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The sarco(endo)plasmic reticulum Ca^{2+}-ATPase mRNA isoform, SERCA 3, is expressed in endothelial and epithelial cells in various organs

Marielle Anger[a], Jane-Lyse Samuel[b], Françoise Marotte[b], Frank Wuytack[c], Lydie Rappaport[b], Anne-Marie Lompré[a,*]

[a]*INSERM U 275 – LOA – ENSTA, Ecole Polytechnique, Centre de l'Yvette, 91120 Palaiseau, France*
[b]*INSERM U 127, Hôpital Lariboisière, 41 Bd de la Chapelle, 75010 Paris, France*
[c]*Laboratorium voor Fysiologie, Universiteit Leuven, Campus Gasthuisberg, B-3000 Leuven, Belgium*

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The sarco(endo)plasmic reticulum Ca^{2+}-ATPase mRNA isoform, SERCA 3, was previously shown to be expressed in a great variety of muscle and non-muscle tissues [(1989) J. Biol. Chem. 264, 18561–18568] but its cellular localization within these organs was unknown. We have used in situ hybridization and RNase protection techniques to demonstrate that SERCA 3 mRNA is expressed in specific cell types, namely the endothelial and epithelial cells.

Sarcoplasmic reticulum; Ca^{2+}-ATPase mRNA isoform; In situ hybridization; Endothelial cell; Epithelial cell

1. INTRODUCTION

The sarco(endo)reticulum (SR/ER) Ca^{2+}-ATPase is involved in the transport of Ca^{2+} from the cytosol to various intracellular Ca^{2+} stores, such as the sarcoplasmic reticulum, the endoplasmic reticulum and the calciosomes. It is present in several cell types and plays an important role in controlling cellular functions, such as relaxation and secretion. Multiple Ca^{2+}-ATPase isoforms have been identified by cDNA cloning. They are encoded by three different genes (SERCA 1, 2 and 3) and result from alternative splicing of pre-mRNA transcripts [1–3]. The SERCA 1 gene is exclusively expressed in fast skeletal muscle [4,5]. The SERCA 2 gene gives rise to two alternatively spliced transcripts, one of which, SERCA 2a, has been detected in adult cardiac muscle, in slow skeletal muscle and in some smooth muscle tissues whereas the other, SERCA 2b, is found in adult smooth muscle and in non-muscle tissues [6–12]. SERCA 3, originally isolated from adult rat kidney, is highly homologous to SERCA 1 and SERCA 2 mRNAs; it was shown to be expressed in a broad variety of muscle and non-muscle tissues [1] but its cellular localization remained unknown. We have used in situ mRNA hybridization and RNase protection assays to demonstrate that, in several organs, SERCA 3 is expressed in specific cell types, namely the arterial endothelial cells and some epithelial cells.

*Corresponding author. Fax: (33) (1) 60 10 60 85.

2. MATERIALS AND METHODS

2.1. *Complementary DNA probes*

SERCA 3 and SERCA 2b cDNA probes were prepared by PCR amplification. They correspond to nucleotides 1,107 1,523 and 3,489 3,630 of the sequences published by Burk et al. [1] and Gunteski-Hamblin et al. [7], respectively. For SERCA 3, first-strand cDNA was synthesized from 1 μg of poly(A)$^+$ RNA isolated from spleen using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies) at 37°C. For SERCA 2b, 10 μg of total RNA from rat aortic smooth muscle cells in subculture were transcribed at 42°C using avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim) and the 3′-end primer. DNA amplification was performed in the presence of *Taq* polymerase (2.5 U) and the PCR conditions for amplification of SERCA 3 mRNA were 1 min at 94°C, 2 min at 45°C, 1 min at 72°C followed by 40 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The amplified SERCA 3 fragment was blunt-end ligated to the *Sma*I site of pGEM-7ZF(+) vector. After an initial incubation of 2 min at 92°C, SERCA 2b mRNA was amplified by 30 cycles of 1 min at 92°C, 2 min at 55°C and 3 min at 72°C. *Hin*dIII and *Xho*I restriction sites were included in the primers and the amplified fragment was inserted in the corresponding sites of the pB II KS vector (Stratagene). Double-stranded DNA was sequenced using the Sequenase kit (USB) to confirm the amplification of the expected product. The α-smooth muscle actin probe was a gift from Dr. Gabbiani (Geneva), a 130 nucleotide *Dde*I–*Hin*dIII DNA fragment corresponding to a part of the 3′ untranslated region of the α-smooth muscle (α-Sm) actin mRNA that was subcloned in the pSP64 vector. Its specificity had been determined previously [13].

