{2-b} isoform.

Abbreviations used: SERCA, sarco-endoplasmic reticulum Ca²⁺ATPase; HEL, human erythroleukaemia; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; Ca²⁺ATPase, Ca²⁺-activated Mg²⁺-dependent ATPase; PBS, phosphate-buffered saline; MMLV-RT, murine Moloney-virus reverse transcriptase; 1 × SSC, 15 mM-sodium citrate/150 mM-NaCl.

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EXPERIMENTAL

Cell culture

HEL cells [25] were cultured at 37 °C in RPMI 1640 medium (Boehringer, Mannheim, Germany) supplemented with penicillin, streptomycin and 10% (v/v) heat-inactivated fetal calf serum in a fully humidified atmosphere with 5% CO₂. Cells were passaged every 3 days and studied in the exponential phase of growth.

Preparation of the platelet-membrane fraction

Membrane vesicles were prepared as previously described [11]. Briefly, fresh human platelets were isolated and washed in modified Tyrode's solution. The cells were then disrupted by controlled ultrasonication in a lysis buffer containing 0.8 mg of benzamide/ml, 0.1 unit of aprotinin/ml, 4.8 µg of leupeptin/ml, 1 mM-phenylmethanesulphonyl fluoride (PMSF), 1 mM-dithiothreitol (DTT) and 1 mM-EGTA. Lysed platelets were centrifuged at 19000 *g* for 30 min to eliminate intact platelets, mitochondria and granules. The supernatant was centrifuged at 100000 *g* for 1 h and the membrane fraction was derived from the pellet. This fraction contained both plasma and intracellular membranes [11].

Preparation and characterization of the antibodies

Antiserum against rabbit skeletal-muscle Ca²⁺ATPase of sarcoplasmic reticulum was raised in guinea pigs. The preparation and characterization of purified immunoglobulins have already been described [19]. The present immunoglobulin fraction reacted with both muscle sarcoplasmic-reticulum Ca²⁺ATPase and platelet Ca²⁺ATPases. In contrast, no cross-reactivity was observed between the immunoglobulins in this fraction and erythrocyte or cardiac sarcolemmal Ca²⁺ATPases.

Antiserum directed against the SERCA_{2-b} Ca²⁺ATPase isoform was raised against a synthetic peptide corresponding to the last 12 amino acids of the C-terminal region of the Ca²⁺ATPase [33]. This antiserum gave a positive reaction with the peptide and recognized a 100 kDa protein band in different smooth-muscle preparations. Its specificity was established by its cross-reactivity with the SERCA_{2-b} Ca²⁺ATPase isoform from the pig stomach smooth muscle and liver, but not with the SERCA_{2-a} cardiac isoform or the skeletal sarcoplasmic-reticulum SERCA₁ isoform.

Western-blot procedures

Purified rabbit skeletal-muscle Ca²⁺ATPase, HEL-cell lysate and human platelet membrane proteins were solubilized in 50 mM-Tris/HCl buffer, pH 6.8, containing 2.3% (w/v) SDS, 0.01% (w/v) Bromophenol Blue and 25% (v/v) glycerol. The samples were reduced with 5% (v/v) 2-mercaptoethanol by boiling for 10 min at 100 °C and applied to an SDS/11% polyacrylamide gel as described by Laemmli [34]. Electrophoresis was performed at 30 V overnight. Electrophoretic transfer of proteins from unstained polyacrylamide gels to nitrocellulose membranes was performed as described earlier [35]. The nitrocellulose was incubated with the antibody against the Ca²⁺ATPase from rabbit muscle sarcoplasmic reticulum as previously described [19]. Ca²⁺ATPase was detected by using 5 µg of purified immunoglobulins/ml of phosphate-buffered saline (PBS; 10 mM-phosphate buffer, pH 8, containing 140 mM-NaCl) and 0.2% Tween 20. Antibody binding was revealed by incubating the nitrocellulose with 1200 MBq of ¹²⁵I-labelled protein A/mg (30000 c.p.m./strip; Amersham International, Amersham, Bucks., U.K.) at 37 °C for 1 h. After they had been washed, autoradiography of the dried nitrocellulose membranes was performed with Kodak X-Omat AR films with intensifying screens.

