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Evidence for two isoforms of the endoplasmic-reticulum Ca^{2+} pump in pig smooth muscle

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cDNA clones coding for the endoplasmic reticulum Ca^{2+}-transport ATPase have been cloned from a pig smooth-muscle cDNA library. The transcripts can be divided into two classes which differ in their 3′ ends due to alternative splicing of the primary gene transcript. The class 1 cDNA encodes a protein of 997 amino acids (M_r 110 000). The class 2 protein (1042 amino acids; M_r 115 000) is completely identical to the class 1 protein except that the four *C*-terminal amino acids of the class 1 protein are replaced in the class 2 protein with a tail of 49 amino acids. Comparison of these sequences with other Ca^{2+} pump sequences reveals that the class 1 isoform corresponds to the sarcoplasmic reticulum Ca^{2+} pump of slow-twitch skeletal/cardiac muscle, whereas the class 2 protein corresponds to a Ca^{2+} pump recently detected in non-muscle tissues.

INTRODUCTION

It has been amply documented that smooth muscle contains two distinct ATP-driven Ca^{2+} transport systems [1–3]. One Ca^{2+} pump (M_r 130 000) extrudes Ca^{2+} across the plasma membrane (PM) and the second (M_r 100000) reaccumulates Ca^{2+} into an intracellular Ca^{2+} store, generally assumed to be the endoplasmic reticulum (ER). Recently we have demonstrated that the porcine smooth-muscle ER Ca^{2+} pump strongly resembles the sarcoplasmic reticulum (SR) Ca^{2+} pump of slower-twitch skeletal muscle and of cardiac muscle [4].

Recombinant DNA techniques have proven to be very powerful tools for analysing the molecular structure and function of the different SR/ER Ca^{2+} pumps. It has been shown that the SR Ca^{2+} pump of fast skeletal muscle and the SR Ca^{2+} pump of slow-twitch skeletal/cardiac muscle are encoded by two separate genes [5–7] and that each gene transcript can be alternatively spliced [7–10]. In the fast skeletal muscle, a developmentally regulated alternative splicing process results in a neonatal and an adult isoform [7,8], whereas non-muscle tissues express a Ca^{2+} pump which results from alternative splicing of the slow-twitch/cardiac SR Ca^{2+} pump gene transcript [9, 10]. We now present evidence that the slow-twitch/cardiac SR Ca^{2+} pump gene is also expressed in the smooth-muscle layer of the pig stomach and that the gene transcript is spliced along the muscle-specific and non-muscle-specific pathway.

MATERIALS AND METHODS

RNA isolation

The stomach of a 2-day-old piglet was removed immediately after killing. The gastric mucosa and the serosa were carefully removed to minimize contamination with non-smooth-muscle tissues. Total RNA was extracted from the smooth-muscle layer according to the Chirgwin procedure [11]. A poly(A^+)-enriched RNA fraction was isolated via oligo(dT) cellulose column chromatography [12].

cDNA library synthesis

This poly(A^+) RNA (5 μg) was oligo(dT)-primed and reversely transcribed with avian-myeloblastosis-virus reverse transcriptase. Second-strand cDNA was synthesized according to the Gubler & Hoffman method [13]. The cDNA was processed following a standard protocol [14] and was ligated into *Eco*RI-cut and dephosphorylated lambda gt11 arms (Promega, Madison, WI, U.S.A.). The cDNA library was plated on *Escherichia coli* Y1090 [14]. Approx. 10^6 independent clones were obtained from 190 ng of cDNA. The cDNA library was amplified and stored at 4 °C.

Screening procedures

Appropriate DNA restriction fragments (an internal *Eco*RI fragment of the rabbit cardiac SR Ca^{2+} pump clone (nt 764-1445) [5] and probes A and B (see Fig. 1)) were nick-translated with [α-^{32}P]dCTP (400 Ci/mmol) up to a specific activity of 10^8 c.p.m./μg of DNA [15]. Aliquots of the phage stock were plated on agar plates and transferred in duplicate to nylon filters (Hybond-N; Amersham, U.K.). Filters were prehybridized for 4 h at 42 °C in a mixture containing 5 × SSPE, 0.1 % SDS, 5 × Denhardt's solution, denatured salmon sperm DNA (50 μg/ml) and 50 % formamide. The probe was added to the prehybridization mixture to an approximate concentration of 10^6 c.p.m./ml and hybridization was performed overnight at 42 °C. Filters which were hybridized with the heterologous rabbit probe, were washed with a final stringency of 1 × SSC/0.1 % SDS at 42 °C. Filters which were hybridized with the homologous probes A and B were washed with a final stringency of 0.1 × SSC/0.1 % SDS at 42 °C.

