

Characterization of the Gene Encoding Human Sarcolipin (SLN), a Proteolipid Associated with SERCA1: Absence of Structural Mutations in Five Patients with Brody Disease

Alex Odermatt,* Peter E. M. Taschner,† Stephen W. Scherer,‡ Barbara Beatty,§
Vijay K. Khanna,* David R. Cornblath,¶ Vinay Chaudhry,¶ Won-Chee Yee,||
Bertold Schrank,** George Karpati,†† Martijn H. Breuning,†‡‡
Nine Knoers,§§ and David H. MacLennan*,1

*Banting and Best Department of Medical Research, University of Toronto, Charles H. Best Institute, 112 College Street, Toronto, Ontario, Canada M5G 1L6; †Department of Human Genetics, Sylvius Laboratories, Leiden University, P.O. Box 9503, Wassenaarseweg 72, 2300 RA Leiden, The Netherlands; ‡Department of Genetics, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8; §Department of Pathology, University of Toronto, Ontario Cancer Institute, Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario, Canada M5G 2M9; ¶Department of Neurology, Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, Maryland 21205; ||Department of Neurology, Washington University School of Medicine, 660 Euclid, St. Louis, Missouri 63110; **Neurologische Universitätsklinik, Josef-Schneider-Strasse 11, 97080 Würzburg, Germany; ††Montreal Neurological Institute, McGill University, 3801 Rue University, Montreal, Quebec, Canada H3A 2B4; ‡‡Department of Clinical Genetics, Erasmus University, Dr. Molewaterplein 50, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands; and §§Department of Human Genetics, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

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Sarcolipin (SLN) is a low-molecular-weight protein that copurifies with the fast-twitch skeletal muscle sarcoplasmic reticulum Ca^{2+} ATPase (SERCA1). Genomic DNA and cDNA encoding human sarcolipin (SLN) were isolated and characterized and the *SLN* gene was mapped to chromosome 11q22–q23. Human, rabbit, and mouse cDNAs encode a protein of 31 amino acids. Homology of SLN with phospholamban (PLN) suggests that the first 7 hydrophilic amino acids are cytoplasmic, the next 19 hydrophobic amino acids form a single transmembrane helix, and the last 5 hydrophilic amino acids are luminal. The cytoplasmic and transmembrane sequences are not well conserved among the three species, but the luminal sequence is highly conserved. Like SERCA1, SLN is highly expressed in rabbit fast-twitch skeletal muscle, but it is expressed to a lower extent in slow-twitch muscle and to an even lower extent in cardiac muscle, where SERCA2a and PLN are highly expressed. It is expressed in only trace amounts in pancreas and prostate. *SLN* and *PLN* genes resemble each other in having two small exons, with their entire coding sequences lying in exon 2 and a large intron separating the two segments. Brody dis-

ease is an inherited disorder of skeletal muscle function, characterized by exercise-induced impairment of muscle relaxation. Mutations in the *ATP2A1* gene encoding SERCA1 have been associated with the autosomal recessive inheritance of Brody disease in three families, but not with autosomal dominant inheritance of the disease. A search for mutations in the *SLN* gene in five Brody families, four of which were not linked to *ATP2A1*, did not reveal any alterations in coding, splice junction or promoter sequences. The homozygous deletion of C438 in the coding sequence of *ATP2A1* in Brody disease family 3, leading to a frameshift and truncation following Pro₁₄₇ in SERCA1, is the fourth *ATP2A1* mutation to be associated with autosomal recessive Brody disease. © 1997 Academic Press

INTRODUCTION

Sarcolipin

Sarcolipin (SLN) was first isolated as a low-molecular-weight proteolipid from rabbit fast-twitch skeletal muscle sarcoplasmic reticulum and from preparations of the Ca^{2+} ATPase (SERCA1) purified from that source (MacLennan *et al.*, 1972). Attempts were made to discover a function for SLN, by reconstitution of the proteolipid with purified preparations of SERCA1 and measurement of any alteration in SERCA1 function.

Sequence data from this article have been deposited with the GenBank/EMBL Data Libraries under Accession Nos. U96091, rabbit cDNA; U96092, U96093, human gDNA; U96094, human cDNA.

¹To whom correspondence should be addressed. Telephone (416) 978-5008. Fax: (416) 978-8528. E-mail: david.maclennan@utoronto.ca.

SLN was reported to enhance the coupling ratio between Ca^{2+} uptake and Ca^{2+} -dependent ATP hydrolysis when reconstituted with SERCA1, but, when purified SLN was added to the exterior of these reconstituted liposomes, Ca^{2+} accumulation was diminished, implying that SLN might act as a Ca^{2+} ionophore (Racker and Eytan, 1975). MacLennan *et al.* (1980) fractionated SLN away from SERCA1, reconstituted the highly purified SERCA1 in lipid bilayers, and found no alteration in Ca^{2+} /ATP coupling ratios before and after removal of SLN. Later studies by Maruyama and MacLennan (1988) showed that SERCA1 cDNA, expressed alone in heterologous cell culture, yielded microsomal membranes containing fully functional Ca^{2+} uptake and Ca^{2+} -dependent ATPase activities, suggesting that SLN plays no essential role in Ca^{2+} uptake. The protein was sequenced by Wawrzynow *et al.* (1992) and named sarcolipin.

In studies of the yeast H^+ ATPase, Navarre *et al.* (1992) discovered that two low-molecular-weight proteolipids are associated with this P-type ATPase. In double mutants, lacking both proteolipids, the V_{max} of the H^+ ATPase was reduced to 50% of that of wild type strains (Navarre *et al.*, 1994). The mechanism of interaction of these proteolipids with the H^+ ATPase has not been determined.

Phospholamban (PLN) is a 52-amino-acid protein located in the sarcoplasmic reticulum of cardiac, slow-twitch, and smooth muscles, where it acts as an inhibitor of SERCA2 by lowering its apparent affinity for Ca^{2+} (Tada and Katz, 1982). The protein has a hydrophilic N-terminal sequence of 30 amino acids, which would alter its solubility by comparison with low-molecular-weight proteolipids, but, in respect to its interaction with a P-type cation pump, it resembles other proteolipids.

In this paper, we characterize the sarcolipin (SLN) gene and demonstrate structural similarities between the SLN and PLN genes, as well as homology between the peptide sequences of their products. SLN gene transcription is shown to be high in fast-twitch skeletal muscle, where SERCA1 expression predominates. By contrast, PLN gene transcription was shown earlier to be high in cardiac muscle, where SERCA2a expression predominates (Fujii *et al.*, 1988).

Brody Disease

Brody disease is an inherited disorder of skeletal muscle function, characterized by exercise-induced impairment of muscle relaxation. Studies of the sarcoplasmic reticulum from Brody patients have shown decreased Ca^{2+} uptake and Ca^{2+} -dependent ATP hydrolysis. (Brody, 1969; Karpati *et al.*, 1986; Danon *et al.*, 1988; Taylor *et al.*, 1988; Wevers *et al.*, 1992; Poels *et al.*, 1993; Benders *et al.*, 1994). In the studies of Karpati *et al.* (1986), no immunologically reactive ATPase was observed in fast-twitch fibers of probands from two

Brody families. In later studies, Benders *et al.* (1994) were unable to measure a decrease in SERCA1 protein content in skeletal muscle from seven Brody families, but SERCA1 activity was reduced by 50%.

