Structure and expression of mitsugumin29 gene

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Abstract Recently mitsugumin29 unique to the triad junction in skeletal muscle was identified as a novel member of the synaptophysin family; the members of this family have four transmembrane segments and are distributed on intracellular vesicles. In this study, we isolated and analyzed mouse mitsugumin29 cDNA and genomic DNA containing the gene. The mitsugumin29 gene mapped to the mouse chromosome 3 F3-H2 is closely related to the synaptophysin gene in exon-intron organization, which indicates their intimate relationship in molecular evolution. RNA blot hybridization and immunoblot analysis revealed that mitsugumin29 is expressed abundantly in skeletal muscle and at lower levels in the kidney. Immunofluorescence microscopy demonstrated that mitsugumin29 exists specifically in cytoplasmic regions of the proximal and distal tubule cells in the kidney. The results obtained may suggest that mitsugumin29 is involved in the formation of specialized endoplasmic reticulum systems in skeletal muscle and renal tubule cells.

Key words: Mitsugumin29; Synaptophysin family; Endoplasmic reticulum; Triad junction; Skeletal muscle; Kidney

1. Introduction

In skeletal muscle cells, excitation-contraction coupling requires the signal transduction system to convert depolarization of the cell-surface transverse (T-) tubule into Ca^{2+} release from the sarcoplasmic reticulum (SR) [1]. The two major molecules participating in this signal conversion are the dihydropyridine receptor as the T-tubular voltage sensor and the ryanodine receptor as the SR Ca^{2+} release channel [2,3]. Both receptor proteins are thought to be mechanically linked to each other at the triad junction where the T-tubule is facing the SR membrane on both sides [4]. However, previous studies have suggested that an as yet unidentified component(s), other than the dihydropyridine and ryanodine receptors, is essential for the formation of the triad junction and may provide a foundation for the direct interaction of the receptor molecules [5–7].

To further skeletal muscle excitation-contraction coupling at the molecular level, we have begun to identify protein components unique to the triad junction using the monoclonal antibody technique and have recently identified mitsugumin29, a protein with a relative molecular mass of 29 kDa, which is thought to be localized on the junctional SR in rabbit [8]. The primary structure deduced by cDNA cloning revealed that mitsugumin29 is a novel member of the synaptophysin family. The synaptophysin family members are known to be major transmembrane proteins on synaptic, secretory or other related intracellular vesicles in a wide variety of cell types including neurons. Although their contribution to the systems of neurotransmitter release and secretion has been suggested, physiological roles of the members have not yet been established. The structural characteristics and subcellular localization suggest that mitsugumin29 may participate in specific communication between the T-tubular and junctional SR membranes in the triad junction. This report deals with the primary structure of mouse mitsugumin29, the structural organization and chromosomal location of the gene, and the tissue distribution of mitsugumin29.

2. Materials and methods

2.1. DNA cloning

A skeletal muscle cDNA library derived from C57BL/6J mice was screened using the 0.8-kb *Not*I/*Apa*I fragment deriverd from the 5′-terminal region of the rabbit mitsugumin29 cDNA as a probe [8]. Of several clones obtained, λMMG29-2 and λMMG29-8 carrying ~3.3-kb cDNA inserts were used for sequence analysis to determine the primary structure of mouse mitsugumin29. For isolation of genomic segments containing the mitsugumin29 gene, a genomic DNA library derived from 129-strain mice (Stratagene, USA) was screened with the 2.3-kb cDNA insert of λMMG29-2 containing the entire protein-coding sequence as a probe, to yield λMMG29G-7 and λMMG29G-4 in which the cloned genomic DNAs do not overlap. To obtain the genomic DNA segment connecting the cloned DNA segments, a polymerase chain reaction was performed using the 31-mer forward primer (GGAATTCCGCTGGGCTTCATCAAAGTTCTCC derived from the exon 2 sequence), the 29-mer reverse primer (CGGGATCCGACTCACCTGAAGGGATAGCC derived from the exon 3 sequence), mouse genomic DNA from J1 embryonic stem cells [9] as a template, and LA Taq polymerase (Takara Co., Japan). The nucleotide sequences of the cloned DNAs thus obtained were analyzed [10] using a DNA sequencer (ALF Express, Pharmacia Biotech).

