All three splice variants of the human sarco/endoplasmic reticulum Ca^{2+} -ATPase 3 gene are translated to proteins: a study of their co-expression in platelets and lymphoid cells

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The molecular cloning of two previously unknown human sarco/endoplasmic reticulum Ca^{2+} -ATPase 3 (SERCA3) 3'-end transcripts, 3b and 3c, has been recently published. Data were lacking, however, for the presence of these SERCA3 variants in different tissue or cell types at the protein level. Here we report the co-expression of three human SERCA3 protein isoforms in platelets and T lymphoid Jurkat cells. Isoform-specific polyclonal anti-peptide antibodies have been generated that recognize specifically the SERCA3a, 3b or 3c splice variants at their C-termini, and this has been confirmed by peptide-competition experiments as well. None of these antibodies cross-reacted with the housekeeping SERCA2b isoform co-expressed endogenously with SERCA3 proteins in non-muscle cells. Although all three SERCA3 isoforms could be detected in platelets, the 3a form was the most abundantly expressed species. Its size matched the

apparent size of SERCA3a over-expressed in HEK-293 cells. Immunoprecipitation of the SERCA3 variants from platelet membranes using a PL/IM 430-affinity matrix provided evidence that the putative pan-anti-SERCA3 antibody, PL/IM 430, recognizes all SERCA3 protein isoforms. The epitope for the PL/IM 430 antibody could be localized in a 40 kDa N-terminal tryptic fragment common to all three SERCA3 variants. Comparative Western-blot analysis showed that the expression level of the SERCA3a, 3b and 3c isoforms was more than 10 times lower in Jurkat cells than in platelets, whereas expression of the ubiquitous SERCA2b was nearly identical. This work highlights new Ca^{2+} -transporting proteins of haematopoietic cells and provides specific antibodies for their detection.

Key words: antibody, expression pattern, SERCA3 isoform.

INTRODUCTION

Cellular Ca^{2+} signalling plays a pivotal role in signal-transduction processes, which result in specific cellular responses, via regulation of different enzymes and transcription factors [1,2]. Cell activation promotes Ca^{2+} signals generated by the concerted operation of different Ca^{2+} -transporting and Ca^{2+} -binding proteins. Stimuli evoke Ca^{2+} liberation from intracellular Ca^{2+} -storage compartments and Ca^{2+} penetration into the cells, thus elevating the Ca^{2+} concentration in the cytosol [3–5]. The uncontrolled increase in cytosolic Ca^{2+} concentration would lead to cell death. Thus restoration of resting cytosolic Ca^{2+} levels following cellular activation is of vital importance for all cell types. Among the mechanisms that contribute to Ca^{2+} elimination from the cytosol are the sarco/endoplasmic reticulum Ca^{2+} -ATPases (SERCAs) localized in the membrane of the sarco/endoplasmic reticulum [6–8]. These proteins mediate Ca^{2+} uptake into intracellular Ca^{2+} stores and, as shown by Yu and Hinkle [9], substantial refilling of the sarco/endoplasmic reticulum by these Ca^{2+} -ATPases occurs continuously even during agonist-induced signalling. There are data emphasizing that SERCA pump over-expression modulates the frequency of Ca^{2+} waves and consequently the kinetics of cell Ca^{2+} signalling [10,11]. The biological significance of the SERCA proteins resides also in the control of luminal Ca^{2+} concentration in the sarco/endoplasmic reticulum. Adequate sarco/endoplasmic reticulum Ca^{2+} concentration is necessary for normal protein processing [12] and nuclear transport processes [13].

Proteins of the SERCA family are encoded by three distinct genes, *SERCA1*, 2 and 3. Alternative splicing of the primary gene transcripts results in different isoforms, the distribution of which is tissue-specific and developmentally regulated (for a review see [14]). In human non-muscle tissues the housekeeping SERCA2b [15,16] and a SERCA3 isoform [17,18] (SERCA3a according to the recent nomenclature) have been identified. The presence of a SERCA protein recognized by PL/IM 430, a monoclonal anti-SERCA antibody raised against highly purified platelet intracellular membranes, has also been demonstrated [19,20]; however, the identity of this SERCA form has long been debated [21–23]. While two alternatively spliced variants have long been known for both SERCA1 and SERCA2 [14–16,24,25], alternative splicing of the human SERCA3 pre-mRNA has only recently been reported by three independent groups [26–28]. The human *SERCA3* gene contains 22 exons. Exon 21 can be alternatively excluded, partially included or totally included, thus generating the SERCA3a, 3b or 3c isoforms with different C-termini. Tissue Northern-blot analysis and mRNA dot-blot hybridization showed that the human SERCA3 transcripts are widely expressed in non-muscle tissues and that their expression level varies dramatically from tissue to tissue [27,28]. However, no data are available for the presence and the expression pattern of the recently described SERCA3b and 3c variants in different tissue or cell types at the protein level.

The aim of our work was to document that the two human SERCA3 3'-end transcripts, 3b and 3c, are translated to proteins and are co-expressed with the 3a form in several haematopoietic

Abbreviations used: SERCA, sarco/endoplasmic reticulum Ca^{2+} -ATPase; ECL[®], enhanced chemiluminescence; NP-40, Nonidet P-40.

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cell types. We developed isoform-specific polyclonal anti-peptide antibodies against the unique C-termini of the human SERCA3 splice variants and used these antibodies to monitor the expression pattern of all three SERCA3 isoforms in human platelets and T lymphoid Jurkat cells. To clarify the identity of the PL/IM 430-recognizable SERCA form(s), we performed immunomagnetic protein precipitation with a PL/IM 430-affinity matrix, and characterized the captured SERCA proteins using different isoform-specific anti-SERCA antibodies. Finally, the epitope for the PL/IM 430 antibody was also localized, namely in an N-terminal proteolytic fragment common to all three SERCA3 protein isoforms.