2.2. *cRNA probes*

Complementary RNA probes were transcribed in vitro from *Hin*dIII-linearized plasmids in the presence of T_7 (SERCA 3) or T_3 (SERCA 2b) RNA polymerases (Promega) or from *Eco*RI-linearized plasmid and SP_6 RNA polymerase (α-Sm). [^{35}S]UPT (1,000 Ci/mmol; Amersham) was used as the labeled nucleotide triphosphate. Unincorporated nucleotides were removed by two ethanol precipitations. All probes were diluted to a final specific activity of about 60,000 cpm/μl

in 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10 mM sodium phosphate (pH 8.0), 10% dextran sulfate, 1 × Denhardt's, 0.5 mg/ml yeast tRNA and 20 mM dithiothreitol (DTT). Labeled sense sequences synthesized in vitro from the same plasmids were used to test the specificity of the respective probes.

2.3. *RNase protection analysis*

Tissues from adult Wistar rats were excised and quickly frozen in liquid nitrogen. Rat aortic endothelial cells and rat aortic smooth muscle cells were collected after two and four passages, respectively. The cells were collected by centrifugation and the pellets frozen in liquid nitrogen. Total RNA was extracted by the guanidinium isothiocyanate procedure [14]. 10 μg of total RNA were used for RNase protection analysis. cRNA probes were labeled as described above using [α-^{32}P]CTP (1,000 Ci/mmol; Amersham) as the labeled nucleotide triphosphate. The full-length fragments, purified on acrylamide–urea denaturing gels, were hybridized to total RNA in 80% formamide at 42°C overnight. After hybridization, the unreacted cRNA probes were digested in the presence of 0.5 μg of RNase A and 10 U of RNase T1 at 37°C for 30 min following the RPA II protocol (Ambion; Clinisciences; France). Each experiment included a control reaction in which total RNA was replaced by 10 μg of yeast tRNA. After digestion, the protected probes were separated by electrophoresis on 6% polyacrylamide gels. The gels were dried and exposed to X-ray films (Kodak; France).

2.4. *In situ hybridization*

The tissues were fixed in 2% paraformaldehyde in PBS (phosphate-buffered saline) for 2 h at 4°C, then washed in PBS containing 30% sucrose for 4 h at 4°C, embedded in OCT (RUA; Torcy; France), and frozen in liquid nitrogen-cooled isopentane. Serial cryosections (5-μm thick) were transferred to chromium alum gelatin-coated slides, dried a few minutes at room temperature, fixed in 4% paraformaldehyde for 5 min, washed in PBS (2 × 5 min), then dehydrated in ethanol and stored at −70°C with dessicant until use.

In situ hybridization procedures were similar to those described by Wilkinson et al. [15]. About 7 μl of hybridization mixture containing the cRNA probe (400,000 cpm) were applied to each section. The slides were incubated at 50°C overnight. After one washing at 50°C in 5 × SSC (1 × SSC = 0.15 M sodium chloride and 0.015 M sodium citrate) and 10 mM DTT, the slides were incubated in 50% formamide, 2 × SSC and 0.1 M DTT at 65°C for 20 min, treated with 20 μg/ml RNase A at 37°C for 30 min, and washed twice in 2 × SSC and twice in 0.1 × SSC at room temperature. After dehydration, the slides were immersed in Kodak NTB2 Nuclear track emulsion (Eastman Kodak) drained of excess fluid and autoradiographed. After 15 days, sections were developed in Kodak D19, mounted, and examined by light- and dark-field illumination.

