Characterization of the 3′ end of the mouse SERCA 3 gene and tissue distribution of mRNA spliced variants

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Abstract The sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) type 1 and 2 genes are alternatively spliced at their 3′ end. We hypothesized that similar mechanism may occur for SERCA 3. Two spliced variants were identified by RNase protection analysis. We then isolated and sequenced the 3′ end portion of the mouse SERCA 3 gene, and confirmed the presence of an alternative mRNA transcript by sequencing a cDNA fragment obtained by RT-PCR. Tissue distribution of the alternatively spliced mRNAs was studied by RT-PCR: SERCA 3b was the only isoform expressed in endothelial cells from aorta and heart and also was the major isoform in lung and kidney whereas SERCA 3a and 3b were coexpressed in trachea, intestine, thymus, spleen, and fetal liver.

Key words: Sarco(endo)plasmic reticulum; Ca^{2+} ATPase mRNA isoform; SERCA; Alternative splicing; Gene expression

1. Introduction

The sarco(endo)plasmic reticulum Ca^{2+} ATPases (SERCA) play a crucial role in Ca^{2+} homeostasis and signalling by mediating re-uptake of Ca^{2+} from the cytosol into intracellular Ca^{2+} stores. Several SERCA isoforms, encoded by three different genes, i.e. SERCA 1, SERCA 2 and SERCA 3 have been identified ([1,2] for reviews). These pumps have a very high degree of identity but a different pattern of expression. SERCA 1 is exclusively expressed in fast skeletal muscles, and gives rise to two alternatively spliced transcripts: SERCA 1a in adult and SERCA 1b in neonatal muscles. The last eight C-terminal amino acids of SERCA 1b are replaced by a single glycine residue in SERCA 1a [3,4]. The SERCA 2 gene also encodes two alternatively spliced transcripts: SERCA 2a is expressed in heart, slow skeletal and some smooth muscles in the adult, and also in embryonic skeletal muscles, whereas SERCA 2b is a housekeeping isoform present in all cell types [5–7]. The last four C-terminal amino acids of SERCA 2a are replaced in SERCA 2b by 49 residues [8–10]. In the adult, SERCA 3 is expressed in a broad variety of tissues [11] but in specific cell types such as endothelial cells, epithelial cells from trachea, spleen, lung, pancreas, stomach, salivary glands, adrenal gland, thyroid, thymic cortex, cells of the intestinal crypt, and purkinje cells in cerebellum [7,12–14]. SERCA 3 is also present in the hemopoietic cell lineage and platelets, various lymphoid cell lines and mast cells [15,16]. This isoform is always coexpressed with SERCA 2b. Furthermore, the sequences of two mouse SERCA 3 cDNAs [17] and of the 3′ end portion of the human SERCA 3 gene [13] were recently published. In human, two mRNA populations differing by their size in Northern blot were detected, suggesting alternative splicing but the 3′ end sequence of the gene did not reveal the existence of an alternative exon [13].

The purpose of this work was to test whether the SERCA 3 gene, like the two other SERCA genes, can give rise to several mRNA transcripts by alternative splicing at its 3′ end and to study tissue distribution of the various transcripts.

2. Material and methods

2.1. Library screening and DNA sequencing

A 516 bp *Pst*I fragment from the 3′ untranslated region of the rat SERCA 3 cDNA clone RK 8-13 [11], corresponding to nucleotides (nt) 3277 to 3792, was used to screen a mouse genomic library (129SVJ in Lambda Fix vector, Stratagene). The selected genomic clones were subcloned into pBKS vector and digested with various enzymes. A restriction map was constructed after hybridization of the digested fragments with ^{32}P-labeled oligonucleotides designed from the rat SERCA 3 cDNA sequence [11]. Fragments were sequenced in both directions (Sequenase kit, USB).