EXPERIMENTAL

Materials

RPMI 1640 medium, L-glutamine, foetal calf serum and *Eco*RI were purchased from Gibco-BRL Life Technologies (Paisley, Scotland, U.K.). Penicillin and streptomycin were from Boehringer Mannheim (Germany). Keyhole-limpet haemocyanin, BSA, soya-bean trypsin inhibitor and bovine pancreas trypsin inhibitor were from Sigma-Aldrich Kft. (Budapest, Hungary). Dynabeads® M-280 (Tosylactivated) and a Dynal® Magnetic Particle Concentrator were obtained from Dynal A. S. (Oslo, Norway). The IID8 monoclonal anti-SERCA2 antibody [29] was from Affinity BioReagents (Neshanic Station, NJ, U.S.A.). The PL/IM 430 antibody [30], a monoclonal anti-SERCA antibody raised against highly purified platelet intracellular membranes, was purified from hybridoma supernatant by Protein A chromatography [31]. The N89 polyclonal anti-SERCA3 antibody [18], directed against rat SERCA3 proteins, was a generous gift of Professor F. Wuytack (Katholieke Universiteit, Leuven, Belgium). The horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA, U.S.A.). Enhanced chemiluminescence (ECL®) Western-blot reagents and Rainbow® coloured protein molecular-mass markers were from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Optitran BA-S 85 Reinforced NC membranes were from Schleicher & Schuell (Dassel, Germany). Electrophoresis reagents were from Bio-Rad (Richmond, CA, U.S.A.). All other chemicals used were of analytical or molecular-biological grade.

Cells

Human platelets were prepared from freshly drawn human blood as described by Pászty et al. [32]. The PL/IM 430 hybridoma was a generous gift of Professor N. Crawford (Royal College of

Surgeons of England, London, U.K.). KATO III cells (gastric carcinoma), Jurkat cells (JurE6-1 clone) and HEK-293 cells were purchased from the A. T. C. C. (Rockville, MD, U.S.A.). All cells were maintained in RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated foetal calf serum and 2 mM L-glutamine. For the PL/IM 430 hybridoma cells and the Jurkat cells the culture medium was supplemented with 50 units/ml penicillin and 0.05 mg/ml streptomycin. All cell lines were incubated at 37 °C with 5 % CO₂ in a humidified atmosphere.

Expression construct

The cDNA encoding the human SERCA3a splice variant (construct huS3-I in [28], GenBank accession no. Z69881) in pMT2 expression vector was a kind gift of Dr J. Lytton (University of Calgary, Calgary, AB, Canada). The human SERCA3a cDNA was excised with *Eco*RI and subcloned into an *Eco*RI-cut and dephosphorylated mammalian expression vector, pcDNA3 (Invitrogen, Groningen, The Netherlands). Nucleic acid sequence of the subcloned cDNA was confirmed by fluorescent dye-terminator-labelled sequencing on an Applied Biosystems ABI 100 model 377 automated sequencer at Genome Express, Grenoble, France.

Expression of the SERCA3a cDNA in HEK-293 cells

The SERCA3a cDNA construct in the pcDNA3 vector was purified using the EndoFree Plasmid Kit (Qiagen, Hilden, Germany) before transfection. HEK-293 cells were plated at a density of about 1 × 10⁴ cells/cm² in culture dishes of 10 cm diameter. The following day, cells at about 60 % confluence were transfected with 10 µg of the cDNA construct for 1 h, in a small volume of serum-free RPMI 1640 at 37 °C, using the transfection agent ExGen 500 (Euromedex, Souffelweyersheim, France) according to the instructions of the manufacturer. The cells were harvested at 48 h after transfection.

Preparation of whole-cell lysates and mixed microsomal membranes

For preparation of whole-cell lysates from control and transfected HEK-293 cells, the cells were washed with PBS, pH 7.4, and then concentrated by precipitation with ice-cold 6 % (w/v) trichloroacetic acid. The precipitates were then centrifuged for 15 min at 12000 g at 4 °C and the pellets resuspended in electrophoresis sample buffer [32,33]. Preparation of platelet membranes was carried out as in [32]. Microsomal membranes from Jurkat and KATO III cells were prepared as described in [34] for HL 60 cells.

Carboxyl termini of human SERCA3 variants	Antibody
<div> <div>991</div> <div>999</div> <div>hSERCA3a: ...<u>HMH*EEMSQK</u></div> </div>	ChS3a
<div> <div>991</div> <div>1025</div> <div>1043</div> <div>hSERCA3b: ...HMH*ACLYPGLLRTVSQAWSRQPLTTSWTPDHTG*<u>RNEPEVSAGNRVESPVCTSD</u></div> </div>	ChS3b
<div> <div>991</div> <div>1021</div> <div>1029</div> <div>hSERCA3c: ...HMH*ACLYPGLLRTVSQAWSRQPLTTSWTPD<u>HTG*LASLKK</u></div> </div>	ChS3c

Figure 1 Epitopes and nomenclature of the isoform-specific polyclonal anti-human SERCA3 antibodies

The underlined and bold sequences represent the amino acid stretches of the peptides used for immunization of rabbits. Asterisks mark the splice sites in the sequences.