3. RESULTS AND DISCUSSION

The expression of the SERCA 3 mRNA isoform in different organs was determined by RNase protection analysis (Fig. 1). SERCA 3 mRNA was highly expressed in the trachea, was present at lower level in the liver and the aorta, but was undetectable in either the lung or the heart. The results confirm that SERCA 3 is expressed in several muscular and non-muscular organs as already reported by Burk et al. [1] using Northern blot analysis. Some discrepancies in the level of expression of SERCA 3 in the various organs were observed between both sets of data. Due to the cell-specific expression of SERCA 3 in these organs (see below), heterogeneity in the sample used to prepare the RNA may well explain these differences. Alternatively, some cross-

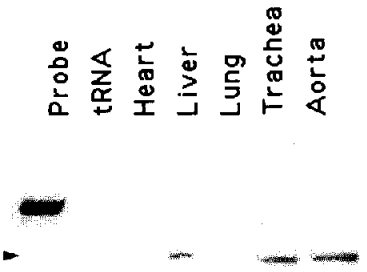

Fig. 1. RNase protection analysis of total RNA from heart, liver, lung, trachea and aorta of adult rat hybridized with the SERCA 3 probe. 10 μg of total RNA from various tissues and 10 μg of yeast tRNA were hybridized with the radiolabeled SERCA 3 cRNA probe. After ribonuclease digestion, the protected fragments were separated on 6% sequencing gel. 'Probe' indicates the position of the undigested probe. The position of the partially protected fragment is indicated by an arrow; it was smaller than the undigested probe since the probe contained plasmid sequences which did not hybridize to mammalian RNA. Furthermore control hybridization and digestion in the presence of tRNA alone clearly indicated the specificity of the probe and the complete RNAse digestion of single-stranded cRNA. The gels were exposed to X-ray films for 4 days.

hybridization of the SERCA 3 probe with other SERCA mRNA species in the Northern hybridization may also lead to over-estimation of SERCA 3 expression.

To determine precisely the cellular specificity of expression of SERCA 3 in these organs, SERCA 3 mRNA was labeled in situ with a specific cRNA probe. Sections from aortic trunk (Fig. 2A–C), myocardium (Fig. 2D–F), trachea (Fig. 2G,H) and liver (Fig. 2I,J) were labeled with the SERCA 3 probe (Fig. 2A,D,G,I) and the α-Sm actin probe (B,E). The α-Sm actin probe was used as a marker of the smooth muscle cells to delineate the medial layer in the vessels. The structures were also visualized in bright-field (Fig. 2C,F,H,I). On sections from either the aortic trunk (Fig. 2A,C), or the myocardium (Fig. 2D,F) SERCA 3 mRNA was detected only in the luminal part of the arterial wall, whereas the α-Sm actin probe clearly hybridized with the medial layer. A strong accumulation of SERCA 3 mRNA transcripts is observed in the layers bordering the lumen of the trachea corresponding to epithelial cells (Fig. 2G,H). In adult liver, SERCA 3 mRNA was detected only at the luminal side of the arterial wall (Fig. 2I,J). The specific expression of SERCA 3 in heart, aorta, liver and trachea demonstrated by in situ hybridization is in agreement with the levels of mRNA detected by RNase protection assay. In order to further confirm the expression of