We have reported the association of some cases of Brody disease with the autosomal recessive inheritance of mutations in the *ATP2A1* gene, encoding SERCA1, in two Brody families (Odermatt *et al.*, 1996), but *ATP2A1* was ruled out as a candidate gene in three other Brody families in an earlier study (Zhang *et al.*, 1995), demonstrating that the cause of the disease is heterogeneous. Some of the non-*ATP2A1*-linked families were characterized by dominant inheritance of the disease. Since SERCA1 content was reported to be normal, but with reduced activity in seven Brody patients studied by Benders *et al.* (1994), we deduced that a candidate gene for Brody disease might encode a protein that would interact with SERCA1 and, when mutated, become a dominant inhibitor of SERCA1 function. Accordingly, the human SLN gene was evaluated as a candidate gene for Brody disease. Unfortunately, we were unable to find any alterations in coding, splice junction, or promoter sequences that would support SLN as a candidate gene for Brody disease in five patients.

MATERIALS AND METHODS

Preparation of RNA and RT-PCR. Total RNA was isolated from normal human skeletal muscle using guanidium-isothiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). A 5- μg aliquot of total RNA was reverse transcribed in 50 μl of a reaction mix (Stratagene cDNA synthesis kit) containing 50 U of MMuLV reverse transcriptase at 37°C in the presence of RNase Block Inhibitor and 0.5 μg of random primers. Following first-strand cDNA synthesis, RNA was hydrolyzed by the addition of 50 μl of 0.3 N sodium hydroxide and 30 mM EDTA and boiling for 5 min. The pH was then neutralized with 25 μl of Tris-HCl, pH 8.3. After extraction with phenol and chloroform, the cDNA was precipitated with ethanol and washed, and the pellet was dissolved in 30 μl of H_2O . Aliquots of 1 μl of first-strand cDNA were used as the template in subsequent PCR amplifications.

PCR cloning of partial SLN cDNA based on known peptide sequence. Reverse translation of the amino acid sequence of rabbit sarcolipin (Wawrzynow *et al.*, 1992) permitted the synthesis of two fully degenerate oligonucleotide primers, SALF7 and SALR7 (Table 1), based on the 5' and 3' ends of the cDNA coding sequence. The oligonucleotide primers also contained nine nucleotide *Bam*HI restriction endonuclease sites at their 5' ends to facilitate subsequent cloning of PCR products. PCR amplification with primers SALF7 and SALR7 was carried out using either 100 ng of DNA from a full-length rabbit skeletal muscle cDNA library (MacLennan *et al.*, 1985) constructed in the pcDX vector (Okayama and Berg, 1983) or, alternatively, using 1 μl of first-strand cDNA derived from normal human skeletal muscle as a template. *Taq* polymerase and *Taq* Extender (Stratagene) were used for PCR amplification in the buffer supplied. Denaturation was for 1 min at 94°C, annealing was at 57°C for 2 min, and extension for 30 cycles was at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Amplified DNA obtained using either rabbit or human template was cloned into the *Bam*HI site of the Bluescript vector BSKs(+) (Stratagene). The coding sequence between degenerate primers was 45 bp long for both the rabbit and the human SLN genes.

TABLE 1

Primer		Sequence		Description
SALF7	5'	ACAGGATCCATGGA (AG) (AC) G (ACGT) (AT) (CG)		(Degenerated PCR)
		(ACGT) AC (ACGT) (AC) G (ACGT) GA (AG) C	3'	
SALR7	5'	ACAGGATCCTG (AG) TA (ACGT) (CG) (AT) (ACGT)		(Degenerated PCR)
		C (GT) (ACGT) AC (ACGT) A (AG) (ACGT) A (AG) CC	3'	
SALF9	5'	CTTGATCCGTTGTCCTTATTACAGTGATCC	3'	(Inverse PCR,
SALR9	5'	CAAGGATCCACAGTGAAGTTGAGACACAGC	3'	rabbit <i>SLN</i>)
RSAF7	5'	TAAGGATCCACACAGCCTGGGAGATGAGCC	3'	(cDNA cloning,
RSAR8	5'	TAAGGATCCGTTGGTTGACTTAACCTTTATTACATG	3'	rabbit <i>SLN</i>)
RACE1	5'	CAGCCTTGACTTGAGCAGTAGCTGAGACTCAAGTCG		
		TGTTTTTTTTTTTTTTTTTTTT	3'	(3' RACE)
RACE3	5'	GTTGGATCCAGTAGCTGAGACTCAAGTCGTG	3'	(3' RACE)
SALF10	5'	CAAGGATCCGAGCTGTGTCTCAACTTCAC	3'	(Inverse PCR,
SALR10	5'	CAAGGATCCAGGATCACTGTAATAAGGACAAC	3'	human <i>SLN</i>)
HSAF5	5'	GTTGGAGCTCAAGTTGGAGACAGCGAG	3'	(cDNA cloning,
HSAR5	5'	GTTGAGCTCGTTGACTTAACCTTTATTACATGATAG	3'	human <i>SLN</i>)
HSAF1	5'	GTTGGATCCTTGATTACGGTTATCTTATGTG	3'	(Inverse PCR, human
HSAR1	5'	GAAGGATCCACAATAGTGAAGTTGAGAAACAG	3'	3' flanking gDNA)
HSAF8	5'	GATTCTAGAGTCTTTGATCCTCTTCAGGTAAGT	3'	(Inverse PCR, human
HSAR8	5'	TACTCTAGACTATGGCAGGTTTCTCCTCGCTG	3'	5' flanking gDNA)
				(Primer extension)
HSAF3	5'	GTCCTGGGATTGACTGAG	3'	(Detection of a minor
HSAR11	5'	CCTCTGCCCTTCAAGACTAG	3'	human mRNA transcript)
HSAF12	5'	AAACCCACATCAATTGCAC	3'	(5' region probe)
HSAF9	5'	AGAACGGGCATTGTTATACAGG	3'	(Mapping, human <i>SLN</i>)
HPE1	5'	TTTCTGAGGGCACACCAAGGACCTCTGGCTTG	3'	(Primer extension)

Cloning of rabbit *SLN* cDNA. Knowledge of the 45-bp sequence of rabbit *SLN* cDNA lying between primers SALF7 and SALR7 (Table 1) permitted the design of additional primers (SALF9, SALR9) for inverse long PCR amplification in which the cDNA and the whole pcDX vector sequence were included. PCR amplification was carried out with *Taq* polymerase:*Pfu* polymerase (5:1 ratio), using 100 ng of the full-length rabbit skeletal muscle cDNA library in pcDX as a template. An initial hot start at 94°C for 2 min preceded 30 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 1 min, and elongation at 72°C for 10 min. A single product of about 4.5 kb was purified on a 1% agarose gel and extracted with a gel extraction kit from Qiagen. About 10 ng of the product was then blunt ended by incubation with 0.7 U/ μ l mung bean nuclease for 30 min at 37°C; the DNA was extracted with phenol and chloroform, precipitated, and self ligated for 12 h at 12°C using 5 U of T4 DNA ligase in a volume of 200 μ l. Transformation of competent DH5 α cells and selection for ampicillin resistance conferred by the amplified pcDX vector yielded over 1000 colonies. The sequence of the full-length rabbit muscle cDNA was determined using a T7 sequencing kit (Pharmacia). To exclude any PCR cloning artifacts, rabbit *SLN* cDNA was also amplified with two oligonucleotides, RSAF7 and RSAR8 (Table 1), using the pcDX library as a template. The sequence of the 0.7-kb product was verified by cycle sequencing.