The nitrocellulose membranes were incubated with the SERCA_{2-b} antiserum as already described [33]. Ca²⁺ATPase was detected using a 1:120 antiserum dilution. Antibody binding was revealed by using an anti-rabbit biotin-avidin-peroxidase system [36]. The quenching solution and the anti-SERCA_{2-b} antibody were supplemented with 25% (v/v) heat-inactivated human serum to increase staining specificity.

Detection of the phosphorylated intermediate of the platelet Ca²⁺ATPases

Membrane Ca²⁺ATPases were labelled by the method of Sarkadi *et al.* [36]. For this purpose, the membrane preparation described above was slightly modified: platelets were disrupted in lysis buffer without MgSO₄ or ATP. After the different centrifugation steps, membranes were resuspended in buffer without MgSO₄, benzamide or ATP, and with 1 mM-DTT. The membrane fractions were phosphorylated for 1 min at 4 °C with [γ -³²P]ATP (final concentration 0.05 µM) in a buffer containing, in mM, 40 Tris/HCl, pH 7.0, 0.014 EGTA, 88 KCl, 0.050 CaCl₂ and 0.050 LaCl₃. The reaction was stopped by adding a solution containing 11 mM-NaH₂PO₄, 1 mM-ATP and 6% (v/v) trichloroacetic acid (final concentrations), and the resulting preparation was kept for 30 min on ice. The precipitate was washed twice with the same solution and dissolved in 150 mM-Tris/HCl (pH 6.8)/2% SDS/10 mM-EDTA (sodium salt)/60% (w/v) sucrose/0.014% (w/v) Bromophenol Blue/5% (v/v) 2-mercaptoethanol. The proteins were then submitted to acidic SDS/PAGE [7.5% (w/v) gels] and electroblotted on to nitrocellulose membranes overnight at 4 °C in a buffer containing 25 mM-Tris/HCl, pH 8.3, 192 mM-glycine and 20% (v/v) methanol. Autoradiography of the dried blots was performed with Kodak X-Omat AR films with intensifying screens.

Isolation and characterization of cDNA clones

A cDNA library was constructed from HEL cells as reported previously [37]. Recombinant phages (3 × 10⁸) from the amplified library were plated and transferred on to nitrocellulose membranes which were then processed and prepared for plaque hybridization *in situ* [38]. The membranes were hybridized overnight at 42 °C with cDNA probes (see the Results section), radio-labelled by random priming (random-primed DNA labelling kit; Boehringer) to achieve a specific radioactivity of 1 × 10⁸ d.p.m./µg, in a solution containing 50% (w/v) formamide, 5 × Denhardt's solution [0.1% BSA/0.1% Ficoll/0.1% poly(vinylpyrrolidone)], 5 × SSC (where 1 × SSC = 15 mM-sodium citrate/150 mM-NaCl), 0.1% SDS and 100 µg of denatured salmon-sperm DNA/ml. Membranes were then washed at different stringencies (see the Results section) and autoradiographed.

A human platelet cDNA expression library was constructed as previously described [39]. Briefly, total RNA was extracted from isolated platelets (the ratio of platelets to contaminating leucocytes was about 20000:1). After isolation of poly(A⁺) RNA, the platelet library was constructed in λgt11. It was amplified and shown to express the specific platelet glycoprotein 1b. This library was screened as described above.

DNA sequencing

DNA was sequenced by the dideoxy chain-termination method of Sanger *et al.* [40] with [³⁵S]-thio]dATP. The cDNA inserts were subcloned in either M13mp18 or 19 vectors, or in pBluescript KS⁺ phagemid (Stratagene, La Jolla, CA, U.S.A.). Single- or double-stranded DNA was sequenced by using a commercial kit (Sequenase version 1 and 2; USB, Cleveland, OH, U.S.A.). Some template-specific 20- or 23-mer primers synthesized according to known DNA sequences were also used (Institut

Pasteur, Paris, or Appligène, Strasbourg, France). The sequence was obtained by analysing either strand of the longest clone.

Northern-blot procedure

Total RNA was extracted from fast skeletal muscle (tensor fasciae latae), cardiac ventricle and soleus muscle of adult Sprague-Dawley rats. After dissection, the tissues were immediately frozen in liquid nitrogen and RNA was isolated by the method of Chirgwin *et al.* [41]. For Northern-blot analysis, total RNA was size-fractionated on 1% agarose containing 3% formaldehyde and blotted on to membranes (Pall Biodyne TM-A transfer membranes; Glen Cove, NY, U.S.A.). The membranes were prehybridized at 42 °C in 50% formamide/0.1% BSA/0.1% Ficoll/0.1% poly(vinylpyrrolidone)/50 mM-sodium phosphate buffer (pH 6.5)/0.1% SDS/250 µg of denatured salmon sperm DNA/ml and then hybridized under the same conditions with a random primed labelled probe. Membranes were washed in 1 × SSC/0.1% SDS at 55 °C for 1 h and exposed on films for 48 h.