2.2. Chromosomal mapping and RNA blot analysis

Fluorescence in situ hybridization was carried out as described previously [11]. A mixture of DNA fragments derived from λMMG29G-4 and λMMG29G-7 was used as the probe.

Hybridization filters blotted with RNA preparations from mouse tissues (Multiple Choice Northern Blot, OriGene Technologies Inc., USA) were reacted with the 2.3-kb cDNA insert of λMMG29-2 as a probe and were analyzed as described previously [12].

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Abbreviations: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; T-tubule, transverse tubule

The sequences reported in this paper have been deposited in DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession numbers AB010140–AB010144.

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2.3. Immunohistochemical analysis

Immunoblot analysis for mitsugumin29 in microsomal preparations from rabbit tissues was performed as previously described [8] except that peroxidase-conjugated secondary antibody was detected with ECL Western blotting reagents (Amersham). The hybridoma supernatant containing mAb1007 [8] was reacted with cryosections of rabbit kidney fixed with acetone and then the labelled antibodies were detected with affinity purified secondary antibody conjugated with fluorescein isothiocyanate (DAKO, Denmark). Fluorescence microscopic observation was performed using a photomicroscope (IX-FLA, Olympus, Japan).

3. Results and discussion

3.1. cDNA cloning and primary structure of mouse mitsugumin29

A mouse skeletal muscle cDNA library was screened by hybridization with a DNA fragment comprising part of the rabbit mitsugumin29 cDNA [8]. Nucleotide sequence analysis of the clones thus obtained revealed an open reading frame of 792 nucleotides encoding a sequence of 264 amino acid residues, the calculated molecular weight of mouse mitsugumin29 being 29 223 (including the initiating methionine). As shown in Fig. 1, the amino acid sequence of mouse mitsugumin29 shows 95% and 46% identity with those of rabbit mitsugumin29 and human synaptophysin [13], respectively. The hydropathicity profile of mouse mitsugumin29 predicts no amino-terminal signal sequence and four transmembrane segments (M1–M4) and is similar to those of the members of the synaptophysin family (data not shown). The similarity in primary sequence and characteristic structural features confirms our previous proposal that mitsugumin29 spans the membrane four times and both its amino- and carboxyl-termini are located in the cytoplasmic side as in the case of synaptophysin [14].

As previously reported [14], sequence comparison of synaptophysin from three animal species showed that the transmembrane and cytoplasmic regions are highly conserved, while substitutions cluster in the two intravesicular loops. Functional sites within the synaptophysin molecule are therefore proposed to reside in transmembranous and cytoplasmic regions. In contrast, in mitsugumin29, the amino-terminal cytoplasmic region and the intravesicular loop between M1 and M2 contain clustered substitutions but the other portions are highly conserved between mouse and rabbit. These observations might suggest that the positions of functional sites, for example sites for protein-protein interaction, are not well conserved among the members of the synaptophysin family.

3.2. Structural organization of mouse mitsugumin29 gene

A mouse genomic library was screened with a probe from the mouse mitsugumin29 cDNA, and two clones carrying distinct parts of the mitsugumin29 gene were isolated. The genomic DNA fragment containing the gap between the cloned segments was amplified using a polymerase chain reaction

