Mitsugumin23, a novel transmembrane protein on endoplasmic reticulum and nuclear membranes

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Abstract We report the identification using monoclonal antibody and the primary structure by cDNA cloning of mitsugumin23, a novel transmembrane protein with a molecular mass of ~23 kDa from skeletal muscle sarcoplasmic reticulum. Mitsugumin23 possesses three putative transmembrane segments, and its carboxy-terminal hydrophilic region exhibits sequence similarity with the tail-end portion of the myosin heavy chain. Immunochemical analysis showed that this protein is distributed throughout the outer nuclear membrane and the sarcoplasmic reticulum including the terminal cisternae at the triad junction in skeletal muscle cells. Furthermore, RNA blotting and immunohistochemical experiments demonstrated that mitsugumin23 is distributed among a wide variety of cell types in various tissues. The distribution and primary structure indicate the possibility that mitsugumin23 interacts with cytoplasmic protein(s) and participates in a housekeeping function on the intracellular organelle membranes.

Key words: Endoplasmic reticulum; Sarcoplasmic reticulum; Nuclear membrane; cDNA cloning; Monoclonal antibody; Immunohistochemistry

1. Introduction

In skeletal muscle excitation-contraction (E-C) coupling, depolarization of transverse (T) tubules of the invaginated cell surface membrane causes Ca^{2+} to be released from the sarcoplasmic reticulum (SR) [1]. This signal transduction is the province of the triad junction, at which T-tubular and junctional SR membranes are closely associated [2]; the dihydropyridine receptor as the T-tubular voltage sensor and the ryanodine receptor as the Ca^{2+} release channel on the junctional SR participate in the signal conversion physiologically [3,4]. On the other hand, the SR has two major domains, the terminal cisternae responsible for Ca^{2+} release and the longitudinal tubules playing a role in Ca^{2+} uptake. Despite the essential functions of the SR and triad junction, their organization at the molecular level is poorly understood. Furthermore, recent studies have suggested the presence of as yet unidentified protein(s) contributing to the structural characteristics of the triad junction [5,6]. To understand E-C coupling, therefore, it is important to examine the molecular components of the T-tubular and junctional SR membranes at the triad junction.

Previous biochemical studies have identified triadin and junctin as SR transmembrane proteins specific to the triad junction [7,8], but their functions are not yet established. Recently we started to examine the molecular components at the triad junction using monoclonal antibodies (mAbs) and identified mitsugumin29, a novel member of the synaptophysin family [9]. In this paper we report the identification of mitsugumin23, a novel transmembrane protein located on the SR and nuclear membranes in skeletal muscle. Based on the tissue distribution and primary structure, we propose a possible housekeeping function of mitsugumin23 in the intracellular organelles.

2. Materials and methods

2.1. Monoclonal antibody production and immunochemical analysis

Heavy SR membranes were prepared from adult rabbit skeletal muscle [10], and mice were immunized with this preparation to produce mAbs [11]. The resulting antibodies were selected based on immunofluorescence microscopic observations as described previously [9]. Immunoblot analysis was performed as described previously [12] using prestained markers (Gibco-BRL) as size standards. For large-scale preparation of antibody numbered 2595 (mAb2595), the hybridoma cells were injected into BALB/c mice and the antibody was purified from the ascites fluid using a protein G-affinity column (Pharmacia Biotech).

2.2. Purification and amino acid sequence analysis of mitsugumin23

Isolated microsomes were solubilized at a protein concentration of 0.5 mg/ml with 1% Triton X-100 in a buffer containing 10 mM Tris-HCl, pH 7.4. The solubilized proteins were reacted with mAb2595 coupled to NHS-activated Sepharose (Pharmacia Biotech). The resin was extensively washed with a buffer containing 0.2% Triton X-100, 0.3 M NaCl and 10 mM Tris-HCl, pH 7.4, and the absorbed proteins were recovered with an elution buffer containing 1.5% SDS and 10 mM Tris-HCl, pH 7.4. The eluted proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a Problot membrane (Applied Biosystems). The blotting membrane containing mitsugumin23 was subjected to amino acid sequence analysis, as described previously [12]. The determined amino acid sequences of the peptides were identical to the sequences of the following amino acid residues deduced from the nucleotide sequence of the cDNA (see Fig. 2 for amino acid numbers and sequences): peptide T3+4-1, 216–221; T3+4-2, 222–227; T5+6-1, 38–47.