Generation and characterization of the isoform-specific polyclonal anti-human SERCA3 antibodies

The antibodies were generated by immunizing New Zealand rabbits with BSA-conjugated synthetic peptides for SERCA3a and SERCA3c or with keyhole-limpet-haemocyanin-conjugated synthetic peptide for SERCA3b. The peptides selected correspond to the extreme C-termini of the human SERCA3 variants and have uniqueness to the particular SERCA isoform against which the polyclonal antibody was being raised. Peptides derived from SERCA3a (CGHMHEEMSQK) and SERCA3c (CGHTGLASLKK) were synthesized by MoBiDic (Uppsala, Sweden), and the peptide derived from SERCA3b (NEPEVSA-GNRVESPVCTSD) was synthesized at the Mayo Clinic (Rochester, MN, U.S.A.). The additional cysteine and glycine residues (underlined in the sequences above) were added to the N-terminal part of the peptides derived from SERCA3a and 3c to allow coupling of these peptides to BSA by means of the 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester method [35]. The peptide derived from SERCA3b was conjugated to the keyhole-limpet haemocyanin carrier by means of the glutaraldehyde-mediated hapten-carrier conjugation protocol [35]. Production of the three polyclonal anti-human SERCA3 antibodies was performed by the Crosslink Laboratory (Budapest, Hungary). The nomenclature of these new antibodies is given in Figure 1. Portions of all three antibodies were purified from the final sera on the corresponding peptide-Sepharose® 4B affinity columns. The bound Igs were eluted from the PBS (pH 7.4)-washed affinity matrix by sequential additions of 100 mM glycine/HCl, pH 2.5, and 100 mM ethanolamine/HCl, pH 11, buffers and then neutralized with 1 M Tris/HCl, pH 7.4. Finally, antibodies were desalted, concentrated, sterile-filtered and stored in a buffer containing 140 mM NaCl, 25 mM Tris/HCl, pH 7.4, 0.1% (v/v) Nonidet P-40 (NP-40), 0.1% (v/v) Tween 20, 10 mg/ml BSA, 0.1 µg/ml bovine pancreas trypsin inhibitor and 0.02% (w/v) sodium azide. The sensitivity of the antibodies was analysed by the Crosslink Laboratory by performing both ELISA and dot-blot experiments using the corresponding peptide-ovalbumin (for anti-SERCA3a and anti-SERCA3c) and peptide-BSA or peptide-ovalbumin (for anti-SERCA3b) conjugates. The cross-reactivity of the antibodies was also checked by performing both ELISA and dot-blot experiments using peptide-ovalbumin conjugates (results not shown). The suggested working dilutions of these new anti-SERCA3 antibodies for Western blots are the following: 1:5000 for ChS3a (final serum); 1:1000 for ChS3a (purified IgG); 1:2000 for ChS3b (purified IgG); 1:1000 for ChS3c (purified IgG).

Electrophoresis and immunostaining

Samples were separated on 7.5%, 10% or 12% (as indicated in the Figure legends) Laemmli-type SDS/polyacrylamide gels and electrotransferred on to nitrocellulose membranes. Saturation of the membranes was performed overnight in a buffer containing 150 mM NaCl, 10 mM Tris/HCl, pH 7.4 (TBS), 0.1% (v/v) Tween 20 and either 5% (w/v; for monoclonal antibodies), or 10% (w/v; for polyclonal antibodies) dried skimmed milk. Then the membranes were incubated with primary antibodies diluted in the same buffer containing 5% dried skimmed milk for (i) 90 min for the IID8 and PL/IM 430 monoclonal antibodies, (ii) 2 h for the three novel anti-SERCA3 polyclonal antibodies and (iii) 3 h for the N89 polyclonal antibody. Dilutions of 1:1000 of the IID8 antibody and 1:500 of the N89 antibody were used. The PL/IM 430 antibody was generally used at 0.5–1 µg/ml final concentration. The new anti-SERCA3 antibodies were used at the dilutions indicated above, except for immunostaining

presented in Figure 3 (lane 9, see below), where the ChS3a, ChS3b and ChS3c antibodies were mixed at 1:480000, 1:80000 and 1:640 final dilutions, respectively. After incubation with primary antibodies, the blots were washed extensively in TBS/0.1% Tween 20 without (for monoclonal antibodies) or with (for polyclonal antibodies) 5% milk. Then the blots were treated either with horseradish peroxidase-conjugated anti-mouse IgG (90 min for the IID8 and PL/IM 430 monoclonal antibodies at 1:2000 dilution) or with horseradish peroxidase-conjugated anti-rabbit IgG (2 h for all polyclonal antibodies used, at 1:4000–1:2000 dilutions). Finally, the blots were washed and developed using the ECL® Western-blot reagents. Immunostaining was performed at room temperature, except for the N89 antibody, where the entire procedure was performed at 4 °C to eliminate background staining.

In peptide-competition studies, the antibodies used at the suggested working dilutions (see above) were preincubated for 90 min at room temperature with the corresponding peptides at a 10 µM peptide concentration, and immunostaining was performed with these pretreated antibodies as described above. Luminograms were scanned using a Bio-Rad Gel Doc 1000 densitometer.

Trypsin treatment

Trypsin treatment of platelet membranes was carried out in a medium containing 160 mM KCl, 17 mM K-Hepes (pH 7.2), 2 mg/ml membrane proteins and 50 µg/ml trypsin for 2.5 min at 4 °C. The reaction was stopped with a 10-fold excess (w/w) of soya-bean trypsin inhibitor.

Immuno-magnetic protein precipitation

Purified PL/IM 430 monoclonal antibody was immobilized on to Dynabeads® M-280 (Tosylactivated) according to the instructions of the manufacturer. The amount of antibody bound on to the Dynabeads® was determined by means of Western blotting using horseradish peroxidase-conjugated anti-mouse IgG as secondary antibody, and the purified PL/IM 430 antibody as standards. The amount of the bead-bound antibody was approx. 5 µg/ml.