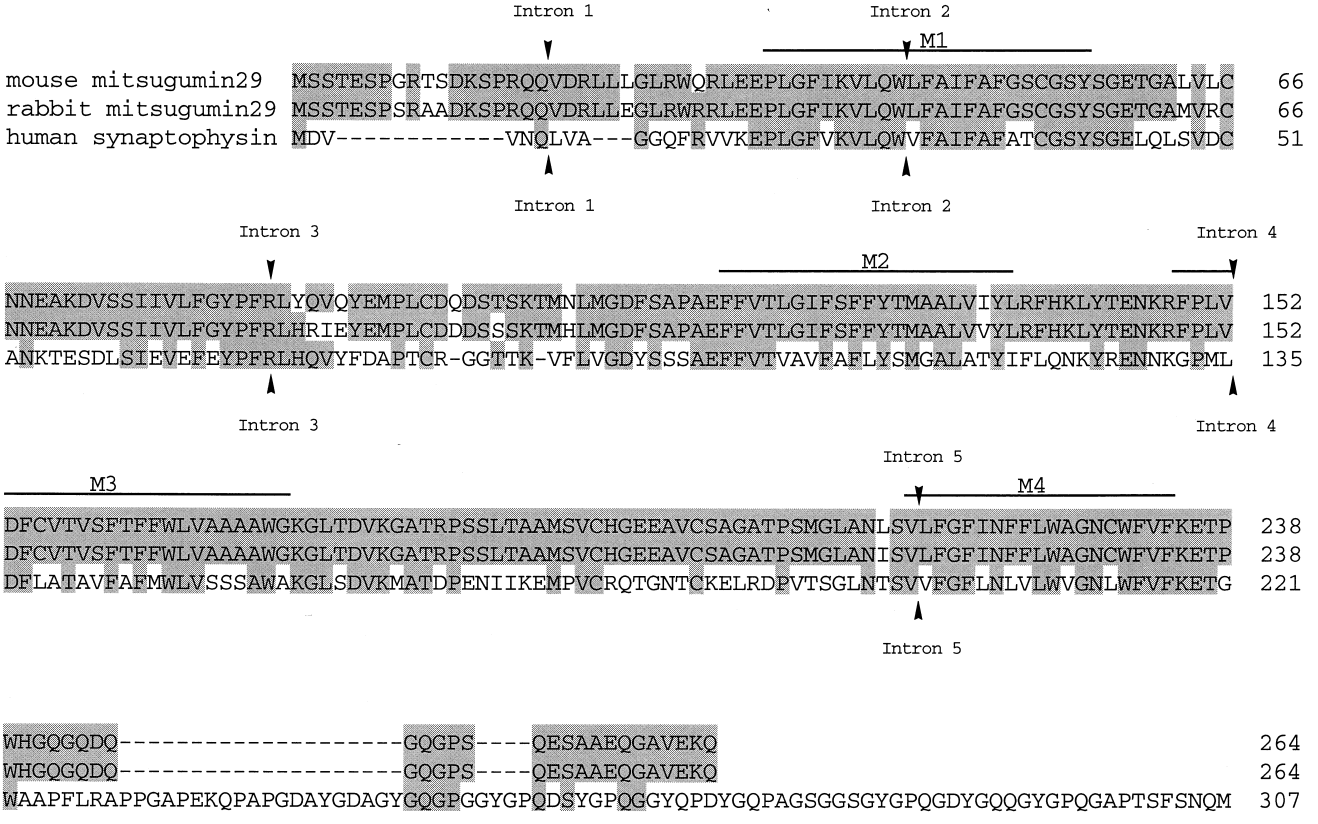

Fig. 1. Deduced amino acid sequence of mouse mitsugumin29 and its alignment with those of rabbit mitsugumin29 and human synaptophysin. The one-letter amino acid notation is used and gaps (−) are inserted to achieve maximum homology. Amino acid residues are numbered from the initiating methionine and the numbers of the residues are given at the right-hand end of the individual lines. The predicted transmembrane segments (M1–M4) are indicated; the termini of each segment have been tentatively assigned. The positions of introns interlapping the protein-coding sequence in the mouse mitsugumin29 gene and the human synaptophysin gene are indicated with arrows. The cDNA sequence data for mouse mitsugumin29 will appear in the DDBJ, EMBL and GenBank databases under accession number AB010140.