Abbreviations used: PM, plasma membrane; ER, endoplasmic reticulum; nt, nucleotide(s); SR, sarcoplasmic reticulum.

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The nucleotide sequence data will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X15073 and X15074.

Subcloning and sequencing procedures

Positive clones were picked and purified. Clones ER-1, ER-3 and ER-6 were subcloned into pGEM-7Zf(+) (Promega) for sequence analysis. Subcloned fragments were progressively deleted with Exonuclease III/S1 Nuclease (Erase-a-Base kit; Promega) to generate sets of overlapping deletion clones [16]. Sequence analysis was performed on double-stranded plasmid DNA [17] according to the modified chain-termination method [18] (Sequenase kit; United States Biochemical Corporation, Cleveland, OH, U.S.A.).

Unless stated otherwise, routine techniques were carried out and solutions (SSPE, SSC, Denhardt's solution) were prepared following standard procedures [19].

RESULTS AND DISCUSSION

A heterologous probe derived from a cDNA clone coding for the SR Ca^{2+} pump of rabbit cardiac/slow-twitch-skeletal muscle [5] was used to screen a pig stomach smooth-muscle cDNA library. In a first attempt 10^5 plaques were screened and five positive clones were retained. Restriction enzyme mapping and partial sequence analysis revealed that these clones could be classified into two related yet distinct classes which had similar 5′ ends but which differed in their 3′ termini. Clone ER-3, which belonged to class 1, was 3795 nucleotides (nt) long and ended with a poly(A) tail that was preceded by a poly-adenylation signal (AATAAA: nt 3764–3769 in Fig. 2) [20]. An *Eco*RI–*Pst*I restriction fragment corresponding to the 5′ end of this clone (probe A in Fig. 1) was used as a probe to detect transcripts which were further extended towards the 5′ terminus. Another 2×10^5 plaques of the same cDNA library were screened and four additional positive clones were detected. One of them, clone ER-6, also hybridized with probe B which was derived from the unique 3′ terminus of the class 2 clone ER-1 (see Fig. 1). Clone ER-6 was 3898 nt long. It extended the ER-1 clone with 998 nt towards the 5′ end and with 26 nt towards the 3′ end which contained a poly-adenylation signal (AATAAA: nt 3943–3948 in Fig. 2 [20]) followed by a poly(A) tail.

Sequence analysis was performed on clones ER-3, ER-1 and ER-6 as indicated in Fig. 1. The nucleotide sequences and the deduced amino acid sequences are shown in Fig. 2. Class 1 and 2 cDNAs were found to be completely identical up to nt 2980 (see Fig. 2) after which they diverged. This suggests that the class 1 and 2 transcripts arise from the same primary gene transcript which is then alternatively spliced at its 3′ end (see below). The first ATG codon of clone ER-3 (class 1 cDNA) is situated in a consensus sequence for initiation of eukaryotic translation [21] and is followed by an open reading frame of 2991 nt. The class 1 cDNA encodes a protein of 997 amino acids (M_r 110 000). The class 2 cDNA has an open reading frame of 3126 nt encoding a protein of 1042 amino acids (M_r 115 000). This class 2 protein is identical to the class 1 protein except for its *C*-terminus: the four ultimate amino acids of the class 1 protein (ARNYLEP/*AILE*) are replaced in the class 2 protein with an additional tail of 49 amino acids (ARNYLEP/*GKEC*..41 *AA*..*MFWS*). Sequence comparison of the class 1 and 2 proteins with previously sequenced SR/ER Ca^{2+} pumps reveals that the class 1 protein is nearly identical to the SR Ca^{2+} pump of rabbit