Platelet RNA PCR

Total platelet RNA was isolated from human platelets using a previously described fast method [42]. This method is based on total RNA isolation from small blood samples by the isolation of platelets and then of RNA, by a single-step acid/guanidinium thiocyanate/phenol/chloroform extraction. The contamination of platelets by other blood cells was one leucocyte in 5000 platelets. The RNA preparation obtained was shown by Northern-blot analysis to contain significant amounts of platelet-specific transcripts, but no lymphocyte transcripts were detected. Synthetic 22-mer primers from two regions of the mRNA were designed for the PCR. Total platelet RNA template (200 ng) was denatured by heating to 75 °C for 3 min and annealed to 7 pmol of the 3'-oligonucleotide by slow cooling to 30 °C. First-strand cDNA was then synthesized by adding 200 units of murine-Moloney-virus reverse transcriptase (MMLV-RT; BRL, Bethesda, MD, U.S.A.), incubated for 1 h at 42 °C in PCR buffer containing 10 mM-Tris/HCl, pH 8.3, 50 mM-KCl, 2.5 mM-MgCl₂, 0.001% gelatin and the 4-deoxynucleotide triphosphates at 2.5 mM each in a total volume of 20 µl. The MMLV-RT was heat-inactivated for 10 min at 95 °C and the reaction volume adjusted to 90 µl with the same PCR buffer containing 7 pmol of the 5'-oligonucleotide. PCR was initiated by adding 2.5 units of *Taq* polymerase (*Thermus aquaticus* DNA polymerase; Amersham) and 35 cycles were carried out in an automated thermocycler (Hybaid, Teddington, Middx., U.K.), using cycle times of 1 min at 55 °C, 2 min at 70 °C and 45 s at 93 °C. PCR products (15 µl) were visualized on both 2.5% (w/v) ethidium bromide-stained agarose gel and by Southern blotting of the DNA transferred to a nitrocellulose membrane.

RESULTS

Comparative immunoblots of purified Ca^{2+} ATPase from sarcoplasmic reticulum, platelet membranes and HEL-cell lysate

To determine whether HEL cells express a Ca^{2+} ATPase similar to that identified in human platelets, two preliminary biochemical experiments were performed. Firstly, immunoblot experiments were conducted using an affinity-purified antibody raised against rabbit skeletal-muscle sarcoplasmic-reticulum Ca^{2+} -ATPase which recognized both the cardiac sarcoplasmic-reticulum and platelet Ca^{2+} ATPases. As shown in Fig. 1, the HEL-cell lysate (lane 2) exhibited a radiolabelled band of about 95 kDa which co-migrated with the Ca^{2+} ATPase from skeletal muscle (lane 1) and platelets (lane 3). Secondly, we tested the effect of trypsin on Ca^{2+} ATPase from the HEL cells, and again

found the same proteolytic profile as that previously characterized for platelet Ca^{2+} ATPase (results not shown). The results of these immunochemical and proteolytic characterization experiments prompted us to use HEL cells for the molecular cloning of platelet-type Ca^{2+} ATPase.

Fig. 1. Comparative immunoblots of purified sarcoplasmic-reticulum Ca^{2+} -ATPase from rabbit skeletal muscle, HEL-cell lysate and platelet membranes

The Ca^{2+} ATPase from rabbit skeletal muscle was purified as described by MacLennan [57]. The platelet-membrane fraction was prepared as described in the Experimental section. An HEL-cell lysate was obtained by incubating the cells for 30 min at 4 °C in 50 mM-Tris/HCl buffer, pH 8, containing 150 mM-NaCl, 1% Triton X-100 and 1 mM-PMSF. Cells were then centrifuged at 12000 *g* for 1 min and the resulting supernatant fraction was used for immunoblotting. The three sources of Ca^{2+} ATPase, i.e. 2 µg of purified Ca^{2+} ATPase (lane 1), 300 µg of HEL-cell lysate (lane 2) and 50 µg of platelet membranes (lane 3), underwent electrophoresis on 11% (w/v) polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes and immunodetected as described in the Experimental section. Molecular mass was determined by using the following markers: phosphorylase *b* (92.5 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), soya-bean trypsin inhibitor (21 kDa) and lysozyme (14.5 kDa).