Cloning of human *SLN* cDNA. The 3' end of the human *SLN* cDNA was cloned by application of the 3' RACE technique (Innis *et al.*, 1990) in which 5 μ g of total RNA from human skeletal muscle was reverse transcribed with primer RACE1 (Table 1) as described above. Subsequent PCR amplification with primers RACE3 and HSAF1 using *Taq* polymerase:*Pfu* polymerase (5:1 ratio) yielded a single product of 550 bp, which was cleaved with *Bam*HI and cloned into the Bluescript vector. Sequence analysis indicated that all eight clones analyzed were identical. The 5' end of human *SLN* cDNA was cloned by PCR amplification from a λ gt11 5' stretch cDNA library, kindly provided to us by Drs. Peter Ray and Perry Howard, Hospital for Sick Children, Toronto. Phage DNA was prepared according to the λ phage library protocol from Stratagene, in which 1 μ g of λ gt11 DNA is cleaved by *Bs*WI (which cuts at both sides of the cDNA inserts) and self ligated. An aliquot con-

taining 50 ng of circularized *Bs*WI DNA was used as a template in an inverse PCR amplification with primers SALF10 and SALR10 (Table 1). The PCR product of 1.1 kb was cleaved with *Bam*HI, cloned into Bluescript, and the sequence of the 5' end of human *SLN* cDNA was determined. All eight clones analyzed started at the A residue at position +6 of the cDNA sequence (Figs. 1B and 2). In addition, cDNA was reamplified with primers HSAF5 and HSAR5 from human first-strand cDNA, and the sequence of the 0.7-kb product was verified by cycle sequencing.

Determination of the human *SLN* gene structure. Two primers at both ends of the full-length cDNA (HSAF5 and HSAR5) were used to amplify human genomic DNA. A single DNA product of 4.5 kb with a single intron in the 5' untranslated region of the cDNA was obtained. Flanking genomic DNA sequences were obtained by inverse PCR, in which 4 μ g of human genomic DNA was cleaved with *Eco*RI, *Bgl*II, or *Hind*III, extracted with phenol and chloroform, precipitated with 50% isopropanol containing 250 mM sodium acetate, washed once with 70% ethanol, and self ligated. The inverse long PCR amplification used 50 ng of circularized DNA with a primer pair corresponding to the 5' (HSAF8, HSAR8) or the 3' (HSAF1, HSAR1) segments of the cDNA, respectively. For the 3' end, single products of 5.7 and 3.5 kb were obtained from the *Bgl*II and *Hind*III circularized genomic DNA fragments, but no product was observed when *Eco*RI circularized DNA was used as a template. For the 5' region, 5-, 4-, and 0.9-kb products were obtained for *Bgl*II, *Hind*III, and *Eco*RI, respectively (Fig. 1A).

Primer extension. Total RNA was isolated from normal human skeletal muscle of two unrelated individuals as described above. The oligonucleotides HSAR8 or HPE1 (Table 1) were labeled with polynucleotide kinase and [γ -³²P]ATP to a specific activity of 300 nCi/ng. Thirty micrograms of total RNA in 30 μ l of hybridization buffer (40 mM Pipes (1,4-piperazine-diethanesulfonic acid), pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide) was denatured for 5 min at 90°C and annealed overnight at 30°C with 3 ng of the labeled oligonucleotide. The nucleic acids were ethanol precipitated, washed, and reverse transcribed as described above. The final pellet was dissolved in 4 μ l of water, and 6 μ l of formamide loading buffer was added.

Three microliters of the product, corresponding to 10 μ g of reverse transcribed total RNA, was analyzed by electrophoresis on a 7.2% denaturing polyacrylamide sequencing gel.

Northern blots. Northern blots, containing 8 μ g of total RNA from rabbit fast-twitch (psoas) and slow-twitch (soleus) skeletal muscle, were prepared and treated as described by Ausubel *et al.* (1994). Multiple tissue Northern (MTN) blots, loaded with 2 μ g per lane of poly(A)⁺ RNA derived from a number of human tissues, were purchased from Clontech. A gel-purified, 0.5-kb PCR fragment amplified with primers HSAF1 and HSAR5 from human cDNA and a 2-kb human β -actin cDNA probe (Clontech) were labeled with [³²P]dCTP using random primers and T7 polymerase. Hybridization was carried out for 1 h at 68°C using ExpressHyb solution (Clontech) containing the labeled probe at a final concentration of 5 ng/ml (1×10^6 CPM/ml). The MTN blots were subjected to autoradiography for 2, 16, and 72 h, at -70°C using Kodak β -max film and two intensifying screens. The blots were stripped and probed for β -actin mRNA according to the protocol of the manufacturer. Autoradiography was for 4 h.

Southern blots. Multiple species Southern (Zoo) blots, loaded with 4 μ g of genomic DNA cleaved with the restriction endonuclease *Eco*RI, were purchased from Clontech and hybridized according to the instructions of the manufacturer. Either the 0.5-kb HSAF1-HSAR5 probe, specific for exon 2 (described above), or the 350-bp probe obtained with primers HSAF12 and HSAR8, specific for the 5' region of the *SLN* gene, was used. The blot was washed at low stringency (2 \times SSC, 0.05% SDS; 60°C).

Chromosomal mapping of the *SLN* gene. To determine the chromosomal localization of the *SLN* gene, a 4.5-kb, PCR-amplified genomic DNA fragment (HSAF5 and HSAR5) was used for fluorescence *in situ* hybridization (FISH) experiments. FISH was performed following the procedure of Lichter *et al.* (1990), by counterstaining the chromosomes with propidium iodide and DAPI. The biotinylated probe was detected with avidin-FITC. Images of metaphase preparations were captured by a thermoelectrically cooled, charge-coupled camera (Photometrics, Tucson, AZ). Separate images of DAPI-banded chromosomes (Heng and Tsui, 1993) and of FITC-targeted chromosomes were obtained. Hybridization signals, pseudocolored yellow for FITC and pseudocolored blue for DAPI-stained chromosomes, were overlaid electronically and merged using Adobe Photoshop 3.0 software. The band assignment was determined by measuring the fractional chromosome length and by analyzing the banding pattern generated by the DAPI counterstained image.

The chromosomal localization of the *SLN* gene was confirmed by screening the NIGMS somatic cell hybrid panel 2 (Dubois and Naylor, 1993) and the GeneBridge 4 radiation hybrid (RH) panel (Walter *et al.*, 1994), purchased from Research Genetics. Radiation hybrid mapping experiments were carried out in duplicate and the data were analyzed at a lod score of >3.0 using the RH server from the Whitehead Institute/MIT Center for Genomic Research. The primers used for this specific analysis were HSAF9 and HSAR11 (Table 1), which amplified a 900-bp human-specific band using the following conditions: denaturation at 94°C for 30 s; annealing at 62°C for 2 min; extension at 72°C for 2 min, for 35 cycles. The complete set of RH data is available through the NCBI radiation hybrid mapping service at <http://www.ncbi.nlm.gov/SCIENCE96/>.

Analysis of mutations in *ATP2A1* of patients from Brody families BD3 and BD6. Genomic DNA was amplified, and the 23 *ATP2A1* exons, plus 1500 bp upstream of the transcription initiation site containing the putative promoter region, were sequenced, as described by Odermatt *et al.* (1996). At least 30 bp of flanking sequence was determined for each exon.