2.2. RNA isolation and RNase protection analysis

Total RNA from various rat and mouse tissues were isolated using the guanidinium thiocyanate procedure (RNA instapure, Eurogentec). RNase protection assay was performed on 20 μg of total RNA from adult rat aorta and trachea. A 807 bp *Sma*I-*Pst*I fragment corresponding to nt 2471–3277 of the rat cDNA RK 8–13 [11] was subcloned into pBKS vector. The cRNA probe was prepared from the *Bam*HI linearized plasmid transcribed in vitro in the presence of T_3 RNA polymerase (riboprobe system, Promega) and [α-^{32}P]-CTP (800 Ci/mmol, NEN life Science). The full-length probe, purified on denaturing polyacrylamide gel, was hybridized to total RNA at 39°C overnight. Unreacted cRNA was digested by 50 U of RNase T1 at room temperature for 30 min, following RPA II procedure (Ambion, Clinisciences). Yeast tRNA was used as a negative control. After digestion, protected fragments were separated by electrophoresis and exposed to X-ray film (Kodak, France).

2.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Five μg of total RNA from mouse tissues were used for reverse transcription using Ready-To-Go You-Prime first-Strand beads (Pharmacia Biotech) and 0.2 μg of random hexamers (Boehringer Mannheim). Reaction was performed for 1 h at 37°C. First-strand DNA was amplified in presence of 2.5 U of AmpliTaq DNA polymerase (Thermus aquaticus; Perkin Elmer) and 40 pmoles of each specific primer (Genset) for 105 s at 95°C and 35 cycles of 15 s at 95°C, 30 s at 64°C, followed by 15 cycles of 15 s at 95°C, 1 min at 64°C and finally 7 min at 72°C. Controls without reverse transcription were performed in each experiment. PCR products were separated on 12% polyacrylamide gel, stained with vistra green (Amersham Life Science) and then electroblotted onto nylon membrane (Parablot NY plus; Ma-

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cherey-Nagel). Prehybridization was performed in 6×standard saline citrate (1×SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate), 0.1% ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.5% sodium dodecyl sulfate (SDS), 100 μg/ml salmon sperm DNA at 42°C for 1 to 2 h. Hybridization was done in 6×SSC, 0.5% SDS and 100 μg salmon sperm DNA at 39°C overnight with an internal oligonucleotide (nt 2925–2941) ^{32}P-labeled using T_4 kinase and [γ^{32}P]-ATP. After final washing for 1 h in 1×SSC, 0.1% SDS at 40°C, the membrane was exposed to the phosphorimager screen which was scan on a Storm (Molecular Dynamics).

3. Results and discussion

It has been clearly demonstrated that several mRNA isoforms are generated by alternative splicing at the 3′ end of SERCA 1 and SERCA 2 genes ([1,2] for reviews). Furthermore, in human platelets a novel SERCA 3 isoform recognized by the PL/IM 430 antibody but different from SERCA 3 RK 8–13 [11] was also identified [18]. Thus, we hypothesized that the SERCA 3 gene could also give rise to multiple isoforms by splicing at its 3′ end. In a first approach, we searched for the presence of multiple RNA populations by RNase protection assay (RPA) using total RNA from tissues expressing high level of SERCA 3, aorta and trachea. In aorta SERCA 3 is expressed in endothelial cells whereas in trachea it is present in epithelial cells [12]. When we used as a probe a fragment of cDNA covering the 3′ end of the coding sequence and the beginning of the untranslated region, nt 2471 to 3277, two mRNA populations were observed: the fully protected

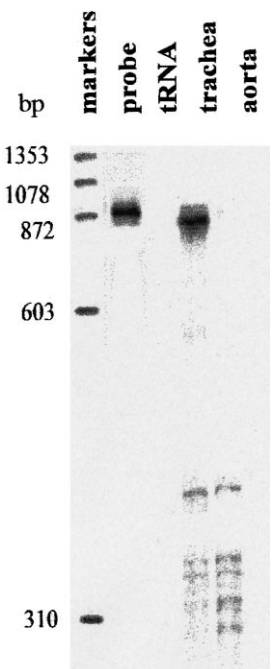

Fig. 1. RNase protection analysis of the expression of SERCA 3 mRNA variants. Total RNA rat from aorta and trachea as well as yeast tRNA were hybridized to a cRNA probe generated from a 807 bp *Sma*I-*Pst*I fragment corresponding to nt 2471–3277 of the rat cDNA RK 8–13 [11]. The upper fragment corresponds to fully protected probe SERCA 3a, and lower fragments to SERCA 3b. Due to insertion of an additional exon in SERCA 3b, two major fragments of approximately 500 bp and 300 bp are expected for SERCA 3b (see below). The undigested probe, lane probe, is slightly higher than the fully protected fragment due to insertion of vector sequences. Positions of DNA markers (bp) are indicated.