Fig. 2. Schematic representation of probes and cDNA clones of the human SERCA_{2-b} mRNA

The top line defines the heterologous rat cDNA [46] insert used to clone HEL-cell Ca^{2+} ATPase. Human SERCA_{2-b} mRNA [47] is shown in the line below: the open box represents the coding sequence and the line, the untranslated regions. The third line down indicates the position of the first insert (HEL-cell clone 1). The bottom line represents the longest insert of 3.9 kb (HEL-cell clone 2) defined after sequencing the cDNA.

Fig. 3. Northern blotting of fast-skeletal-muscle, rat cardiac and smooth-muscle RNAs

RNA-blot analysis of total RNA from fast skeletal muscle (lane 1), cardiac muscle (lane 2) and soleus muscle (lane 3) hybridized with the probe corresponding to the 5' end portion of the HEL-cell Ca^{2+} -ATPase mRNA (HEL-cell clone 1); 10 μg of total RNA was loaded per lane. The blot was washed in $1 \times \text{SSC}/0.1\%$ SDS at 55°C and autoradiographed for 48 h at -80°C .

Isolation, characterization and identification of HEL-cell Ca^{2+} -ATPase cDNA

As platelet and cardiac Ca^{2+} -transport ATPases were thought to be related on the basis of biochemical and functional evidence [43–45], we decided to screen the HEL-cell library with a cardiac cDNA probe. In all, 300 000 recombinant phages from this library were screened, using λ RH ATP 411, a 1510 bp fragment of rat heart cDNA (Fig. 2 and [46]). One positive clone termed HEL-cell clone 1 was obtained after three screening rounds at low stringency using $1 \times \text{SSC}/0.1\%$ SDS for 30 min at 37°C . The 0.9 kb insert isolated from HEL-cell clone 1 was sequenced and displayed a 100% homology with the SERCA_2 sequence. HEL-cell clone 1 mapped at the 5' end of the mRNA (Fig. 2).

This human HEL-cell clone 1 insert was then used to rescreen another 300 000 recombinant phages at higher stringency ($0.2 \times \text{SSC}/0.1\%$ SDS for 30 min at 55°C) and 23 new clones were isolated. The restriction map and the sequence of the longest insert (3.9 kb) termed HEL-cell clone 2 cDNA (Fig. 2) were 100% identical with the map and sequence of SERCA_{2-b} , one of the two alternative transcripts of the human SERCA_2 gene which is expressed in non-muscle tissue [47]. The two sequences were identical because the cDNA extended from nucleotides 42 to 3909 of the mRNA, thus covering both the coding portion from nucleotides +42 to 3126 and the part of the 3' untranslated region of the molecule extending from nucleotides 3126 to 3909. This cDNA thus contained the portion of the sequence that extends 3' downstream from the alternative splicing site at bp 2980 and is specific for SERCA_{2-b} .

Finally, since alternative SERCA_2 transcripts other than SERCA_{2-a} and SERCA_{2-b} have recently been described [48], we conducted Northern-blot experiments to detect transcripts homologous with HEL-cell clone 1. Fast-skeletal-muscle, cardiac muscle and non-muscle total RNAs from rat were studied (no equivalent human tissues were available) under moderate stringency ($1 \times \text{SSC}/0.1\%$ SDS, 55°C for 1 h). As Fig. 3 shows, the

Fig. 4. PCR analysis of platelet RNAs

(a) Location of primers. The diagram represents the two alternative transcripts of the SERCA_2 gene (SERCA_{2-a} and SERCA_{2-b}). A first primer couple was designed to detect the RNA species of SERCA_2 (SERCA_{2-a} and SERCA_{2-b}) in platelets (1 and 2). The second primer couple (3 and 4), was specific for the SERCA_{2-a} isoform. A refers to the 266 bp amplification product using primers 1 and 2. B refers to the 142 bp amplification product using primers 3 and 4. (b) Analysis of SERCA_2 mRNAs by PCR. The left-hand side shows the results obtained on platelet RNA with primers 1 and 2, and the right-hand side shows the results obtained with primers 3 and 4. Rat heart total RNA was used as control. No amplification product was detected in the absence of RNA or reverse transcriptase (not shown). The amplification products were analysed by both ethidium bromide staining of a gel (G) and Southern blotting (S). HEL-cell clone 1 cDNA (Fig. 2) was used as probe for Southern blotting of the amplification products of the primer couple 1 and 2. The cDNA from the platelet clone was used as probe for Southern blotting of the amplification products of couple 3 and 4. Owing to the strong signal of heart RNA with couple 1 and 2, the corresponding autoradiograph shown here was obtained using X-Omat MA film, whereas the other results were obtained with X-Omat AR film.