Analysis of *SLN* sequence in patients of Brody families BD3 (F3) to BD7 (F7). Muscle biopsies were available from Brody patients F4P3 and F5P3 (Table 2). First-strand cDNA, prepared as described above, was amplified with primers HSAF5 and HSAR5, and the sequence of the 0.7-kb product was determined by cycle sequencing. Genomic DNA was isolated from blood from patients F3P3, F6P3, and F7P3, using the Qiaamp blood kit (Qiagen). A 5-kb DNA fragment was amplified with primers HSAF12 and HSAR11. PCR ampli-

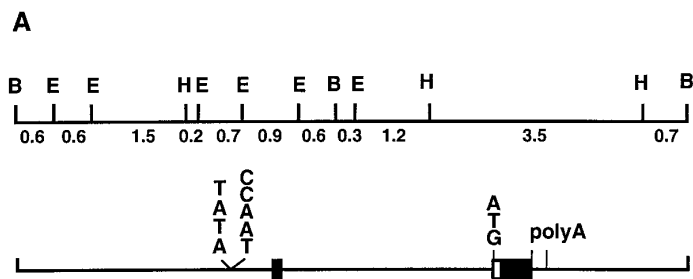


FIG. 1. (A) Structure and restriction map of human *SLN*. The upper line shows the restriction endonuclease map of the gene derived from clones obtained by PCR amplification. Restriction endonuclease sites are: E, *Eco*RI; B, *Bgl*II; H, *Hind*III. The locations of the two exons within the gene (solid boxes) and the location of the coding sequence within exon 2 (white box) are indicated on the lower line. The positions of the TATA and CCAAT sequences, the ATG initiation codon, and polyadenylation signal sequences are marked. (B) Nucleotide and deduced amino acid sequences of human *SLN*. Upstream nucleotides, in lowercase, are numbered negatively, on the left, to the transcription initiation site. Exon sequences are in uppercase and numbered positively thereafter. The sequence of the single intron is presented in lowercase and is not numbered. Numbers relative to the cDNA at the ends of both exons are in parentheses below the sequence. Putative DNA binding elements for eukaryotic transcriptional factors are underlined and identified below the sequence. The GenBank accession number for these sequences are U96092-U96094.

fication and cycle sequencing were performed as described by Odermatt *et al.* (1996).

Case histories. Brody patients F3P3, F4P3, and F5P3 were described as patients 1-3 in Zhang *et al.* (1995). Patient F3P3 was also described as patient 1 in Karpati *et al.* (1986). Patient F6P3 had a long history of exercise- and cold-induced muscle stiffness, pain, and cramps. Treatment with Verapamil produced impressive improvement in his symptoms. His mother and sister were said to suffer from similar symptoms, suggesting dominant inheritance of the disease. No myotonia was observed either clinically or on electrophysiological testing. Creatine kinase was mildly elevated. Ischemic exercise testing showed normal elevation of serum lactate and ammonia. A muscle biopsy showed nonspecific, mixed myopathic and neuroopathic features. Patient F7P3 was described as patient 6 in Benders *et al.* (1994).

RESULTS

Characterization of the Human *SLN* Gene

Figure 1A shows the structure and restriction map of human *SLN*, and Fig. 1B provides a partial DNA sequence of the human *SLN* gene. The partial sequence includes: 967 bp of the 5' region containing the probable promoter of the *SLN* gene, nucleotides -967 to -1; exon 1 nucleotides +1 to +99, which form part of the 5' untranslated region of the cDNA; about 300 bp of sequence at both the 5' and the 3' ends of the single 3.9-kb intron; exon 2 nucleotides +100 to +719, which form the remainder of the 5' untranslated region, the coding sequence for *SLN*, and the 3' untranslated region of the *SLN* gene; and 556 bp of downstream sequence. Primer extension analysis using total RNA isolated from skeletal muscle of an individual with no

B[illegible]

FIG. 1—Continued

tide +719 in Fig. 1B. The total length of the gene is about 5.6 kb.

A search for potential DNA binding elements for known eukaryotic transcriptional factors revealed a cluster of three possible TATA box sequences between -745 bp and -783 bp (Fig. 1B). Two additional TATA boxes were found between -945 and -949 bp and between -633 and -636 bp. A CCAAT box was found 45 bp downstream of the TATA box cluster between -700 and -704 bp. The CCAAT box of the *SLN* gene, CTC-CCAATCT, differed from the consensus sequence GGY-CCAATCT (Lewin, 1994) in having pyrimidines rather than purines upstream of the YCCAATCT sequence. Several E box sequences (CANNTG), recognized by the MyoD family of basic helix-loop-helix transcriptional activators (Piette *et al.*, 1990; Weintraub *et al.*, 1990), were identified in the 5' region of the *SLN* gene. One pair of E boxes, separated by 11 bp and lying between -587 and -609 bp, and a second pair of E boxes, separated by 10 bp and lying between -198 and -219 bp, were located downstream of the CCAAT box. In addition, a potential binding site for the myocyte enhancer factor MEF-2 (CTA(A/T)₄TAG) was identified upstream of the TATA box cluster between -805 and -814 bp. The sequence CTTGGTTACAA, with similarity to the MEF-3 enhancer element (TCAGGTTT(T/A)CA) described by Hidaka *et al.* (1993), was found just upstream of the MEF-2 motif between -828 and -838 bp. An additional MEF-3-like motif (TCAGTTTTTGAA) was found between -497 and -505 bp. Unlike the MEF-3 consensus sequence, the motifs found in the *SLN* gene had palindromic ends. We identified a possible site for the transcriptional activator protein AP-

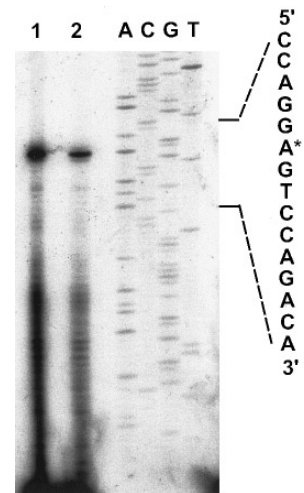


FIG. 2. Primer extension. 10 μ g of total RNA from normal human skeletal muscle of control individuals 1 (lane 1) or 2 (lane 2) were reverse transcribed using 32 P-labeled oligonucleotide HSAR8. (A, C, G, T) Sanger sequencing reactions with the same primer. The reaction products were resolved on a 7.2% DNA sequencing gel, and radioactivity was visualized by autoradiography. The relevant DNA sequence is given on the right. The transcription initiation site is indicated by an asterisk.

history of genetic disease was carried out to determine the transcription initiation site. A single product was generated using primer HSAR8 (complementary to a sequence within exon 1; Fig. 2) or HPE1 (complementary to a sequence within exon 2; not shown). The SLN transcription initiation site was assigned to an A residue 175 bases upstream from the translation initiation site in mRNA (+1 in Figs. 1B and 2). The polyadenylation site in the major transcript was located at nucleo-

A

Human SLN	gccgtctgaaa	ATG	<u>GGG</u>	<u>ATA</u>	<u>AAC</u>	ACC	CGG	GAG	CTG	TTT	CTC	AAC	TTC	ACT	<u>ATT</u>	GTC
		Met	<u>Gly</u>	<u>Ile</u>	<u>Asn</u>	Thr	Arg	Glu	Leu	Phe	Leu	Asn	Phe	Thr	<u>Ile</u>	Val
Rabbit SLN	ccacgctgaga	ATG	GAG	<u>CGA</u>	TCC	ACC	CGG	GAG	CTG	<u>TGT</u>	CTC	AAC	TTC	ACT	GTT	GTC
		Met	Glu	Arg	Ser	Thr	Arg	Glu	Leu	<u>Cys</u>	Leu	Asn	Phe	Thr	Val	Val
Mouse SLN	acattctgaag	ATG	GAG	<u>AGG</u>	<u>TCT</u>	<u>ACT</u>	<u>CAG</u>	GAG	CTG	TTT	<u>ATC</u>	AAC	TTC	<u>ACA</u>	GTT	GTC
		Met	Glu	Arg	Ser	Thr	<u>Gln</u>	Glu	Leu	Phe	<u>Ile</u>	Asn	Phe	Thr	Val	Val
Human SLN	<u>TTG</u>	ATT	<u>ACG</u>	GTT	<u>ATT</u>	CTT	ATG	TGG	CTC	CTT	GTG	AGG	TCC	<u>TAT</u>	CAG	TAC
	Leu	Ile	Thr	Val	Ile	Leu	Met	Trp	Leu	Leu	Val	Arg	Ser	Tyr	Gln	Tyr
Rabbit SLN	CTT	ATT	ACA	<u>GTG</u>	ATC	CTT	<u>ATT</u>	TGG	CTC	CTT	GTG	AGG	TCC	TAC	CAG	TAC
	Leu	Ile	Thr	Val	Ile	Leu	<u>Ile</u>	Trp	Leu	Leu	Val	Arg	Ser	Tyr	Gln	Tyr
Mouse SLN	<u>CTC</u>	<u>ATC</u>	<u>ACC</u>	GTT	<u>CTC</u>	CTT	ATG	TGG	CTC	<u>CTC</u>	GTG	AGG	TCC	TAC	<u>CAA</u>	TAC
	Leu	Ile	Thr	Val	<u>Leu</u>	Leu	Met	Trp	Leu	Leu	Val	Arg	Ser	Tyr	Gln	Tyr

B

						+	-				
Human SLN	Met	Gly	Ile	Asn	Thr	Arg	Glu	Leu	...		
		-	+			+	-				
Rabbit SLN	Met	Glu	Arg	Ser	Thr	Arg	Glu	Leu	...		
		-	+				-				
Mouse SLN	Met	Glu	Arg	Ser	Thr	Gln	Glu	Leu	...		