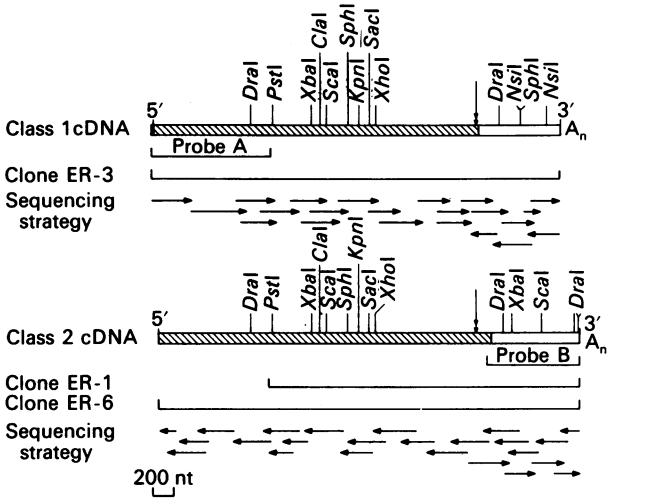

Fig. 1. Restriction map and sequencing strategy of the pig class 1 and 2 ER Ca^{2+} pump clones

cDNAs of class 1 (clone ER-3) and class 2 (clones ER-1 and ER-6) are shown schematically. Hatched boxes represent open reading frames, open boxes represent 5′ and 3′ untranslated regions. The class 1 and 2 cDNAs are completely identical up to nt 2980 of clone ER-3, but they have different 3′ ends. The point of divergence is indicated by a vertical arrow. Sequence analysis was performed in both directions. The common part (5′ to the site of divergence) was sequenced on the coding strand of ER-3 and on the non-coding strands of ER-1 and ER-6. The unique 3′ ends were sequenced in both directions on clones ER-3 and ER-1, as indicated in the Figure. Restriction enzyme sites are indicated with vertical lines. Probe A is an *Eco*RI–*Pst*I fragment corresponding to the 5′ end of clone ER-3, whereas probe B is an *Xba*I–*Eco*RI fragment corresponding to the 3′ end of clone ER-1.

slow-twitch skeletal/cardiac muscle (98% identity) [5], whereas the class 2 protein is very similar to a Ca^{2+} pump which has recently been described in rat brain, kidney and stomach (98% identity) [10] and also in human kidney (99% identity) [9]. The presence of two ER Ca^{2+}-pump clones in a pig-smooth-muscle cDNA library may therefore indicate that smooth muscle expresses two distinct ER Ca^{2+} pumps: a 'muscle isoform' (997 amino acids; M_r 110 000) and a 'non-muscle isoform' (1042 amino acids; M_r 115 000). However, an alternative explanation should be considered. It is possible that the starting material for our 'smooth-muscle' cDNA library (see the Materials and methods section) was still contaminated by non-smooth-muscle cells. If so, the detection of two ER Ca^{2+} pump transcripts could be related to cell-type (smooth muscle versus non-smooth muscle) dependent differences in gene expression. To exclude the latter hypothesis, further experiments such as hybridization *in situ* with specific probes are needed.

The two ER Ca^{2+} pumps differ only in their *C*-terminus. According to a proposed reaction mechanism [5, 6], all the basic functions of the Ca^{2+} pump reside within the proximal two-thirds of the enzyme, and no specific function has been attributed to the *C*-terminus. However, it is interesting to note that the *C*-terminus of the plasma-membrane Ca^{2+} pump is involved in the regulation of the Ca^{2+}-transport activity, as it contains the calmodulin-binding site and a possible phosphorylation site for the