HEL-cell clone 1 hybridized to cardiac muscle 4 kb SERCA_2 mRNA (lane 2) and non-muscle mRNA (lane 3) but not to fast-skeletal-muscle mRNA (lane 1), which was consistent with the known tissue-specific expression of the SERCA_2 gene. No larger or smaller transcripts were detected.

Evidence for the presence of the SERCA_{2-b} isoform in platelets

To show definite proof that HEL-cell and platelet Ca^{2+} -ATPases are identical, we adopted three complementary approaches which by then had become available. 1. We screened a recently constructed platelet $\lambda\text{gt}11$ amplified cDNA library using HEL-cell clone 1 cDNA and obtained four positive clones. These clones were identical, displayed a 100% homology with HEL-cell clone 2 cDNA (results not shown) and extended from nucleotides 2284 to 3112 at the 3' end of the cDNA. Consequently, like HEL-cell clone 2, these clones correspond to the SERCA_{2-b} isoform, since they include its corresponding sequence

Fig. 5. Immunoblot analysis of mixed platelet membranes with a SERCA_{2b} -specific antiserum

Human platelet membrane proteins were phosphorylated as described in the Experimental section, electrotransferred on to a nitrocellulose membrane and autoradiographed (lane 1). The same nitrocellulose membrane was then incubated with the antiserum as described in the Experimental section and immunostained for the detection of antibody cross-reactivity (lane 2).

downstream of the spliced site at bp 2980. 2. We amplified platelet SERCA_2 mRNA using the PCR technique with oligonucleotide primers synthesized from two regions of the mRNA (Fig. 4a). The first couple of primers (bp 1163–1183 for 5' sense primer 1 and bp 1408–1429 for 3' antisense primer 2) amplified a 266 bp fragment common to both the SERCA_{2a} and SERCA_{2b} isoforms. Primer 3 (5' sense, bp 2860–2882) and primer 4 (3' antisense, bp 2981–3002) were respectively generated from exons b and c of the SERCA_2 gene [47]; since exon c was only present in SERCA_{2a} and not in SERCA_{2b} , primer 4 could not anneal to SERCA_{2b} mRNA. The 142 bp fragment generated was therefore specific for SERCA_{2a} . In addition, exons b and c were separated by a large intron (> 3 kb), so that the 142 bp SERCA_{2a} fragment cannot have originated from potentially contaminating genomic DNA. It was not possible to amplify SERCA_{2b} mRNA faithfully because it contains an intronic sequence, which is derived from the intron between exons b and c [47] and is not distinguishable from a contaminating genomic DNA sequence. Fig. 4(b) illustrates the amplification of platelet RNA compared with heart RNA as a control, after agarose-gel electrophoresis (G) and Southern blotting (S). A strong signal was obtained by both ethidium staining and Southern-blot hybridization for the 266 bp product common to the SERCA_{2a} and SERCA_{2b} isoforms in both platelets and control heart RNA (Fig. 4b, left-hand side). This is in agreement with the significant concentration of SERCA_2 transcripts found in platelets. The 142 bp product specific for the alternative SERCA_{2a} transcript was not detectable in platelets after ethidium staining but only after Southern-blot hybridization, as opposed to control heart RNA, in which a strong band was obtained by both methods (Fig. 4b, right-hand side). This is consistent with the very low concentration of SERCA_{2a} transcripts in platelets. However, the comparatively high overall concentrations of SERCA_2 transcripts indicate that SERCA_{2b} concentrations are significantly high in platelets. 3. We attempted to detect the SERCA_{2b} isoform of Ca^{2+} ATPase in the proteins. For this purpose, we combined immunoblotting of platelet membranes using an antibody specific for SERCA_{2b}

(see the Experimental section) with detection of the highly specific 'phosphorylated intermediate' formed during the catalytic cycle of the Ca^{2+} ATPases. As Fig. 5 shows, a 100 kDa band corresponding to the phosphorylated form of the platelet-membrane Ca^{2+} ATPase (lane 1) exactly co-migrated with a band immunostained by the anti- SERCA_{2b} antibody (lane 2).