FIG. 3. (A) Alignment of human, rabbit, and mouse *SLN* nucleotide and deduced amino acid sequences. The first 11 nucleotides, in lowercase, form part of the translation initiation sequence (Kozak, 1987), while the nucleotides in uppercase indicate coding sequences. Stop codons are italicized. Nucleotides and amino acids deviating from the consensus sequence are underlined and boldface. (B) Comparison of charge distribution in the hydrophilic, N-terminal domain of human, rabbit, and mouse *SLN*.

1 (Lee *et al.*, 1987) between -341 and -348 bp. The translation initiation signal sequence (Kozak, 1987) at the end of the promoter sequence (GCTGTGAAA-ATGG) is reasonably conserved compared to the consensus sequence (GCCGCCA/GCCATGG).

Sequencing of the 3' end of the human *SLN* gene revealed the major polyadenylation signal, AATAAA, between nucleotides 701 and 706, with polyadenylation occurring after residue 719. A potential polyadenylation signal sequence, AATAAA, was also found 252 bp downstream of the major polyadenylation signal sequence. To test whether this downstream polyadenylation signal was used, PCR analysis was carried out with a forward primer (HSAF3) in exon 1 and a reverse primer (HSAR11) at the downstream poly(A)⁺ site. Human skeletal muscle first-strand cDNA primed with oligo(dT) was used as a template. A product of 660 bp was amplified (not shown), indicating that the longer transcript was, indeed, synthesized. Contamination by genomic DNA was excluded on the basis of the absence of intronic sequence in the PCR product. We estimate that the level of the longer transcript was less than 5% of that of the major 719-bp transcript. There was no indication of the use of a potential polyadenylation signal sequence, AATTAA, 158 bp upstream of the major signal sequence.

Isolation and Analysis of cDNA Clones Encoding Rabbit and Human *SLN*

Nucleotide sequence. Figure 3 illustrates the nucleotide sequences encoding human, rabbit, and mouse *SLN* and their deduced amino acid sequences. The rabbit sequences were confirmed in eight clones obtained from a cDNA library by circularization, following inverse long PCR amplification from primers in exon 2.

The human sequences were obtained by similar methods, as outlined under Materials and Methods. Sequences from mouse cardiac and skeletal muscle *SLN* were obtained from a computer search of the GenBank EST database, which also identified human *SLN* EST sequences (Washington University School of Medicine-Merck EST project) covering most of the full-length cDNA, but lacking the 5' and 3' tails.

Comparison of human, rabbit, and mouse *SLN* cDNAs revealed 84% nucleotide sequence identity between human and rabbit, 44% nucleotide sequence identity between human and mouse, and 41% nucleotide sequence identity between mouse and rabbit *SLN* cDNAs. The higher level of nucleotide sequence identity between the human and rabbit *SLN* cDNAs is reflected in their identical length and in their use of the same polyadenylation signal sequence for the major transcript, 14 bp upstream of the poly(A)⁺ tail. This polyadenylation site is lacking in the mouse *SLN* ESTs, which contain a consensus polyadenylation signal sequence 150 bp further downstream, with polyadenylation occurring an additional 100 bp downstream. The length of the mouse *SLN* cDNA is at least 950 bp. An AUUUA motif that could act as an RNA degradation signal (Shaw and Kamen, 1986) is present in the major transcript of human and rabbit mRNA, about 250 bp downstream of the stop codon, but is lacking in the mouse transcript. Another potential polyadenylation signal sequence, present in all three species, is located 50 bp downstream of the potential RNA degradation signal. The first 11 nucleotides, which include the translation initiation signal sequence (Kozak, 1987), are not well conserved among the three species.

Amino acid sequence. The *SLN* gene encodes a protein of 31 amino acids that can be divided into three

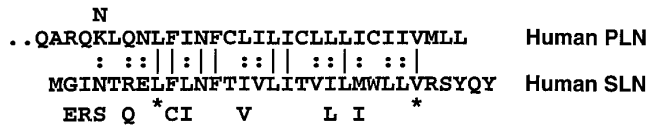


FIG. 4. Alignment of human SLN and PLN peptide sequences. Amino acids 23 to 52 of human PLN are shown in the upper line. The lysine at position 27 of human PLN is replaced by an asparagine in PLN of rabbit, dog, and pig (above). The peptide sequence of human SLN is shown in the lower line. Amino acids different in rabbit or mouse are indicated beneath. The vertical lines in the alignment indicate identical amino acids, whereas conserved amino acids are shown by two vertical dots. The asterisk indicates the first and last amino acid of the predicted transmembrane helix of SLN.

domains: a hydrophilic N-terminal domain of 7 amino acids; a hydrophobic, transmembrane helical domain of 19 amino acids; and a hydrophilic C-terminal domain consisting of 5 amino acids. Neither the hydrophilic N-terminal sequences nor the hydrophobic transmembrane sequences are well conserved among the three species studied, and changes in net charge occur in the N-terminal sequences. The hydrophilic C-terminal sequences are, however, perfectly conserved. The amino acid sequence of the rabbit protein, deduced from the cDNA, is identical to the peptide sequence determined by Wawrzynow *et al.* (1992).

The SLN sequence from residue 4 to residue 26 (4 N-terminal hydrophilic residues and 19 hydrophobic transmembrane residues) shares a striking similarity with residues 28 to 49 of PLN (Fig. 4). This similarity becomes more evident if alternate residues at different points in the sequences of the three species are considered. Asn⁴ in human SLN corresponds to Asn²⁷ in PLN in rabbit, pig, and dog. Gln⁶ in mouse SLN corresponds to Gln²⁹ in human PLN. At human SLN positions 10, 20, and 22 Leu, Ile, and Met differ from the corresponding residue in human PLN, but in other species, correspondence of one or more of these residues is observed. On the basis of these sequence similarities, we propose that SLN and PLN are homologous proteins. Since the orientation of PLN in the membrane is well established, we propose that the N-terminal domain of SLN is cytosolic and that the C-terminal domain faces the lumen of the sarcoplasmic reticulum.

Characterization of SLN Expression

The distribution of *SLN* mRNA in various human tissues was determined by Northern blot analysis using a PCR-amplified probe from the 3' untranslated region of the cDNA that hybridized to a single transcript of 0.7 kb (Fig. 5). This *SLN* transcript was highly expressed in skeletal muscle, but was at least 50-fold less abundant in heart muscle, as determined by densitometry of the exposed film. Only trace amounts were detected in prostate and pancreas. In all other tissues analyzed (brain, placenta, lung, liver, kidney, uterus, colon, small intestine, bladder, and stomach), no *SLN* transcript was detected. A 9-fold higher level of the

SLN transcript was found in rabbit fast-twitch skeletal muscle (psoas) than in slow-twitch skeletal muscle (soleus) (Fig. 5C).