Mixed microsomal platelet membranes at 1 mg/ml final concentration were lysed for 30 min at 4 °C in a buffer containing 160 mM KCl, 17 mM K-Hepes (pH 7.2), 1% (v/v) NP-40, 50 µg/ml aprotinin, 50 µg/ml leupeptin, 20 µg/ml pepstatin-A, 50 µg/ml soya bean trypsin inhibitor and 50 µg/ml Bowman-Birk trypsin/chymotrypsin inhibitor. The lysate was centrifuged at 15000 *g* for 15 min at 4 °C. The pellet was washed with a buffer containing 160 mM KCl and 17 mM K-Hepes (pH 7.2) and dissolved in electrophoresis sample buffer [33]. The supernatant of 1 mg of platelet-membrane lysate was mixed with 10 mg [approx. (6–7) × 10⁸ beads] of prewashed PL/IM 430–Dynabeads® M-280 affinity matrix and gently agitated for 2 h at 37 °C. A Dynal® Magnetic Particle Concentrator was used to separate the beads from the lysate supernatant. The bead pellet was washed at least three times with a washing solution containing 160 mM KCl, 17 mM K-Hepes (pH 7.2) and 0.5% NP-40. Finally, the bound proteins were eluted from the affinity matrix with the elution buffer containing 160 mM KCl, 0.1 M glycine/HCl (pH 2.5) and 0.5% NP-40. In some cases, in a following elution step, 160 mM KCl, 0.5 M acetic acid and 0.5% NP-40 was also used, but the amount of the eluted SERCA proteins by using this buffer did not exceed 10% of that eluted with 160 mM KCl, 0.1 M glycine/HCl (pH 2.5) and 0.5% NP-40. Eluted samples were neutralized by adding 1 M Tris/HCl (pH 8.0). Final washing steps and storage of PL/IM 430–

Dynabeads® M-280 were performed according to the instructions of the manufacturer. When the 40 kDa PL/IM 430-recognizable SERCA fragment was captured using the PL/IM 430-affinity matrix, platelet membranes were first digested by trypsin, then lysed, and immunoprecipitation was performed as described above for the non-proteolysed platelet membranes.

RESULTS

Generating antibodies specific for the human SERCA3 variants a, b and c

Isoform-specific polyclonal anti-peptide antibodies were raised against the C-termini of human SERCA3 splice variants a, b and c. Figure 1 lists the C-termini of these SERCA3 variants, where the amino acid stretches of the peptides used for generating the antibodies are in bold type and underlined. These regions are unique for each SERCA3 variant, and therefore the antibodies raised against these peptides were expected to be highly specific. The nomenclature of these novel polyclonal antibodies is also presented in Figure 1: C refers to the C-terminal region of the enzyme where the epitope is located, h refers to human, S stands for SERCA-type Ca^{2+} -ATPase, 3 stands for the *SERCA3* gene, and the letters a, b and c indicate the spliced variants of the

SERCA3 gene. Out of three attempts at immunization we obtained three useful antibodies, which we called anti-SERCA3a (ChS3a), anti-SERCA3b (ChS3b) and anti-SERCA3c (ChS3c). Portions of the three anti-SERCA3 sera were immunoaffinity purified. In the experiments presented in this paper we used either the final serum or the purified IgG for SERCA3a (as indicated in Figure legends) and the immunoaffinity-purified IgGs for SERCA3b and 3c.

Antibodies specific for the SERCA3 variants recognize distinct SERCA3 proteins in human platelets

Earlier studies demonstrated that human platelets express a SERCA3 protein, which has been identified as the 3a form. Recently, Lacabartz-Porret and her co-workers [36] reported that human platelets express SERCA3a, 3b and 3c at the mRNA level. Therefore, we presumed the co-expression of the SERCA3b and 3c splice variants along with SERCA3a in human platelets at the protein level as well, and used these cells to test the specific recognition of the SERCA3 forms by the newly generated anti-SERCA3 antibodies. Mixed microsomal platelet membranes were resolved by gel electrophoresis, electroblotted and probed using the anti-SERCA3a (Figure 2A, lanes 1–4), anti-SERCA3b (Figure 2A, lanes 5–8) or anti-SERCA3c (Figure

Figure 2 Western blots of microsomal membranes from human platelets and KATO III cells showing the specificity of the anti-SERCA3 antibodies

(A) Human platelet membranes were electrophoresed (SDS/PAGE, 7.5% gel), blotted and immunostained using the isoform-specific polyclonal anti-SERCA3 antibodies: anti-SERCA3a (purified IgG) for lanes 1–4, anti-SERCA3b for lanes 5–8 and anti-SERCA3c for lanes 9–12. Immunostaining with all three antibodies was performed in either the absence or presence of 10 μM of the 3a, 3b and 3c peptides used for antibody production, as indicated at the top of the panel. The quantities of platelet membranes loaded on to the gel were 5 μg for lanes 1–8 and 20 μg for lanes 9–12. (B) Microsomal membranes from KATO III cells (25 μg /lane) and human platelets (5 μg /lane) were electrophoresed (SDS/PAGE, 7.5% gel), blotted and immunostained using anti-SERCA3 antibodies [ChS3a (final serum) for lanes 1 and 2, ChS3b for lanes 3 and 4 and ChS3c for lanes 5 and 6] or the monoclonal anti-SERCA2 (IID8) antibody for lanes 7 and 8. Results shown are typical Western blots performed on at least three different membrane preparations.

Figure 3 Western blots demonstrating that the major SERCA3 form expressed in human platelets is 3a

Whole-cell lysates from either control HEK-293 cells (HEK/C) transfected with empty plasmid (lanes 1 and 4; 5 μ g/lane) or HEK-293 cells containing over-expressed human SERCA3a (HEK/3a; lanes 2 and 5; 5 μ g/lane), and human platelet membranes (lanes 3 and 6; 1.5 μ g/lane) were electrophoresed (SDS/PAGE, 7.5% gel), blotted and immunostained using either the anti-SERCA3a (final serum) (lanes 1–3) or the PL/IM 430 (lanes 4–6) antibodies. For lanes 7 and 8, 5 μ g of platelet membranes was loaded on to the gel and immunostained by either PL/IM 430 (lane 7) or PL/IM 430 pretreated with the mixture of the 3a, 3b and 3c peptides (10 μ M; lane 8). For lanes 7 and 8 films were developed after an extended ECL[®] reaction to detect also the less abundantly expressed SERCA3b and 3c proteins along with SERCA3a. Lane 9 illustrates immunostaining of 10 μ g of platelet membranes with the mixture of the three polyclonal anti-SERCA3 antibodies. Dilutions of the antibodies in that mixture were optimized to obtain staining intensities for the SERCA3a, 3b and 3c proteins all in the same range of signal intensity (see the Experimental section for details). Results shown are typical of at least three immunostainings for each lane.