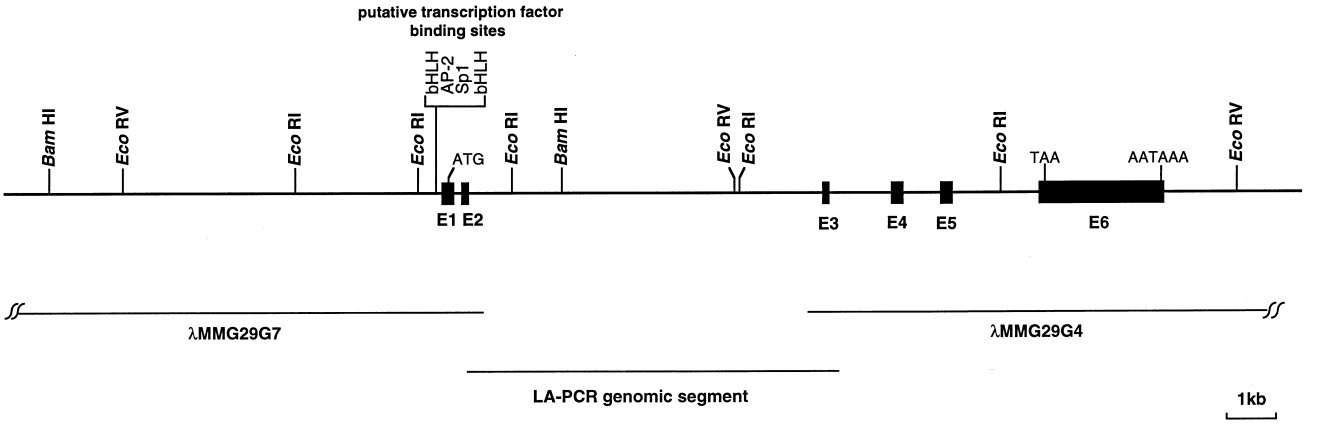

Fig. 2. Restriction enzyme map of genomic segment containing mouse mitsugumin29 gene. Exons (E1–E6) in the gene are indicated by filled boxes. The position of the initiating methionine (ATG), the termination codon (TAA) and the polyadenylation signal (AATAAA) are shown. Consensus sequences for transcription factors found in the putative promoter region (GGGCGG for Sp1, GSSWGSCC for AP-2 and CANNTG for basic helix-loop-helix proteins (bHLH); S = G or C, W = A or T, N = any nucleotides) are also indicated. The DNA sequence data for the mouse mitsugumin29 gene will appear in the DDBJ, EMBL and GenBank databases under accession numbers AB010141–AB010144.

(Fig. 2). Comparison of cDNA and genomic DNA sequences revealed the exon-intron boundaries that follow the GT-AG rule [15] in the gene. Fig. 2 shows the restriction map and exon-intron organization of the mouse mitsugumin29 gene. The protein-coding sequence of the mitsugumin29 gene is split by five introns. A previous study has defined that of six introns in the human synaptophysin gene, five introns divide the protein-coding sequence [16]. Furthermore, between the mitsugumin29 and synaptophysin genes exon-intron junctions locate to completely identical positions in their aligned amino acid sequence (Fig. 1). In both genes, introns 1–5 interrupt the amino-terminal cytoplasmic sequence, the first transmembrane segment, the first intravesicular loop, the third transmembrane segment and the fourth transmembrane segment, respectively, while an additional intron divides the 3′-noncoding region into two fragments in the synaptophysin gene. This observation is consistent with the previous conclusion that mitsugumin29 is highly related to the members of the synaptophysin family in molecular evolution [8] and also demonstrates that the family members arose from a common ancestor.

Specific expression of mitsugumin29 mRNA in skeletal muscle and renal tubule cells (see Section 3.3) may predict a unique promoter sequence in the gene. A survey of known consensus sequences showed that the 5′-flanking region of the gene contains the motif sequences for several transcription factors including Sp1, AP-2 and basic helix-loop-helix proteins as indicated in Fig. 2. Myogenic transcription factors including MyoD and myogenin belong to the basic helix-loop-helix protein family. The 5′-flanking region carrying clustered motif sequences for the transcription factors may participate in the transcription of the mitsugumin29 gene.

To determine the chromosomal localization of the mouse mitsugumin29 gene, metaphase chromosomes from lymphocytes showing typical Q bands were examined using the genomic DNA fragments isolated. Specific labelling with the probe was observed in the chromosome 3 F3-H2 region; 87 out of total 125 signals decorated the region (Student's t-test, $P < 0.01$). The mouse synaptophysin gene was mapped to the X chromosome A–D region [16]. The results indicate that the genes for the synaptophysin family members are not clustered on the genome.