Multiple-Species Genomic DNA Analysis

A Southern blot containing genomic DNA from nine eukaryotic species, digested with restriction endonuclease *Eco*RI, was probed with a 350-bp, PCR-amplified DNA fragment from the 5' exon 1-containing region of the *SLN* gene (Fig. 6). A single band was detected in genomic DNA from each of human (0.9 kb), monkey (0.95 kb), dog (1.5 kb), cow (1.05 kb), and rabbit (4.4 kb). The size of 0.9 kb of the *Eco*RI-digested human genomic DNA fragment confirmed the finding of a 0.9-kb DNA product obtained by inverse PCR amplification with primers specific for the 5' region of the *SLN* gene. Genomic DNA from chicken yielded two bands of 1.6 and 2.6 kb. The hybridization signal of the human probe with mouse and rat genomic DNA was very weak, reflecting the fact that there is much lower similarity between human and mouse *SLN* cDNAs than between human and rabbit *SLN* cDNAs. No hybridization to digested yeast genomic DNA was observed. A similar pattern was obtained using a labeled probe specific for the 3' region of the *SLN* gene (not shown).

Chromosomal Localization

To determine the location of the *SLN* gene, we performed FISH using a 4.5-kb, PCR-amplified DNA probe covering both exons and the single intron. Positive signals were observed on both chromosome 11 chromatids in the region of 11q22–q23. No hybridization to any other chromosome was observed. The assignment of the *SLN* gene to human chromosome 11 was confirmed using monochromosomal rodent–human somatic cell hybrids. To refine the position of the gene on the genetic and physical map of 11q, we screened the GeneBridge 4 RH panel. The results indicate that *SLN* is flanked by the two genetic markers CHLC.GATAGC11 and CHLC.GATA71E06. *SLN* was 7.04 cR from the former marker. Although this entire region appears to be covered by a set of overlapping YACs (Hudson *et al.*, 1995), our analysis failed to detect a positive clone for the *SLN* gene from this collection. Nevertheless, the FISH and RH data are consistent, indicating that the gene maps to human chromosome 11q22–q23.

Sequencing of SLN from Patients of Brody Families BD3 to BD7

No mutations were found in the sequence of the *SLN* cDNA amplified from patients F4P3 (recessive inheritance, Table 2) or F5P3 (dominant inheritance), nor were any mutations found in the sequence of the *SLN* gene amplified from genomic DNA from patients from families BD3 (recessive), BD6 (dominant), or BD7 (dominant) (Table 2). Genomic sequences included each of the two exons

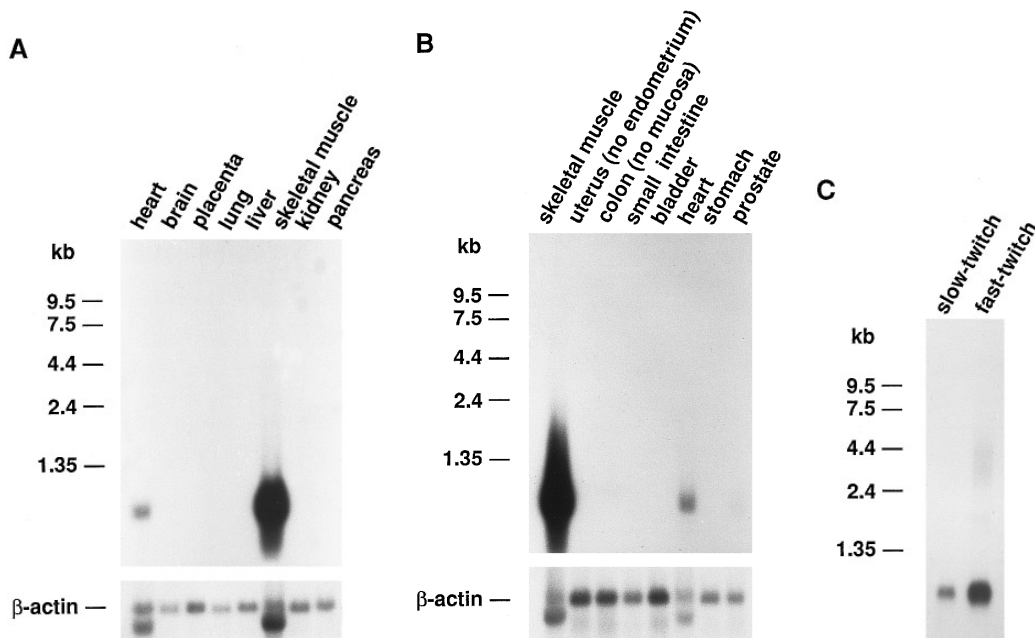


FIG. 5. Analysis of *SLN* gene expression. Human multiple tissue Northern (MTN) blots (Clontech) containing: (A) mRNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas; and; (B) skeletal muscle, uterus (no endometrium), colon (no mucosa), small intestine, bladder, heart, stomach and prostate (**top**) were probed with a 0.5-kb PCR product (HSAF1–HSAR5) derived from human *SLN* cDNA. The positions of RNA size markers (kb) are indicated on the left. Autoradiography was carried out for 16 h at -70°C . (**Bottom**) The same blots were probed with a human β -actin cDNA and exposed for 4 h. The second hybridization signal at 1.6–1.8 kb observed in both heart and skeletal muscle is due to probe hybridization to either the α or the γ form of actin (Giovanna *et al.*, 1991; Lamballe *et al.*, 1991). (C) Analysis of *SLN* expression in rabbit fast-twitch and slow-twitch skeletal muscle. The Northern blot was probed with the 0.7-kb RSAF7–RSAR8 PCR product, specific for rabbit *SLN* mRNA. Autoradiography was for 6 h.

and at least 100 bp of intron sequence flanking each exon and 850 bp of the proposed promoter region. The substitution of G for C11 in the 5' untranslated region of exon 2 was detected in patient F3P3. The insertion of a T in both alleles at position -118 was observed in patient F6P3. This mutation did not affect the structure of *SLN* and was not considered significant.

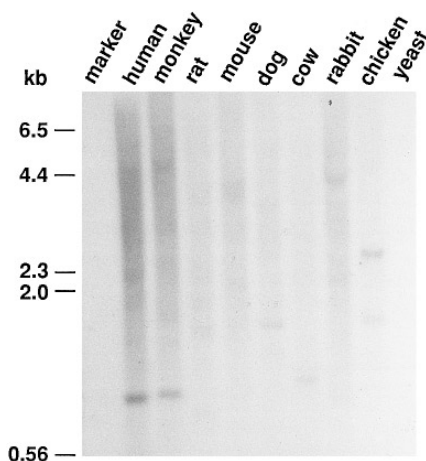


FIG. 6. Southern blot analysis of the *SLN* gene. A multiple species Southern blot (Clontech) was probed with the 350 bp HSAF12–HSAR8 PCR product, specific for the 5' region of the human *SLN* gene, and washed under low-stringency conditions (60°C ; $2\times$ SSC, 0.05% SDS). Positions of DNA size markers are indicated on the left.

Sequencing of *ATP2A1* from Genomic DNA of Patients F3P3 and F6P3

Sequence analysis of *ATP2A1* from genomic DNA of patient F6P3 (dominant) did not reveal any mutations in the putative promoter region, in the two exons, or in 10–100 bp of intronic sequence flanking each exon. The substitution of T for C678, a polymorphism that does not change the amino acid sequence (Zhang *et al.*, 1995), was detected in patient F6P3.