2A, lanes 9–12) antibodies. Where indicated, the corresponding antibodies were pretreated with 10 μ M of 3a, 3b or 3c peptides used for immunization. The ChS3a antibody immunostained a protein band at about 97 kDa, and the only peptide that inhibited this staining was the one derived from SERCA3a. The ChS3b antibody immunostained a protein at a slightly slower electrophoretic mobility, and the only peptide that inhibited this staining was the one derived from SERCA3b. Finally, the ChS3c antibody immunostained a protein that migrated between the protein bands visualized by ChS3a and ChS3b, and the only peptide that inhibited this staining was the one derived from SERCA3c. The electrophoretic mobility of the proteins recognized by the ChS3a, ChS3b and ChS3c antibodies corresponded well to the predicted sizes of the SERCA3 proteins: 999 amino acids for 3a, 1043 for 3b and 1029 for 3c [27,28].

We explored the potential cross-reactivity of the three polyclonal antibodies with SERCA2b, the only known SERCA isoform co-expressed with SERCA3 proteins in non-muscle cells. In earlier studies we have demonstrated that the KATO III gastric cancer cell line expresses the SERCA2b isoform, but not the SERCA3a protein [19–21]. Therefore, microsomal membranes from KATO III cells (25 μ g/lane) were analysed for immunostaining by the ChS3a, ChS3b and ChS3c antibodies (Figure 2B, lanes 1, 3 and 5, respectively). Platelet membranes (5 μ g/lane) were also immunostained by these antibodies (Figure 2B, lanes 2, 4 and 6). Immunostaining of SERCA2b was performed using the monoclonal IID8 anti-SERCA2 antibody (Figure 2B, lanes 7 and 8). None of the anti-SERCA3 antibodies

Figure 4 Immuno-magnetic protein precipitation of the PL/IM 430-recognizable SERCA protein(s) from human platelet membranes using the PL/IM 430-Dynabeads[®] M-280 affinity matrix

This Western blot presents the immunostaining by PL/IM 430 antibody of the following samples. Lane 1, 2.5 μ g of platelet membranes. Lane 2, pellet of platelet membrane lysates corresponding to 25 μ g of platelet membranes. Lanes 3 and 4, supernatant of platelet membrane lysates corresponding to 2.5 μ g of platelet membranes before (lane 3) and after (lane 4) immunoprecipitation with the PL/IM 430-Dynabeads[®] matrix. Lanes 5 and 6, pattern of protein mixtures obtained by solubilization of proteins bound on the PL/IM 430-Dynabeads[®] used for immunoprecipitation from 20 μ g of platelet membranes before (lane 5) or after (lane 6) elution of the PL/IM 430-recognizable SERCAs (SERCA_{PL/IM}). These samples were heated at 90 °C for 15 min in electrophoresis sample buffer before loading on to the gel. Lane 7, immunoprecipitated PL/IM 430-recognizable SERCA protein(s) obtained from 40 μ g of platelet membranes. Lane 8, 500 ng of purified PL/IM 430 antibody after heating at 90 °C for 15 min in the electrophoresis sample buffer. This sample was used to control for the positions of the heavy and light chains of the IgG of PL/IM 430 (IgG_H and IgG_L) also appearing in the mixtures of solubilized proteins bound to the affinity matrix (lanes 5 and 6). Samples were separated by SDS/PAGE (10% gel). Results shown are typical of at least five separate immunoprecipitations.

cross-reacted with SERCA2b expressed at a relatively high level in KATO III cells. Figure 2(B) also documents that none of the SERCA3 isoforms could be detected in KATO III cells on our Western blots. It is worth mentioning that SERCA3b (1043 amino acids [27]) migrated very close to the SERCA2b isoform (1042 amino acids [14,16]; Figure 2B, lanes 4, 7 and 8), and their electrophoretic mobility appeared to be very similar to that of the 105 kDa recombinant protein component of the Rainbow[®] molecular-mass standard mixture (arrow at the left side of Figure 2B). These results demonstrate that the recently described SERCA3a, 3b and 3c mRNAs encode the expected proteins, and that these SERCA3 protein isoforms are co-expressed in human platelets.

The major SERCA3 protein isoform expressed in human platelets is SERCA3a

A monoclonal antibody, PL/IM 430, recognizes SERCA protein(s) in platelets and other non-muscle cells [19–21]. This antibody has been raised against highly purified platelet internal membranes [30] and has been widely used to study SERCA expression in different non-muscle tissues. Poch and his co-workers [28] reported that the PL/IM 430 antibody immunostains the cloned human SERCA3a and 3c isoforms over-expressed in HEK-293 cells. However, another group found that this monoclonal antibody could not recognize the cloned human SERCA3a over-expressed in COS cells [23]. To solve this discrepancy, we transiently transfected HEK-293 cells with the cDNA of human SERCA3a, and the cell lysates were analysed for their SERCA3a expression using the PL/IM 430 and ChS3a antibodies. Both antibodies recognized the 97 kDa SERCA3a

Figure 5 Recognition pattern of SERCA isoforms in the immunoprecipitates obtained from human platelet membranes using the PL/IM 430–Dynabeads® M-280 affinity matrix (A) and Western blots of the 40 kDa SERCA fragment immunoprecipitated from trypsin-treated platelet membranes (B)