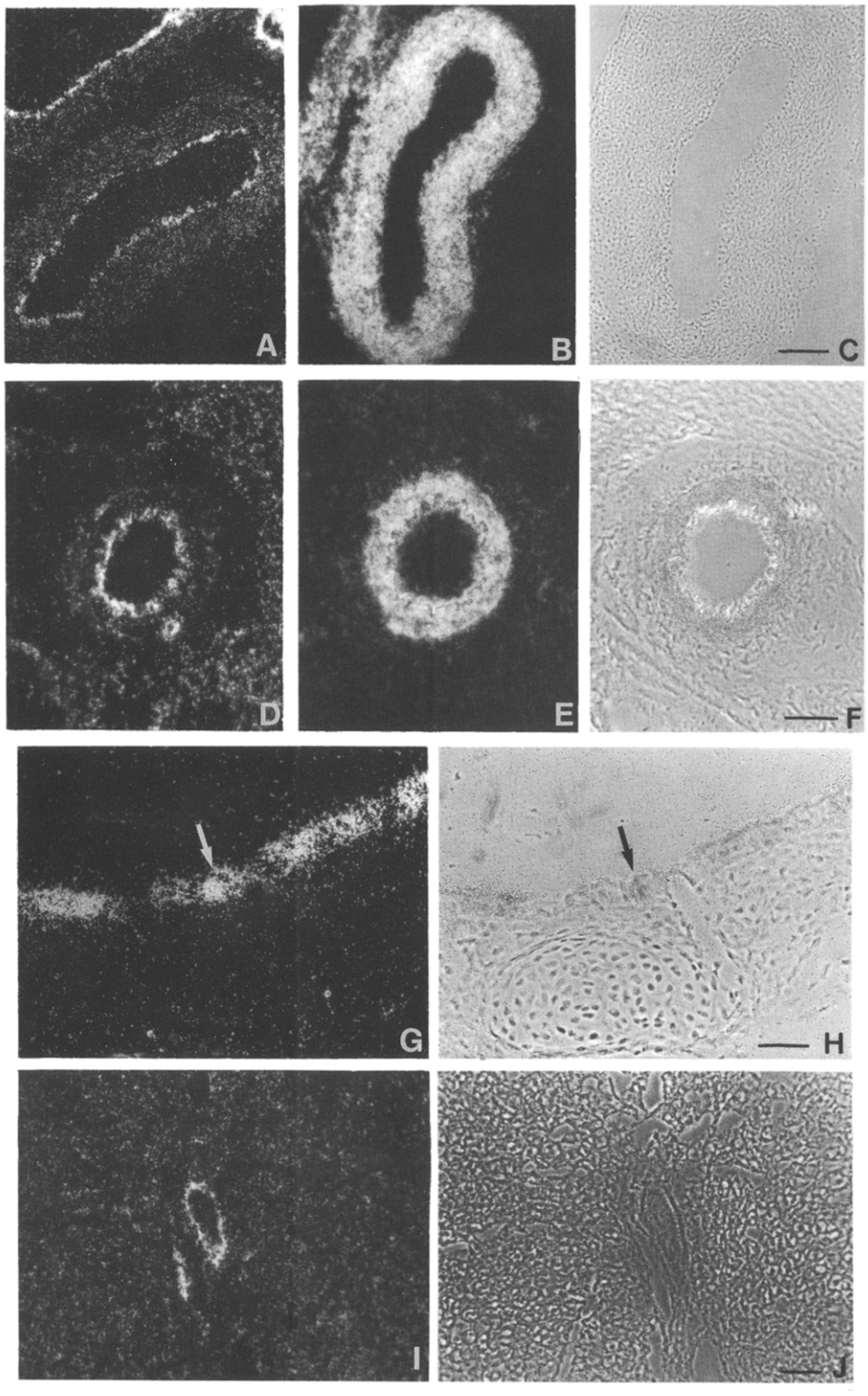

Fig. 2. Localization of SERCA 3 and α-Sm actin mRNAs in different rat tissues. Vascular trunk (A,C), coronary arteries (D,F), trachea (G,H) and adult liver (I,J), hybridized with SERCA 3 (A,D,G,I) and α-Sm actin (B,E) cRNA probes or visualized with bright-field (C,F,H,J). SERCA 3 mRNA was detected in endothelial cells and in epithelial cells (arrow). The α-Sm actin was detected in the medial layer of the vessels. Bar = (A,C) 100 μm; (D,F) 60 μm; (G,H) 50 μm; (I,J) 60 μm. Only background signal was observed with the sense probes.