The fact that no detectable defect in either *SLN* or *ATP2A1* (Zhang *et al.*, 1995) had been found in patient F3P3 (recessive), who had been reported to be devoid of SERCA1 (Karpati *et al.*, 1986), led us to a reexamination of *ATP2A1* in this patient and his brother (patient 2 in Karpati *et al.*, 1986). Haplotyping of the region surrounding *ATP2A1* as described by Odermatt *et al.* (1996) showed that these brothers had the identical haplotype on both chromosomes, indicating homozygosity in this region (not shown). The fact that the parents in this family were first cousins is consistent with this degree of homozygosity.

Resequencing of *ATP2A1* from patient F3P3 showed the presence of the T for C678 polymorphism noted earlier (Zhang *et al.*, 1995), but this time the deletion of a C in a series of three Cs (cDNA nucleotides 437 to 439) was detected (Fig. 7). This deletion led to a homozygous frame shift at Pro¹⁴⁷, which would lead to a stop codon in both transcripts after the incorporation

TABLE 2
Analysis for Mutations in *ATP2A1* and *SLN* of Brody Families

Family	Patient	<i>ATP2A1</i>	<i>SLN</i>	Inheritance	Reference
BD1	F1P3, F1P4	Homozygous Arg ¹⁹⁸ to Stop	n. d.	Recessive	F1P3, ^a P4 ^b
BD2	(F2P1), ^c F2P3, F2P4 (F2P2), ^c F2P3, F2P4	Cys ⁶⁷⁵ to Stop, and intron 3 splice donor GT to CT (compound heterozygote)	n. d.	Recessive	F2P3, ^a P3 ^d
BD3	F3P3, F3P4	Homozygous deletion of a C frameshift at Pro ¹⁴⁷	No mutation	Recessive	P1, ^d P1 ^f
BD4	F4P3	No mutation	No mutation	Recessive	P2 ^f
BD5	F5P3	No mutation	No mutation	Dominant	P3 ^f
BD6	F6P3	No mutation	No mutation	Dominant	
BD7	F7P3	n. d.	No mutation	Dominant	P6 ^b

^a Odermatt *et al.* (1996).

^b Benders *et al.* (1994).

^c Unaffected father.

^d Karpati *et al.* (1986).

^e Unaffected.

^f Zhang *et al.* (1995).

of 33 additional amino acids. This mutation is very similar to the two cases of *ATP2A1*-linked Brody disease described earlier (Odermatt *et al.*, 1996) in that mutations in both alleles led to premature termination codons and to truncation of SERCA1. In three of four cases of autosomal recessive inheritance of Brody disease described to date, SERCA1 protein has been lost due to these truncations (Table 2). Our results suggest that mutations in *ATP2A1*, leading to a predicted functional knock-out of SERCA1, account for the majority of autosomal recessive forms of Brody disease, but not for dominant forms of the disease.

DISCUSSION

Cloning of Rabbit and Human SLN by Application of Inverse Long PCR Several earlier attempts in our laboratory to clone *SLN* by hybridization screening of a skeletal muscle library were unsuccessful, even though

the cDNA is present in our pcDX library, as described here, and is present in other libraries from both skeletal and cardiac muscle (Washington School of Medicine–Merck EST project, GenBank). In this study, we cloned the *SLN* gene from our full-length rabbit skeletal muscle cDNA library in the pcDX vector by a novel application of inverse long PCR. In this method, the vector sequence, containing an antibiotic resistance marker, was coamplified with the full-length cDNA, allowing us to self-ligate the PCR product and use the circularized DNA directly for transformation of competent bacterial cells. Since self-ligation was very efficient, as little as 1 ng of circularized PCR product was sufficient for successful cloning. By inverse long PCR, products up to 8 kb in length have been obtained. Accordingly, this method has potential application for direct cloning of even very long cDNAs inserted into vectors of the usual length of about 4 kb. For example, we have successfully cloned full-length *ATP2A1* cDNAs of

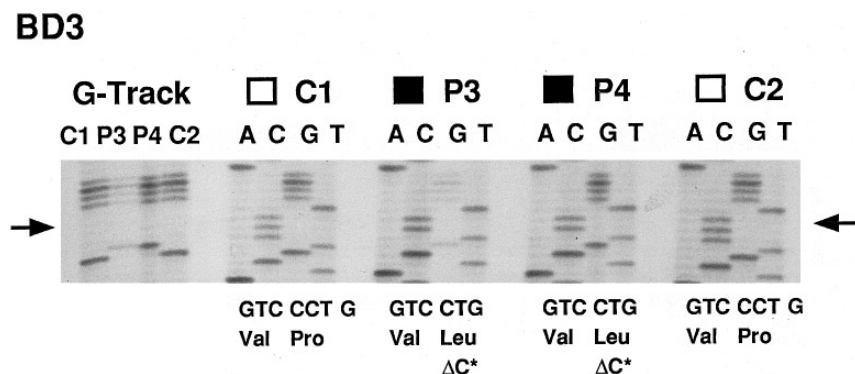


FIG. 7. Analysis of the deletion of one of the three Cs in nucleotide positions 437, 438, and 439, numbered according to the cDNA sequence of the *ATP2A1* gene encoding SERCA1 (Zhang *et al.*, 1995), in family BD3. PCR amplified *ATP2A1* genomic DNA was sequenced, and the deletion of one of the three Cs at Nucleotides 437, 438, or 439, leading to a frameshift beginning with the codon for Pro¹⁴⁷, is indicated by an arrow. The effect of the mutation on amino acid sequences is shown beneath. The G-track and the nucleotide sequences of the homozygous mutant DNAs of the two affected brothers, F3P3 and F3P4, and two unrelated control DNAs, C1 and C2, are indicated.

3.5 kb from our pcDX library. The use of *Pfu* polymerase to avoid PCR artifacts is strongly recommended.

We also applied inverse long PCR to a human skeletal muscle 5' stretch cDNA library in λ gt11 to clone human *SLN*. The phage DNA was digested with an enzyme flanking the cDNA at both sides, and the circularized DNA was used as a template for inverse long PCR. The disadvantage of using phage library templates is the lack of a selectable marker on the amplified product. Accordingly, it is necessary to recover much higher yields of the PCR product for cloning into a plasmid vector.

5' Upstream Region of the Human *SLN* gene

In the *SLN* gene, a cluster of three TATA box elements is located about 40 bp upstream of a CCAAT box, which, in turn, is found 700 bp upstream of our proposed transcriptional start site. A similar unusual arrangement of CCAAT and TATA box elements was described for the skeletal muscle *RYR1* gene (Phillips *et al.*, 1996), with a CCAAT sequence 68 bp upstream of the transcription initiation site and a cluster of five TATA box elements a further 1500 bp upstream. A binding site for the myocyte enhancer factor, MEF2, in combination with a MEF3-like element, follows just upstream of the TATA box cluster in the *SLN* gene. The muscle-specific induction of the aldolase A gene has been shown to be controlled through cooperation between the muscle-specific factor MEF2 and the more ubiquitous factor MEF3 (Hidaka *et al.*, 1993). An additional MEF3-like site is located 200 bp downstream of the CCAAT box in the *SLN* gene. The 5' region of the *SLN* gene contains several E boxes (CANNTG). E boxes, which are recognized by the MyoD family of basic -helix-loop-helix transcriptional activators, occur by chance every 250 bp in DNA sequences. However, MyoD has been shown to bind cooperatively to paired sites with a 19-bp space in the muscle creatine kinase enhancer (Weintraub *et al.*, 1990) and to paired sites with a 12-bp space in the acetylcholine receptor α -subunit enhancer (Piette *et al.*, 1990). In the *SLN* gene, two E box pairs with 11 and 10 bp space between both E boxes follow about 200 and 500 bp downstream of the CCAAT box. These DNA binding elements might be responsible for the muscle-specific expression of the *SLN* gene revealed by Northern analysis. In contrast to the *ATP2A1* gene (Korczak *et al.*, 1988) and the *RYR1* gene (Phillips *et al.*, 1996), Sp1 sites and the proposed muscle-specific promoter element GGC-TGGGG are absent from the *SLN* promoter region.