(A) Immunoprecipitates obtained from 80 µg of platelet membranes (lanes Ip) and 4 µg of platelet membranes (lanes Plt) were electrophoresed (SDS/PAGE, 7.5% gel), blotted and immunostained using five different anti-SERCA antibodies: PL/IM 430 for lanes 1 and 2, anti-SERCA3a (purified IgG), anti-SERCA3b and anti-SERCA3c for lanes 3 and 4, 5 and 6 and 7 and 8, respectively, and anti-SERCA2 (IID8) for lanes 9 and 10. (B) For lanes 1–3, 5 µg of platelet membranes (Plt, lane 1), 5 µg of trypsin-digested platelet membranes (Frg, lane 2) and immunoprecipitates obtained from 15 µg of trypsin-digested platelet membranes using the PL/IM 430–Dynabeads® affinity matrix (Ip/Frg, lane 3) were electrophoresed (SDS/PAGE, 12% gel), blotted and immunostained by PL/IM 430. For lanes 4 and 5, Western blot by the polyclonal anti-SERCA3 antibody (N89) of either 0.5 µg of platelet membranes (Plt, lane 4) or immunoprecipitates obtained from 15 µg of trypsin-digested platelet membranes using the PL/IM 430–Dynabeads® affinity matrix (Ip/Frg, lane 5) is presented. Results shown are typical Western blots performed on at least three different immunoprecipitates.

protein (Figure 3, lanes 2 and 5) that was absent in the control HEK-293 cells transfected with empty plasmid (Figure 3, lanes 1 and 4). Our results are in good agreement with those of Poch et al. [28]. Similarly, both antibodies detected the 97 kDa SERCA3a isoform in human platelets (Figure 3, lanes 3 and 6). Under special conditions, the PL/IM 430 antibody (Figure 3, lane 7) could also detect two additional bands in platelets with slower electrophoretic mobilities. Pretreatment of PL/IM 430 with a mixture of the peptides derived from SERCA3a, 3b and 3c did not influence the recognition of either protein band (Figure 3, lane 8). This indicates that the epitope for the PL/IM 430 antibody is located N-terminal to the first splice site (His-993) in SERCA3 protein(s). Finally, when platelet membranes were probed with a mixture of the ChS3a, ChS3b and ChS3c polyclonal antibodies (see the legend of Figure 3), all three SERCA3 isoforms were stained as close but separate bands at the appropriate electrophoretic mobility (Figure 3, lane 9). Based on the comparison of the electrophoretic mobility of the SERCA3 variants (Figure 3, lane 9) with that of the bands recognized by PL/IM 430 (Figure 3, lanes 7 and 8), we hypothesize that PL/IM 430 recognizes all three SERCA3 isoforms.

The data shown in Figure 3 demonstrate that, although all three SERCA3 splice variants are present in human platelets, the major SERCA3 form is 3a.

The epitope for the PL/IM 430 antibody is located within a 40 kDa N-terminal sequence of SERCA3 that is common to all splice variants

To confirm that all SERCA3 forms are recognized by the PL/IM 430 antibody, we performed immuno-magnetic protein precipitation with PL/IM 430 coupled to Dynabeads® M-280 as an affinity matrix. Immunostaining by PL/IM 430 of samples obtained at different steps of immunoprecipitation is presented in Figure 4. Comparing the staining intensity of the PL/IM 430-coloured protein bands in platelet microsomal membranes (Figure 4, lane 1) and in the immunoprecipitates (Figure 4, lane 7), we conclude that about 8–12% of the total PL/IM 430-recognizable proteins (SERCA_{PL/IM}) could be captured. A relatively high portion of the PL/IM 430-recognizable proteins bound irreversibly to the affinity matrix (Figure 4, lane 6) that could not be eluted even with 0.5 M acetic acid. This might be the

Figure 6 Comparison of the expression of SERCA isoforms in platelets and Jurkat cells: Western-blot analysis

The 100 000 g mixed microsomal membranes (5 µg/lane for platelets and 50 µg/lane for Jurkat cells) were electrophoresed (SDS/PAGE, 7.5% gel), blotted and immunostained using different anti-SERCA antibodies: anti-SERCA3a (purified IgG) for lanes 1 and 2, anti-SERCA3b for lanes 3 and 4 and anti-SERCA3c for lanes 5 and 6; anti-SERCA3c pretreated with 10 µM of the peptide derived from SERCA3c (+P3c) for lanes 7 and 8; and the monoclonal anti-SERCA2 (IID8) for lanes 9 and 10. Results shown are typical of at least three Western blots performed on different membrane preparations.

reason for the relatively low efficiency of purification. The immunoprecipitates (Figure 4, lane 7) were analysed for their SERCA content, and the results are presented in Figure 5(A). In the immunoprecipitates, the protein bands recognized by the ChS3a, ChS3b and ChS3c antibodies (Figure 5A, lanes 3, 5 and 7) corresponded well to those of the SERCA3a, 3b and 3c proteins in platelet membranes, respectively (Figure 5A, lanes 4, 6 and 8). Immunostaining of PL/IM 430-captured proteins by the ChS3a, ChS3b and ChS3c antibodies was inhibited by the peptides derived from SERCA3a, 3b and 3c, respectively (results not shown). These results show co-precipitation of all three SERCA3 variants, a, b and c, by PL/IM 430 and confirm that these SERCA3 forms all contain the epitope for the PL/IM 430 antibody. Lanes 1 and 2 of Figure 5(A) represent immunostaining by PL/IM 430 antibody of both immunoprecipitates and platelet membranes, demonstrating again that the main SERCA3 form in platelets is the 3a isoform, migrating to 97 kDa. This conclusion is based on the observations that PL/IM 430 could stain the immunoprecipitates captured by this antibody preferentially at 97 kDa (Figure 5A, lane 1), and this band co-migrates with SERCA3a also recognized by the ChS3a antibody (Figure 5A, lane 3). Lanes 9 and 10 in Figure 5(A) show that the housekeeping SERCA2b isoform could not be captured by PL/IM 430–Dynabeads® M-280 from platelet membranes, further confirming the specificity of the affinity matrix for SERCA3 proteins.