2.3. cDNA cloning, RNA blot hybridization and cDNA expression

The rabbit skeletal muscle cDNA library [9] was screened using the 5′-^{32}P-labelled probe (8 species-mixed 26-mer: 5′-GA(CT)TT(CT)G-CICCICCIGGICAICAI(CA)G-3′, I = inosine) synthesized on the ba-

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Abbreviations: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; E-C coupling, excitation-contraction coupling; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; T-tubule, transverse tubule

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide databases with the accession number AB013721.

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sis of the sequence of the peptide T5+6-1. Of several clones hybridized with the probe, the following three clones carried the full-length protein coding sequence and were used for sequence analysis [13] with an automatic DNA sequencer (ALF Express, Pharmacia Biotech): λMG23-17 (carrying nucleotides −44 to 1745), λMG23-16 (−16 to 1745) and λMG23-3 (−10 to 1745). RNA blot hybridization analysis was performed as described previously [9] using the cDNA insert of λMG23-12 as a probe and an RNA ladder (Gibco-BRL) as size marker.

The cDNA insert from λMG23-3 was cloned into the *Eco*RI site of the expression vector pcDNA3.1 (Invitrogen), and a polylinker sequence between *Nhe*I and *Bam*HI sites was removed to yield pcDNAMG23Δ-(2). Chinese hamster ovary (CHO) cells were transfected with the expression plasmid, fixed with acetone after 3 days and then reacted with antibody for immunofluorescence staining as in [9].

3. Results and discussion

3.1. Identification of mitsugumin23

To identify novel proteins on the skeletal muscle SR, mice were immunized with heavy SR preparations from rabbit skeletal muscle, and mAbs were prepared [9]. In the course of our screening of mAbs using immunofluorescence microscopy, mAb2595 labelled nuclear membranes, cytoplasmic regions and rows oriented transversely in the longitudinal cryosections of skeletal muscle fibers (Fig. 1A). The labelled rows were probably the triad junctions because the rows were localized at the I-A junction. Immunoblotting of microsomal proteins separated by SDS-PAGE (Fig. 1B) showed that mAb2595 specifically reacted with a single band with an apparent relative molecular mass of ∼23 kDa. Although microsomes prepared from skeletal muscle can be compartmentalized to specific membrane vesicles by sucrose density gradient centrifugation [10], the antigen protein was not enriched in a specific fraction (data not shown). These results indicate that the antigen molecules recognized by mAb2595 are distributed in the outer nuclear membrane and the SR membrane including the longitudinal and terminal regions, in skeletal muscle. No significant effects of reducing agents and *N*-glycosidase F were observed in the migration of the protein on SDS-PAGE (data not shown), suggesting that the protein is not modified with disulfide bridges and *N*-glycosylation. Since no membrane protein with a relative molecular mass of 23 kDa has been reported in skeletal muscle, we named the antigen protein mitsugumin23.

3.2. cDNA cloning and primary structure of mitsugumin23

After solubilization of microsomes prepared from rabbit skeletal muscle using Triton X-100 as a detergent, mitsugumin23 was purified to essential homogeneity by immunoaffinity chromatography using a resin coupled with mAb2595, followed by SDS-PAGE. We could not obtain any information by amino-terminal sequence analysis of the purified material (data not shown), indicating that the amino-terminal of mitsugumin23 is blocked. Therefore, the purified antigen was subjected to proteinase digestion, and the resulting peptides

Table 1
Subcellular localization of mitsugumin23 analyzed by immunofluorescence microscopy in various cell types in rabbit tissues