Conservation of *SLN* in Different Species

Alignment of amino acid sequences of *SLN* from human, rabbit, and mouse (this study; Washington School of Medicine-Merck EST project, GenBank; Wawrzynow *et al.*, 1992) revealed poor conservation of the hydrophilic N-terminal sequence, a relatively well-con-

served hydrophobic transmembrane sequence, and perfect conservation of the C-terminal hydrophilic sequence, which, by analogy with the well-characterized structure of phospholamban (Fujii *et al.*, 1991), should lie in the lumen of the sarcoplasmic reticulum. The functional consequences of the different charge distribution in the cytoplasmic domain of *SLN* in the three species are not clear. By contrast, SERCA1, with which *SLN* seems to interact, is highly conserved between rabbit and human (Zhang *et al.*, 1995). The low level of conservation of the *SLN* cDNA between different species is also reflected in the weak hybridization signal obtained on a Zoo blot using a human *SLN* probe. One or two main bands were detected in all species tested, except yeast (Fig. 6).

Relationship between *SLN* and *PLN* Genes

The human *SLN* and *PLN* (Fujii *et al.*, 1991) genes share common features, consistent with their origin in a common ancestor. Both genes contain two exons and one intron. In both cases, the intron is located about 90 bp downstream of the transcription initiation site, and in both cases it interrupts the 5' untranslated sequence, so that the entire coding sequence is located in exon 2. The introns are relatively large, about 3.9 kb long in *SLN* and about 6 kb long in *PLN*. The 3' ends of both genes contain multiple polyadenylation signals, and more than one polyadenylation site is used in both genes (Fujii *et al.*, 1988). Alignment of human *SLN* and *PLN* cDNA reveals 60% nucleotide identity in the region encoding the predicted transmembrane helices, even though only 30% identity is observed for the N-terminal and C-terminal domains (not shown).

On the basis of the conservation of amino acids in parts of the N-termini and much of the transmembrane helices of *SLN* and *PLN*, we propose that *SLN* and *PLN* are homologous proteins. It is possible that they have achieved this degree of sequence identity by convergent evolution, but, in light of the similarity in gene structure, it seems much more likely that sequence similarity is based on divergent evolution from a common gene.

The differences in the N- and C-terminal sequences of *SLN* and *PLN* must reflect their different interactions with different cytoplasmic proteins (*PLN* is phosphorylated by protein kinase A, but *SLN* is not), but their conserved transmembrane sequences suggest retention of the ability to interact with conserved transmembrane sequences in other proteins. The three SERCA molecules (Burk *et al.*, 1989) are the most likely candidates for such interactions. *PLN* is coexpressed with SERCA2a and interacts functionally with it. *PLN* also retains the capacity to inhibit SERCA1 (Toyofuku *et al.*, 1993), but does not do so because the two proteins are not coexpressed in the same tissue. *SLN* is most highly expressed in fast-twitch muscle, where SERCA1 is highly expressed and the two proteins copurify (Mac-

Lennan *et al.*, 1972). In our most recent *in vitro* coexpression experiments, we have found that SLN inhibits SERCA1 by decreasing its apparent affinity for Ca^{2+} (A. Odermatt, S. Becker, V. K. Khanna, K. Kurzydowski, and D. H. MacLennan, unpublished). Since PLN inhibits the activity of SERCA2a by decreasing its apparent affinity for Ca^{2+} , SLN may act as the counterpart of PLN in fast-twitch skeletal muscle.

Transmembrane helices M4, M5, and M6, which are key to Ca^{2+} binding and translocation in SERCA1 (Rice and MacLennan, 1996), are highly conserved in all three SERCA molecules. Thus the three transmembrane sequences in SERCA molecules and the single transmembrane sequence in SLN/PLN molecules may have coevolved, conferring a degree of evolutionary stability on each other. The cytoplasmic domain of PLN also interacts with a cytoplasmic domain of SERCA2a (Toyofuku *et al.*, 1994). This site is conserved in SERCA1, but is missing from SERCA3.

The *SLN* gene is expressed most abundantly in fast-twitch skeletal muscle (Fig. 5C), the tissue in which SERCA1 is expressed almost exclusively. This is also the tissue from which SLN was first isolated, in association with highly purified SERCA1 (MacLennan *et al.*, 1972). Like SERCA1 expression (Wu and Lytton, 1993), *SLN* expression is 10 times lower in slow-twitch skeletal muscle (soleus) than in fast-twitch skeletal muscle (WST, EDL). In cardiac tissue, the concentration of the *SLN* transcript is about 50-fold less abundant than it is in skeletal muscle (Fig. 5A). *SLN* expression is another order of magnitude lower in pancreas and prostate (Fig. 5B), where SERCA2b predominates. *PLN* is expressed most abundantly in cardiac and slow-twitch skeletal muscle, where SERCA2a is the predominant SERCA molecule, and at much lower levels in fast-twitch skeletal muscle and smooth muscle (Fujii *et al.*, 1988). Thus *SLN* and *PLN* are expressed in muscle-specific and complementary patterns, and the patterns of their expression are directly correlated with the expression of different SERCA molecules in each muscle tissue. On the basis of data presented in this paper, we propose that *SLN* and *PLN* be recognized as two members of a gene family.

A Potential Role for SLN in Brody Disease

Kimura *et al.* (1996) demonstrated that the predicted PLN transmembrane α -helix is the inhibitory domain of the PLN molecule. More recently, Kimura *et al.* (1997) described mutations in the predicted PLN transmembrane α -helix that resulted in a dramatic gain of PLN inhibitory function, manifested as a lowering of the apparent affinity of SERCA2a for Ca^{2+} . They proposed that mutations in PLN or SERCA2a that decreased the apparent affinity of SERCA2a for Ca^{2+} under physiological conditions could lead to cardiomyopathy.

Because a mutant SLN, like a mutant PLN, might

gain inhibitory function leading to the morbidity associated with a highly inhibited form of SERCA1, we initiated a search for SLN mutants in the four Brody families with non-*ATP2A1*-linked Brody disease and with a fifth family available to us. Unfortunately, we did not find any alterations that would support *SLN* as a candidate gene for Brody disease in these five families.

In additional studies of BD, we showed that two brothers in family BD3, in which *ATP2A1* mutations had not been found in an earlier study (Zhang *et al.*, 1995), shared a common haplotype on chromosome 16p12.1–p12.2, suggesting the need for reevaluation of the role of the *ATP2A1* gene in the disease. This led us to resequence *ATP2A1* in this family and to the discovery of a fourth BD mutation that would truncate SERCA1. Currently four mutations in three BD families are known, all resulting in recessive inheritance of BD. The only recessive BD family that has not been shown to have an *ATP2A1* mutation is BD4 (Table 2). We are currently haplotyping two brothers in this family to determine whether resequencing of *ATP2A1* might be warranted. In three other BD families where inheritance is dominant (Table 2), the causal gene has yet to be discovered.

SLN localizes to human chromosome 11q22–q23, a region frequently deleted in several hematological malignancies. In acute leukemias, the *MLL* (*ALL1*, *HRX*, and *HTRX*) gene, which maps to 11q23, is involved in reciprocal translocations with a large number of gene partners (Bernard and Berger, 1995). Leukemias manifesting 11q23 rearrangements have been shown to be associated with very poor prognosis (Rasio *et al.*, 1996). This region also seems to be sensitive to DNA topoisomerase II inhibitors, as the majority of therapy-related leukemias resulting from treatment with these inhibitors demonstrate *MLL* rearrangements (Canaani *et al.*, 1995). Cultured cells harboring such deletions might provide an interesting experimental system for the investigation of the function of *SLN* in cell physiology.

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