SERCA 3 in endothelial cells, we performed RNase protection analysis using RNA from isolated aortic endothelial cells in culture. SERCA 3 mRNA was present in subcultured aortic endothelial cells but not in aortic smooth muscle cells (Fig. 3), whereas SERCA 2b was present in both cell types. Based on the in situ localiza-

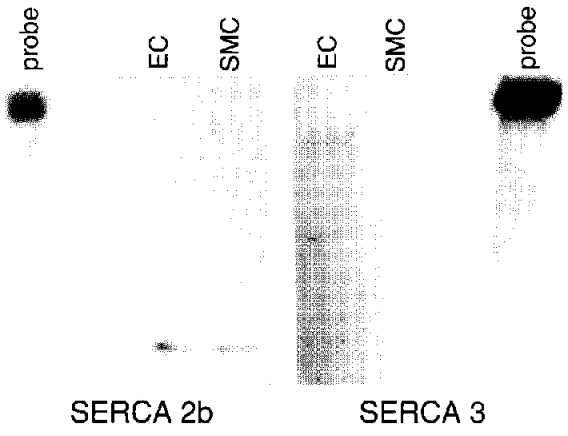

Fig. 3. RNase protection analysis of RNA from subcultured endothelial cells (EC) and subcultured aortic smooth muscle cells (SMC). 5 μg of total RNA hybridized with SERCA 3 and SERCA 2b cRNA probes. 'Probe' indicates the position of the undigested probe. The position of the partially protected fragments is indicated by arrows.

tion of these cells in a thin layer bordering the lumen of the vessels and on the fact that SERCA 3 mRNA is observed in isolated arterial endothelial cells in culture, we conclude that the arterial endothelial cells express the SERCA 3 gene. We had previously demonstrated the presence of SR/ER Ca^{2+}-ATPase in human omental microvascular endothelial cells [16], and the present study indicates that the SERCA 3 isoform is a major SR/ER Ca^{2+}-ATPase expressed in vivo in arterial endothelial cells. The presence of a large amount of an organellar type of Ca^{2+}-ATPase mRNA in endothelial cells is not surprising since ER is abundant in this cell type and the intracellular Ca^{2+} compartment is of major importance in controlling the release of paracrine factors responsible for the regulation of the vascular tone [17]. As shown by both RNase protection analysis and in situ hybridization, SERCA 3 mRNA is also abundant in the tracheal epithelium. In this cell type, SERCA 3 might be important in controlling secretion. The protein composition of the ER in endothelial and epithelial cells has not been well characterized. In addition to the presence of SERCA 3 which we now report, Lesh et al. [18] have demonstrated the presence of ryanodine receptors, the Ca^{2+}-induced Ca^{2+} release channels, throughout the ER of arterial endothelium. It remains to be determined which ryanodine receptor isoform is expressed in endothelial cells and whether SERCA 3 and the ryanodine receptor are present in the same subcellular structure. The presence of both ryanodine receptors and SERCA 3 would constitute a unique Ca^{2+} transport system for arterial endothelium, but the mechanism of activation of Ca^{2+} entry and Ca^{2+} release remains to be established. The low affinity for Ca^{2+} of SERCA 3 compared to all the other SERCA isoforms [19,20] suggests a particular mechanism of activation.

Our results demonstrate for the first time that, in the adult rat, SERCA 3 is expressed in a great variety of organs but that its expression is restricted to a subset of cells, namely the arterial endothelial cells. In the trachea, its expression is restricted to the epithelial cells.

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