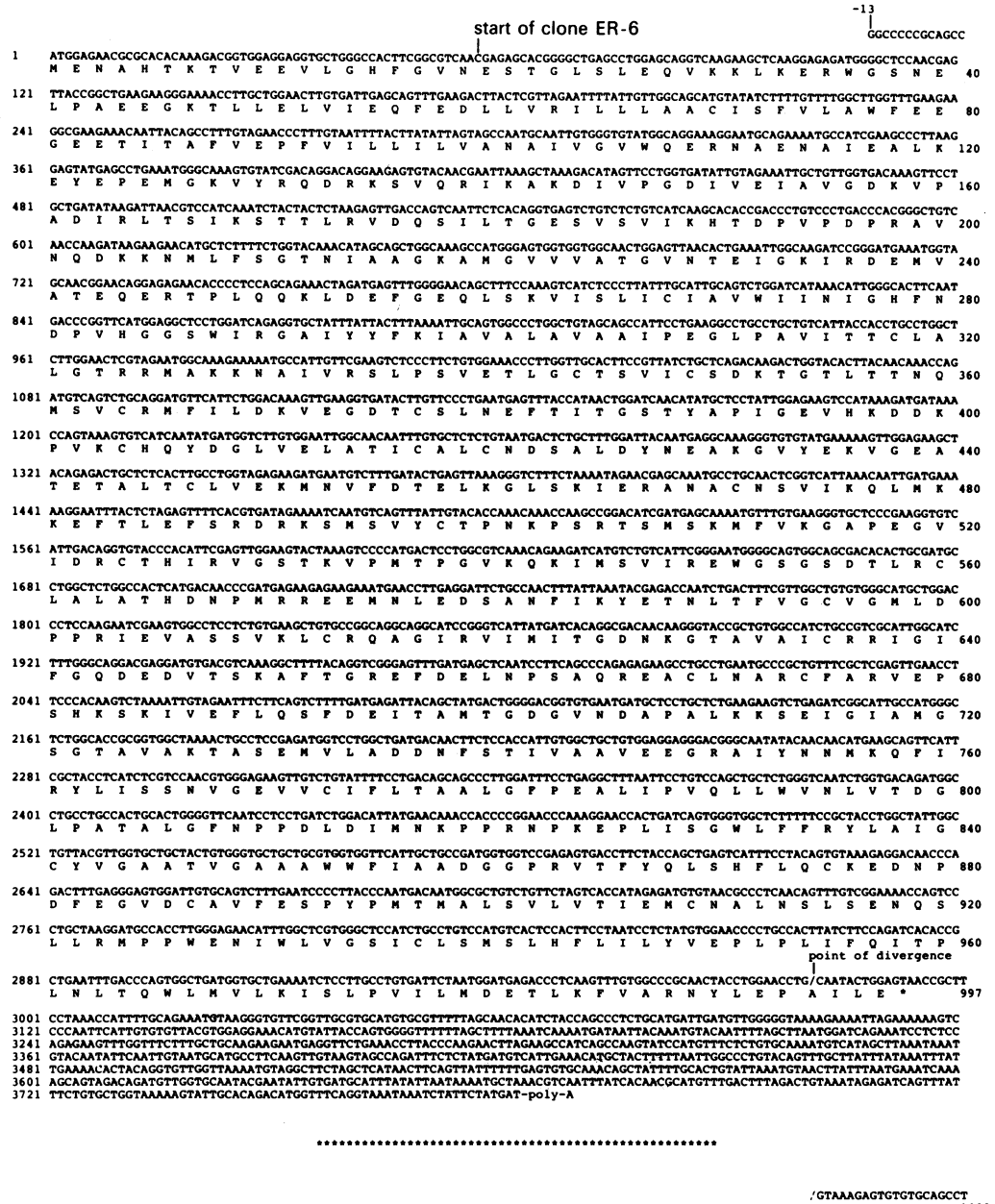

Fig. 2. Nucleotide and deduced amino acid sequences of the pig class 1 and class 2 ER Ca^{2+} pump cDNAs

The nucleotide and derived amino acid sequences of the pig class 1 cDNA are shown continuously. Nucleotides are numbered at the left side with nucleotide 1 being the A of the ATG start codon. Amino acids are numbered at the right. The optional splice site is indicated by a slash within the nucleotide sequence. The unique 3′ nucleotide sequence and *C*-terminus of the porcine class 2 cDNA is shown at the bottom.

cAMP-dependent protein kinase [22, 23]. Therefore the *C*-terminal diversity of the ER Ca^{2+} pump might result in a differential regulation of these ER Ca^{2+} pumps. It has generally been assumed that the cardiac/slow-twitch Ca^{2+} pump is regulated via phospholamban [24], a homopentameric protein present in the SR of cardiac and slow skeletal muscle [25] as well as in the ER of smooth muscle [26]. It will therefore be of interest to determine the phospholamban sensitivity of the two isoforms.