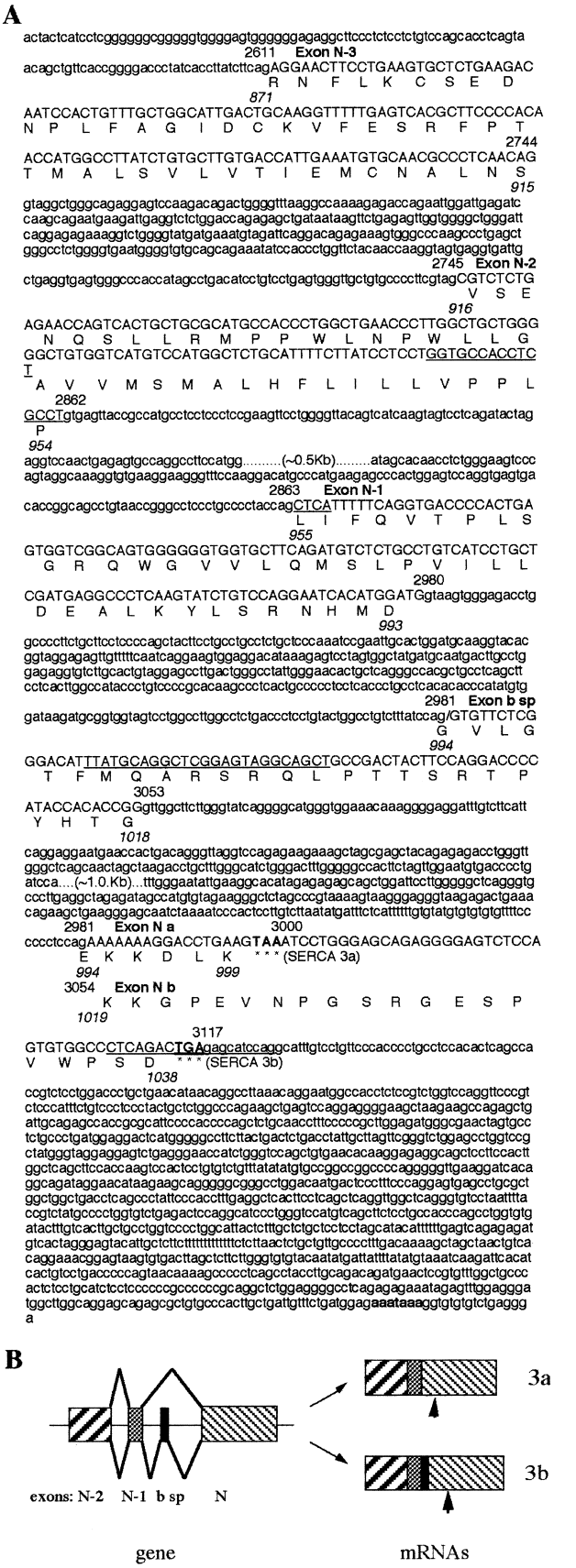

Fig. 2. Sequence and organization of the 3′ end portion of the mouse SERCA 3 gene. A: Sequence of the fifth last exons (upper case) and introns (lower case). Nucleotides are numbered as referred to Tokuyama et al. [17]. Amino acid sequence is presented beneath the nucleotide sequence with the one-letter code. Two mRNA species are generated by addition of a 73 bp additional exon, b sp, and usage of two different stop codons (bold letters and stars). Primers used for PCR are underlined: the upstream is 21S spanning from exons $N-2$ to $N-1$, the middle 21bS, and the downstream 22R. B: Schematic representation of the 3′ end of the mouse SERCA 3 gene and of the two mRNA species. Arrowheads indicate positions of stop codons.
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fragment corresponding to SERCA 3a was present in trachea together with additional shorter fragments (Fig. 1). Only shorter fragments were present in RNA from aorta. One of these fragments, approximately 500 bp, was compatible with the existence of a point of divergency near the end of the coding region and thus with the presence of the two mRNA sequences published by Tokuyama et al. [17].