DISCUSSION

These present results clearly demonstrate that human platelets express the SERCA_{2b} isoform of Ca^{2+} ATPases, the first intracellular-type Ca^{2+} ATPase to be identified in human circulating cells.

During the last three years, cDNA clones have been obtained corresponding to the intracellular-type Ca^{2+} ATPases from animal muscle and non-muscle tissues [49–53]. They all seem to be derived from three distinct genes, termed SERCA_1 , SERCA_2 and SERCA_3 (see the Introduction).

From our results, we conclude that SERCA_{2b} , the non-muscle/smooth-muscle isoform of SERCA_2 , is expressed in megakaryocyte lineage cells, because: (1) only SERCA_{2b} clones were isolated from both the HEL-cell and platelet cDNA libraries and (2) platelet-membrane Ca^{2+} ATPase cross-reacted with a SERCA_{2b} -specific antiserum. Additional indirect evidence that SERCA_{2b} is a major transcript of the SERCA_2 gene in platelets was provided by the poorer amplification of SERCA_{2a} messages than of total SERCA_2 .

The identification of SERCA_{2b} as a platelet Ca^{2+} ATPase agrees with previous biochemical data obtained for the Ca^{2+} -ATPases of platelets and fast skeletal or smooth muscles, such as similar molecular masses of about 100 kDa, and the recognition of platelet Ca^{2+} ATPase by polyclonal antibodies raised against fast-skeletal-muscle sarcoplasmic reticulum.

That platelets express SERCA_2 Ca^{2+} ATPase also emphasizes some other analogies between cardiac, smooth-muscle-cell and platelet Ca^{2+} ATPases, such as their apparent regulation through cyclic AMP, even though different regulatory phosphoproteins are involved, i.e. phospholamban in cardiac cells and an as-yet-unidentified phosphoprotein in the other cell types. Also, the fact that the SERCA_{2b} isoform constitutes a Ca^{2+} ATPase in both platelets and smooth-muscle cells justifies the use of platelets as a model to study the modifications that occur in Ca^{2+} handling during certain smooth-muscle-cell pathologies [54].

Moreover, the present finding lends support to two hypotheses concerning the SERCA_{2b} isoform: first, it corroborates the view that SERCA_{2b} is a 'housekeeping' enzyme because the presence of this isoform has now been demonstrated here in a circulating cell in addition to smooth-muscle/non-muscle tissues. The second hypothesis is that the extended C-terminus of SERCA_{2b} may help to target Ca^{2+} ATPase to a specific subcellular compartment [47], since immunolocalization using polyclonal antibodies raised against purified platelet Ca^{2+} ATPase revealed the presence of the latter enzyme mainly in the cytosol and also in intracellular structures. This may mean that two different pools of intracellular Ca^{2+} ion storage are also present in platelets, and that the SERCA_{2b} might be located in either the sarcoplasmic reticulum or calciosome-like structures [55], as recently suggested in cell types other than platelets.

However, the identification of SERCA_{2b} cannot explain all the characteristics of platelet Ca^{2+} ATPases, such as their specific tryptic-fragmentation patterns. These apparently discrepant patterns might be due to a difference in the post-translational maturation of SERCA_{2b} in platelets and in other cells, and this difference might modify the sensitivity of SERCA_{2b} to trypsin. An alternative possibility is that another Ca^{2+} ATPase not identical with SERCA_{2b} is present in platelets, in agreement with

previous biochemical data showing that platelets contain two similar but distinct Ca^{2+} ATPases [17–19]. In recent experiments with different polyclonal antibodies, we were indeed able to distinguish these two types of Ca^{2+} ATPase and to demonstrate the presence of a specific Ca^{2+} ATPase different from SERCA₁ and SERCA₂. This might be either the SERCA₃ type or an as-yet-unidentified type [56]. Consequently, in this work, we have not only identified a platelet Ca^{2+} ATPase, for the first time, but established the basis for proving that the specificity of the platelet Ca^{2+} ATPase system is due to the presence of two similar but different intracellular-type Ca^{2+} ATPases, and not of a single cell-specific Ca^{2+} ATPase.

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