Lytton & MacLennan [9] have recently demonstrated that the SR Ca^{2+} pump of slow-twitch skeletal/cardiac

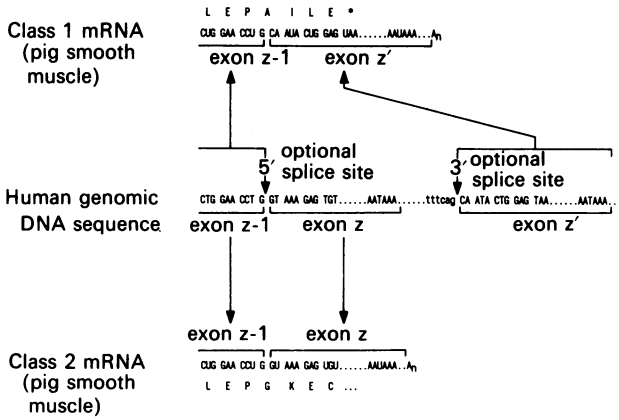

Fig. 3. Alternative splicing of the pig class 1 and 2ER Ca^{2+}-pump transcripts: a comparison of the pig smooth-muscle gene transcripts with the corresponding region of the human gene for the cardiac SR Ca^{2+} pump

The nucleotide sequence in the middle corresponds to a portion of the human cardiac SR Ca^{2+}-pump gene as published by Lytton & MacLennan [9]. The class 1 and 2 cDNAs (starting with nt 2971) and the deduced amino acid sequences (starting with amino acid 991) are aligned with respect to the human genomic sequence. The optional internal donor site is indicated by an arrow. Exon z-1 can be spliced to exon z' generating the class 1 transcript (upper sequence). However, when the splice site is omitted, exon z-1 directly proceeds into exon z which results in a class 2 transcript (lower sequence). The class 2 transcript therefore still contains the unused 5' splice site. Note the 100% identity between the human and porcine sequences.

muscle and the non-muscle Ca^{2+} pump are encoded by the same gene of which the pre-mRNA is alternatively spliced. A structural analysis of the corresponding human gene revealed an optional internal donor site which is used to produce the slow-twitch/cardiac mRNA, but which is neglected in case of the non-muscle mRNA. A comparison of the pig cDNAs with the nucleotide sequence of the human gene suggests that the smooth-muscle class 1 and class 2 transcripts are generated by the same mechanism (internal donor site splicing) (see Fig. 3). Exon z and the subsequent intervening sequences can be excised so that exon z-1 becomes attached to exon z'. This creates a class 1 transcript (upper pathway in Fig. 3). However, if the 5' optional splice site remains cryptic, exon z-1 directly proceeds into exon z and the primary transcript will be cleaved and poly(A)-tailed following the poly-adenylation signal AATAAA which is present in exon z. This results in a class 2 transcript (lower pathway in Fig. 3). The former pathway is the preferred splicing mechanism in slow-twitch skeletal muscle and cardiac muscle, as in these muscle tissues the vast majority of the transcripts are spliced along this pathway [7]. The latter mechanism (omission of the splice site) seems to be the preferred one in non-muscle tissues such as rat brain, kidney and stomach and human kidney [9,10]. Our results may therefore indicate that in smooth muscle both the 'muscle-specific' and the 'non-muscle-specific' pathways are used. de la Bastie and co-workers have recent analysed the smooth-muscle messengers for the ER Ca^{2+} pump by means of S1 Nuclease mapping [27]. They concluded that the smooth-muscle RNA differed from the cardiac mRNA in its 3' end. This mRNA very likely corresponds with our class 2 cDNA, but it remains to be explained why they did not find a fully protected mRNA species which would correspond with our class 1 cDNA.

Our results are in accordance with the previous observation that the smooth-muscle ER Ca^{2+} pump is very similar or even identical to the slow-twitch/cardiac SR isoform [4]. These SR/ER Ca^{2+} pumps are indeed encoded by the same gene which can be alternatively spliced. In contrast, the fast-skeletal muscle Ca^{2+} pump is encoded by a different gene of which the expression seems to be limited to fast-skeletal muscle tissue [6,7].

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