3.3. Tissue distribution of mitsugumin29

To determine the tissue distribution of mitsugumin29, blot hybridization analysis of RNA from mouse tissues was performed using a specific cDNA probe (Fig. 3). Two major hybridizable RNA species of ~4200 and 3350 nucleotides

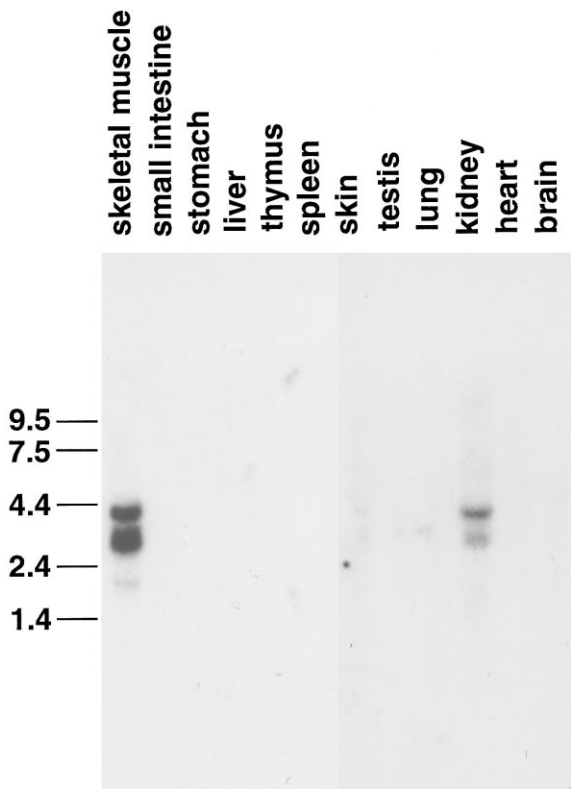

Fig. 3. Blot hybridization analysis of RNA from mouse tissues using probe specific for mitsugumin29 mRNA. Total RNA preparations (20 μg each) were analyzed. The size markers are given in kilobases.

were detected abundantly in skeletal muscle and at lower levels in the kidney, whereas no hybridizable signal was detected in RNA preparations tested. In contrast to the results for mouse, a single mitsugumin29 mRNA band was observed on a blot of RNA from rabbit skeletal muscle [8]. The mechanism for the generation of two mRNAs different in size from the mouse gene was not fully addressed. Based on the following two reasons, it is unlikely that there are two transcription initiation sites in the gene: (i) at least three independent cDNA clones, isolated from the mouse skeletal muscle library with the probe derived from the 5′-terminal region of the rabbit mitsugumin29 cDNA, start from similar positions in the 5′-flanking region of the gene: and (ii) two of these clones carry 3.25-kb cDNA inserts with poly(A) tails. A likely assumption therefore is that the two mRNAs result from alternative use of two polyadenylation signals in the 3′-untranslation region. The polyadenylation signal assigned by the cDNA sequence from skeletal muscle (Fig. 2) most likely corresponds to the tail of the smaller mRNA. Comparison of the signal intensity of the two mRNAs between the tissues suggests that the upstream polyadenylation site in skeletal muscle or the downstream site in the kidney may be preferentially used for the transcriptional termination of the gene.

Despite a high sequence identity between mouse and rabbit mitsugumin29, monoclonal antibody to rabbit mitsugumin29 (mAb1007) [8] did not cross-react with the mouse counterpart (data not shown). The mAb1007-recognition site may reside in the region containing clustered amino acid substitutions between the species (see Section 3.1). To further examine the expression of mitsugumin29 in the kidney indicated by the Northern blot analysis, immunochemical analysis was performed using mAb1007 in rabbit tissues. Mitsugumin29 was detected by the antibody in skeletal muscle at dense levels and kidney at low levels, but the antigen with a molecular mass of 29 kDa could not be detected in other tissues examined in Western blot analysis (Fig. 4). Fluorescence microscopy found that renal tubule cells were labelled specifically with the antibody in the kidney, but we could not detect signals in the glomerulus, Henle's loop or collecting duct system (Fig. 5). Both proximal and distal tubular cells exhibited strong immunofluorescent signals. In accordance with the results in Northern and Western blot experiments, no specific labelling with the antibody was observed in the heart, liver and brain histologically (data not shown).