To better characterize the mechanism of alternative splicing, we have isolated and sequenced the 3′ end portion of the mouse SERCA 3 gene (Fig. 2). By comparing the genomic sequence with that of the cDNAs [17], we could reconstruct the SERCA 3 gene. We confirmed the sequence of SERCA 3b cDNA by sequencing a PCR product obtained by reverse transcription of cardiac RNA (not shown), where SERCA 3 is present in coronary arteries [12]. The additional 73 nucleotides of SERCA 3b were located 387 nt downstream of the 3′ boundary of exon $N-1$ and constitute the b specific exon (b sp). Exons were numbered by comparison with the human SERCA 3 gene [13] and each of them had consensus exon/intron boundaries. Addition of this exon modified the open reading frame of exon N and led to usage of two different stop codons (Fig. 2). This splicing mechanism leads to the generation of two proteins which differ in their C-terminal portion by the substitution of the last six amino acids of SERCA 3a by and extension of 45 amino acids in SERCA 3b. Based on Northern blot detection of two mRNA populations in human tissues (4.0 and 4.8 kb), the possible existence of alternatively spliced isoforms was stressed out but the sequence of the human gene 3′ portion did not reveal the presence of an alternative exon [13]. In fact, the SERCA 3b specific exon was located 349 bp downstream of the 3′ boundary of exon $N-1$ [13,18]. Coding regions of the mouse genomic sequence as well as the mouse cardiac cDNA sequence obtained by PCR were absolutely identical to SERCA 3b cDNA sequence previously published by Tokuyama et al. [17] but human and mouse C-terminal regions were very different [13,18].

We then studied distribution of the alternative transcripts in mouse tissues by RT-PCR using two sets of primers (see Fig. 2A for primer location). Using primers 21S and 22R, two fragments were detected; their size was compatible with the presence of SERCA 3b (303 bp) and SERCA 3a (229 bp) (Fig. 3A). SERCA 3b was the only isoform expressed in aorta and heart and the major one in lung and kidney whereas the two isoforms were detected in all other tissues. No signal was detected in C2C12 skeletal muscle or 10T1/2 fibroblast cell lines and in controls without reverse transcriptase (not shown). The presence of SERCA 3b in aorta was in agreement with the presence of only the short fragments in Fig. 1. Similarly, in trachea, SERCA 3a was slightly more abundant than SERCA 3b as shown by RPA and RT-PCR experiments (Figs. 1 and 3). Note that in Fig. 1 specific activity of the fully protected fragment (3a) is two times higher than that of 3b, whereas in Fig. 3 the same oligonucleotide is unable to detect both isoforms with the same activity. We then used another pair of primers, 21bS and 22R, to detect specifically SERCA 3b or any other potential exon present between exon b sp and exon N (Fig. 2A). These results confirmed that SERCA 3b was expressed in each tissue (Fig. 3B). Furthermore only one fragment was observed with these primers indicating that no other coding sequence was detectable between exons b sp and N.

Our data indicate that SERCA 3b is ubiquitously expressed in tissues containing arterial endothelial cells (aorta, coronary vessels from heart), epithelial (trachea, intestine, thymus, lung, kidney) and lymphoid tissues (thymus, spleen) and that SERCA 3a is present in epithelial and lymphoid tissues but not in endothelial cells. In human, SERCA 3a and 3b were both detected in platelets and in a lymphoid cell line (Jurkat) whereas only SERCA 3a was present in the epithelial-like HeLa cell line [18]. One cannot exclude that the 3b isoform we have seen in epithelial and lymphoid tissues corresponds to endothelial cells of vessels. On the other hand, the presence of only SERCA 3a in HeLa cells may be due to a shift from expression of one isoform to the other during dedifferentiation of cells in culture as we have shown in smooth muscle cells for the SERCA 2 gene [19,20].