To locate more precisely the epitope for the PL/IM 430 antibody, we performed proteolytic digestion experiments of human platelet membranes with trypsin. In earlier studies we demonstrated a unique pattern of proteolysis for the PL/IM 430-recognizable SERCA isoform(s) in trypsin-treated platelet membranes [21]. The PL/IM 430-recognizable 73/68 kDa and 40 kDa SERCA fragments appeared to be different when compared with the trypsin-digested fragments of the SERCA1 and SERCA2 proteins, and the well-known 25 kDa N-terminal or 80 kDa C-

terminal fragments of the SERCA3a [18,19,21]. We could immunoprecipitate a 40 kDa fragment using the PL/IM 430–Dynabeads® M-280 affinity matrix, and we characterized this tryptic fragment by staining it with different anti-SERCA3 antibodies. Lanes 1–3 of Figure 5(B) show immunostaining by PL/IM 430 of the untreated human platelet membranes (lane 1), the trypsin-digested platelet membranes (lane 2) and the immunoprecipitated 40 kDa fragment (lane 3), respectively. The conditions of trypsin treatment favoured the formation of the 40 kDa fragment to the precursor 73/68 kDa doublet. The immunoprecipitated 40 kDa fragment was not recognized by any of our new anti-SERCA3 antibodies raised against the C-termini of the SERCA3 isoforms (results not shown). However, a polyclonal anti-SERCA3 antibody, N89, immunostained both the untreated human platelet membranes at about 97 kDa and the PL/IM 430-captured 40 kDa fragment (Figure 5B, lanes 4 and 5, respectively; N89 was raised against an N-terminal peptide (amino acids 29–39) of the rat SERCA3 protein in the laboratory of Professor F. Wuytack [21]). We have to mention that the 40 kDa fragment appears to be a doublet rather than a single protein band on our Western blots. Thus we presume that proteolytic cleavage occurs at two sites in close proximity within the large cytoplasmic loop of the SERCA3 proteins (Arg-396 and Arg-403).

We conclude that the epitope for the PL/IM 430 antibody is located within the 40 kDa N-terminal part of the molecule that is common to all three human SERCA3 isoforms.

Co-expression of the SERCA3 protein isoforms in T lymphoid Jurkat cells

Dot-blot and Northern-blot hybridization analysis revealed that, among human non-muscle tissues, lymphoid tissues express the SERCA3 transcripts most abundantly [27,28]. Molecular cloning

of two human SERCA3 transcripts (3a and 3c according to the recent nomenclature) using a Jurkat T-cell cDNA library was also described in a recent report [28]. Therefore, we studied further the co-expression of the SERCA3 variants in T lymphoid Jurkat cells. Comparative Western-blot analysis of the expression of the SERCA3 protein isoforms was performed on microsomal membranes of platelets and Jurkat cells (Figure 6). We loaded 10 times more membrane proteins derived from Jurkat cells (50 µg/lane) than from platelets (5 µg/lane) on to the gel. As shown on Figure 6, all three SERCA3 variants could be detected in Jurkat cells. Comparison of the staining intensity of the bands showed that the expression levels of these proteins (Figure 6, lanes 1 and 2, lanes 3 and 4, and lanes 5 and 6, respectively) were more than 10 times lower in Jurkat cells than in platelets. In contrast, the expression of the housekeeping SERCA2b was very similar in the two cell types (Figure 6, lanes 9 and 10), which is in good accordance with previous observations [20]. Figure 6 (lane 6) also shows that the anti-SERCA3c antibody immunostained several low-molecular-mass bands that do not correspond to the intact SERCA3c. The synthetic peptide derived from isoform 3c inhibited immunostaining by ChS3c of the intact SERCA3c and some of the low-molecular-mass protein bands (Figure 6, lanes 7 and 8). These bands may represent proteolytic fragments of SERCA3c, or the ChS3c antibody recognizes other non-related cellular proteins.

Taken together, our data document that the two recently described human SERCA3 3'-end transcripts, 3b and 3c, encode the expected proteins that are co-expressed with the SERCA3a protein isoform in platelets and T lymphoid Jurkat cells.

DISCUSSION

It is well documented that various non-muscle cells are equipped with multiple sarco/endoplasmic reticulum type Ca^{2+} -ATPase isoenzymes [14–21]. SERCA2b has been identified as the ubiquitous housekeeping form, and the presence of a SERCA3 isoform, SERCA3a, has also been demonstrated in some non-muscle cell types. First indications for the alternative splicing of the SERCA3 pre-mRNA came from the EMBL/GenBank Databanks, where two mouse nucleotide sequences coding for SERCA3a and SERCA3b have been deposited (accession numbers U49394 and U49393, respectively). Recently, two additional human SERCA3 transcripts, 3b and 3c, have been cloned by three independent groups [26–28]. In addition, Dode and his co-workers [27] PCR-amplified three SERCA3 (3a, 3b and 3c) cDNAs from mouse pancreatic islets. Martin et al. [37] demonstrated the existence of an additional splice variant of rat SERCA3, termed SERCA3b/3c. Functional analysis of the three mouse SERCA3 isoforms transiently expressed in COS-1 cells [27] and of the human SERCA3a and 3c transiently expressed in HEK-293 cells [28] has also been reported. Studies of the distribution of messages for the SERCA3 isoforms in various cell and tissue types documented that the SERCA3 transcripts were most abundantly expressed in lymphoid tissues, intestine, pancreas and prostate [28], or, as published by another group [27], in thymus, trachea, salivary gland, spleen, bone marrow, peripheral leucocytes and colon. Data were lacking, however, for the presence of endogenously expressed SERCA3b and 3c variants along with other SERCA forms in non-muscle cells at the protein level.