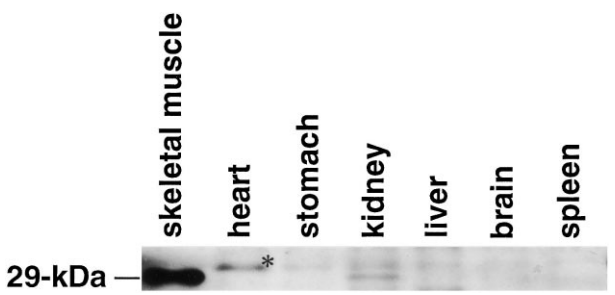

Fig. 4. Immunoblot analysis of microsomal preparations from rabbit tissues using monoclonal antibody against mitsugumin29. Microsomal proteins (30 ng for skeletal muscle, 30 μg for the other tissues) were separated on a 4–20% SDS-polyacrylamide gel and analyzed. The 29-kDa antigen protein recognized with mAb1007 is detected in skeletal muscle and kidney. A slower migrating band found in the heart (asterisk) is probably due to non-specific reaction, since both RNA blot (Fig. 3) and immunohistochemical analyses failed to detect a specific signal in the organ.

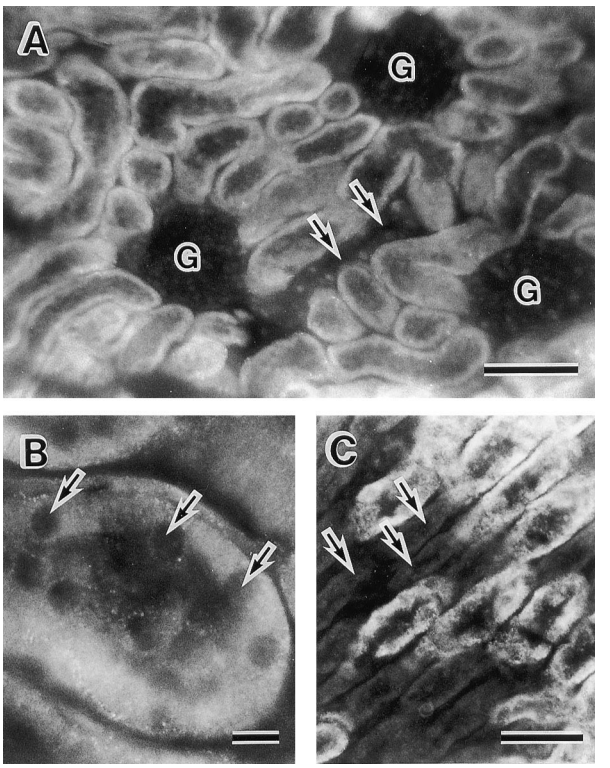

Fig. 5. Immunofluorescence analysis of rabbit kidney tissue using monoclonal antibody to mitsugumin29. Cryosections of rabbit kidney labelled by immunofluoresence were imaged by fluorescence microscopy. A: Renal tubular cells in both proximal and distal tubules are specifically labelled, but the glomerulus (G) and collecting tubule (arrows) are negative. B: Specific labelling is observed in the cytoplasm of renal tubular cells and nuclei (arrows) are negative. C: Cells of the Henle's loop (arrows) are not labelled, and fluorescence-positive cells are proximal or distal tubular cells. Scale bars: 100 μm in A and C, 10 μm in B.

The antibody reacted with the cytoplasm in the renal tubular cells, suggesting that mitsugumin29 is located on the intracellular membranous structures (Fig. 5). In both skeletal muscle and renal tubule cells, the SR or endoplasmic reticulum (ER) is known to be abundant and to form well-organized networks. Moreover, specific structures of the SR or ER associating closely with the plasma membrane are known as terminal cisternae in skeletal muscle cells and as fenestrated saccules in renal tubule cells [4,17]. Mitsugumin29 may be likely to be distributed for forming the specialized ER systems in skeletal muscle and renal tubule cells.

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