When expressed in COS cells, SERCA 3a has a lower affinity for Ca^{2+}, an enhanced sensitivity to vanadate and a

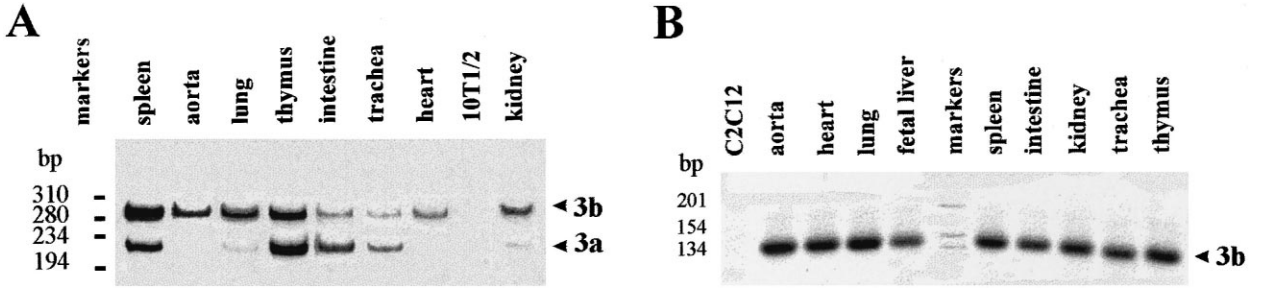

Fig. 3. RT-PCR analysis of SERCA 3 spliced variants expression. A: Southern blot hybridization using an internal oligonucleotide of RT-PCR products obtained with primers 21S and 22R. Two fragments of size compatible with SERCA 3b (303 bp) and SERCA 3a (229 bp) are detected. B: RT-PCR products stained with vistra green. Primers used were 21bS and 22R. A single fragment located between 134 and 154 bp, as expected for SERCA 3b (144 bp), was observed. This figure is representative of at least three experiments performed on different RNA preparations from different mice. Positions of DNA markers are indicated.

higher optimum pH range than the other isoforms [21,22]. In particular, SERCA 3a is less sensitive to Ca^{2+} and more resistant to peroxide than SERCA 2b with which it is coexpressed [21,23]. These unusual biochemical characteristics and the prominence of Ca^{2+} signalling in the cell types in which SERCA 3 is expressed, suggest that it might have a particular role in specialized Ca^{2+}-dependent functions. To test the hypothesis that SERCA 3 has a particular role in endothelial cells and can regulate the vascular tone, G. Shull's group has generated SERCA 3-deficient mice and analyzed contraction and relaxation of aortic rings and Ca^{2+} signalling in endothelial cells [24]. Their results suggest that SERCA 3 plays an important role in mechanisms that mediate endothelium-dependent relaxation of vascular smooth muscle. Our data indicate that SERCA 3b is the only SERCA 3 isoform expressed in endothelial cells. Thus, SERCA 3b should be involved in synthesis or secretion of endothelial vasoactive peptides but a specific role for SERCA 3a remains to be elucidated. We have previously shown that SERCA 3 is expressed at early embryonic stages in the rat, especially in heart, dorsal aorta and liver [6]. However, the knock-out data indicate that SERCA 3 is not absolutely necessary for development since the SERCA 3-deficient mice are viable and fertile. The role of SERCA 3 in embryos is not yet known. Polarized expression of SERCAs was demonstrated in pancreatic acini and in submandibular salivary glands [14]. In pancreatic acini SERCA 2a is expressed at the luminal pole and SERCA 2b at the basal pole and in the nuclear envelope. In acinar and duct cells of submandibular salivary glands, SERCA 2b is present at the luminal pole and SERCA 3 at the basal pole. Furthermore, in platelets, the 97 kDa-PL/IM 430 recognizable Ca^{2+} pump, now identified as SERCA 3b [18], is present only in structures associated with the cytoplasmic face of plasma membranes whereas SERCA 3a and SERCA 2b are in both peripheral and internal compartments [25].

In conclusion, we have shown that the SERCA 3 gene is alternatively spliced using an exon skipping mechanism as SERCA 1 gene. The two alternatively spliced variants are coexpressed in many tissues but only SERCA 3b is present in endothelial cells from aorta and heart.

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