Therefore, we raised isoform-specific polyclonal anti-peptide antibodies against the unique C-termini of the human SERCA3 splice variants (Figure 1), and we have used these antibodies to test the distribution of the SERCA3 proteins in two human haematopoietic cell types, i.e. platelets and T lymphoid Jurkat cells (Figure 2A and Figure 6). All of our anti-SERCA3

antibodies appeared to be specific for the corresponding SERCA3 protein isoform, as shown by peptide competition studies (Figure 2A). None of them cross-reacted with the housekeeping SERCA2b enzyme (Figure 2B), the only known SERCA isoform that is co-expressed endogenously with SERCA3 proteins in non-muscle cells. Other members of the SERCA family, 1a, 1b and 2a, exhibit more restricted tissue distribution and are expressed at high levels in skeletal and/or cardiac muscle [38,39]. We have not studied the potential cross-reaction of the novel anti-SERCA3 antibodies with these latter SERCA variants that do not contain the C-terminal amino acid stretches of the SERCA3a, 3b or 3c variants. We have to mention that the ChS3c antibody often immunostains additional protein bands at lower molecular masses than that of the intact SERCA3c (Figure 6). Whether the ChS3c cross-reacts with other non-related cellular proteins or these bands correspond to proteolytic fragments of SERCA3c is not clear. The synthetic SERCA3c peptide used for immunization, however, inhibited immunostaining by ChS3c of some of the low-molecular-mass protein bands as well as that of the intact SERCA3c variant (Figure 6, lanes 7 and 8).

Our data, obtained by using the isoform-specific anti-SERCA3 antibodies, show that the newly described SERCA3b and 3c transcripts are translated to proteins and are co-expressed with the 3a protein in human platelets as well as in the T lymphoid Jurkat cell line (Figures 2 and 6). The presence of SERCA3a, 3b and 3c mRNAs in human platelets has been demonstrated recently [36] and these SERCA3 transcripts have been detected in Jurkat T-cells as well [26,28]. Although all SERCA3 proteins could be detected in these cell types, the 3a isoform was the most abundantly expressed (results shown only for platelets in Figure 3). This observation is in good accordance with that of Dode et al. [27], who found that both SERCA3b and 3c mRNAs were expressed at lower levels than the SERCA3a mRNA in all human tissues examined. The expression level of the SERCA3 proteins appeared to be at least 10 times higher in platelets than in T lymphoid Jurkat cells (Figure 6). In contrast, the expression of the ubiquitous SERCA2b was very similar in these two cell types and all non-muscle cell types studied so far (Figure 6 and previous results [20,36]). We cannot explain the biological significance of the relatively high expression level of SERCA3 proteins in platelets; however, recent experiments showed that the strong increase in the amount of the SERCA3 variants appears during megakaryocytopoiesis [36]. The authors detected up-regulation of the SERCA3a, 3b and 3c mRNAs during PMA-induced megakaryocytic differentiation of the immature erythroid/megakaryoblastic MEG 01 cell line. Because of the lack of isoform-specific anti-SERCA3 antibodies, they used the monoclonal PL/IM 430 antibody to monitor the expression of a 97 kDa SERCA protein (this SERCA isoform is the SERCA3a variant according to the recent nomenclature). They found an approx. 4-fold increase in the expression of the PL/IM 430-recognizable SERCA protein in PMA-treated MEG 01 cells, whereas that of the housekeeping SERCA2b remained unchanged during maturation. Similar results have also been obtained during all-*trans*-retinoic acid-induced differentiation of acute promyelocytic cells [34]. Whereas an approx. 4-fold over-expression of the PL/IM 430-recognizable SERCA protein could be detected, the expression of the SERCA2b isoform was slightly down-modulated or did not change significantly.

Although the PL/IM 430 monoclonal antibody has been reported to immunostain a 97 kDa SERCA protein in various human non-muscle cells [19,20], the identity of this SERCA variant has long been debated [21–23]. Poch and his co-workers [28] provided evidence that this antibody recognizes both the cloned human SERCA3a and 3c isoforms expressed in HEK-293

cells. Our present data confirm these findings. As shown in Figure 3, PL/IM 430 immunostained all splice variants of SERCA3 (including 3a, 3b and 3c) in human platelets. To further confirm that PL/IM 430 recognizes all SERCA3 splice variants, we performed immuno-magnetic protein precipitation from platelet microsomal membranes by using a PL/IM 430-affinity matrix (Figure 4). Analysis of the captured proteins for their SERCA content showed that all three SERCA3 variants could be precipitated with this affinity matrix, providing evidence that SERCA3a, 3b and 3c all contain the epitope for the PL/IM 430 antibody (Figure 5A).

In contrast with SERCA1 and SERCA2, SERCA3 does not contain the T₁ tryptic cleavage site. Proteolytic digestion of the SERCA3a protein with trypsin occurs at the T₂ site, and results in an approx. 25 kDa N-terminal and an 80 kDa C-terminal fragment [18,19]. Surprisingly, neither of these fragments could be immunostained by PL/IM 430, whereas it recognized a 73/68 kDa doublet and a 40 kDa fragment in trypsin-digested platelet membranes [21]. Here we demonstrate that the epitope for the PL/IM 430 antibody is located within a 40 kDa N-terminal part of SERCA3, which is common to all known splice variants (Figure 5B). Based on these data, it is most probable that the tryptic degradation pattern seen with the PL/IM 430 antibody refers to a specific conformational state of the SERCA3 proteins and not to a distinct SERCA isoform, as suggested previously [21].

In summary, we generated isoform-specific polyclonal antibodies to monitor the novel SERCA3b and 3c proteins along with SERCA3a. By using these antibodies, we demonstrated for the first time the co-expression of the three SERCA3 protein isoforms in two different haematopoietic cell types, i.e. platelets and lymphoid cells. We documented that the generally used monoclonal anti-SERCA antibody, PL/IM 430, recognizes all SERCA3 variants, and that its epitope is located within a common 40 kDa N-terminal region of the SERCA3 proteins. Although the understanding of the functional role of the novel SERCA3 isoforms in cellular Ca²⁺ signalling needs further research, this work highlights new members of the endoplasmic reticulum Ca²⁺-transporting systems, which should be considered in future studies on Ca²⁺ homeostasis of non-muscle cells. The enormous functional diversity of non-muscle cells does require a rather finely regulated Ca²⁺ handling, and this might explain the high complexity of the multi-SERCA system in these cell types.

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