Tissue type: cell type			Nucleus (outer membrane)	Cytoplasm (SR/ER)
Muscle:				
	Skeletal muscle cells		++	++
	Cardiac muscle cells		++	++
	Smooth muscle cells		++	+
Brain:				
	Neuroglial cells		++	−
	Cerebral cortex:	Neurons	++	++
	Cerebellum:	Purkinje cells	−	−
		Granular cells	++	−
		Nuclear neurons	++	++
Secretory organs:				
	Small intestine:	Paneth cells	++	−
		Goblet cells	++	−
	Duodenal gland:	Mucous cells	−	−
		Cerous cells	++	++
	Pancreas:	Acinar cells	++	++
		Endocrine cells	++	+
	Parotid gland:	Serous cells	++	++
	Testis:	Leidig cells	++	++
	Thyroid gland:	Follicle cells	++	++
	Adrenal gland:	Cortical cells	++	++
		Medullary cells	++	−
Epithelium:				
	Small intestine		−	−
	Uriniferous tubules		−	−
	Mucosa		++	−
	Epidermis		++	−
	Choroid plexus		++	++
	Ductus epididymidis		++	++
Others:				
	Hepatocytes		−	−
	Lymphocytes		++	−
	Fibroblasts		++	−
	Endothelial cells		++	−
	Seminiferous epithelial cells		++	−

Regions are graded dense or moderate (++), spare (+) and undetectable (−) according to the immunofluorescence signal densiy observed.

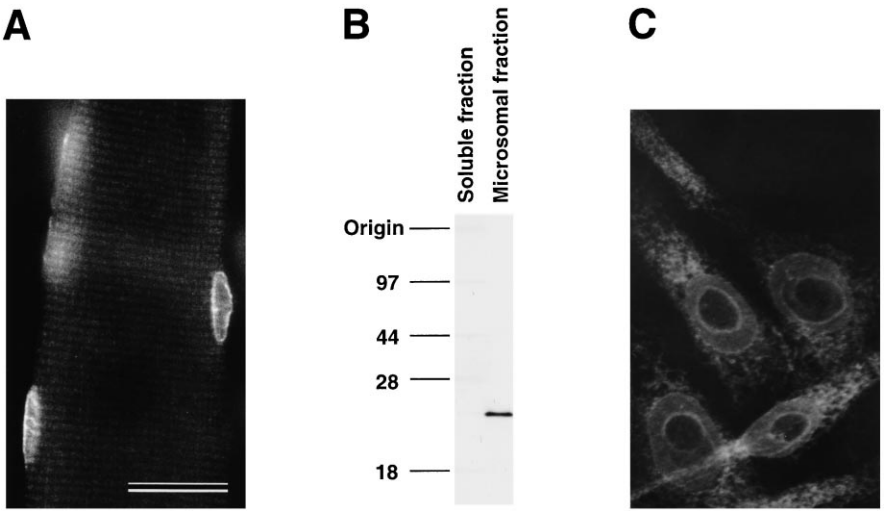

Fig. 1. Immunochemical analysis using mAb2595 against mitsugumin23. A: A longitudinal cryosection of rabbit skeletal muscle labelled with mAb2595 was imaged by fluorescence microscopy. Fluorescence signals were observed in the nuclear membrane and cytoplasmic rows corresponding most likely to triad junctions. Scale bar, 10 μm. B: Soluble and microsomal proteins prepared from rabbit skeletal muscle were separated on an SDS 4–20% polyacrylamide gel and analyzed with mAb2595. Size markers are indicated in kilodaltons. C: CHO cells expressing mitsugumin23 labelled with mAb2595 were imaged by fluorescence microscopy. CHO cells transfected with an expression plasmid for mitsugumin23 were analyzed, and immunofluorescence-positive cells were photographed. No fluorescence-positive cells were detected from the parent cells and cells transfected with a control plasmid.

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                                                5'-----GAAGTCGCACGTGAAGGGTAGCGGTGGCCAGAGCCCGACCCAGT    -1
ATGGCAGGCTCGGGCAGCAGCGCGCCATGGGGCAAGCATCTGCTCCACGCCGTCCTGATGGTGCTAGTGGCCCTCGTCCTCCTTCACTCA     90
MetAlaGlySerGlySerSerAlaProTrpGlyLysHisLeuLeuHisAlaValLeuMetValLeuValAlaLeuValLeuLeuHisSer    30
signal peptide
GCACTGGCCCAGTCCCATCGAGACTTTGCACCTCCAGGTCAGCAGAGGAGGGAGGCCCCAGTTGACCTCCTGACCCAGATAGGTCGGTCT   180
AlaLeuAlaGlnSerHisArgAspPheAlaProProGlyGlnGlnArgArgGluAlaProValAspLeuLeuThrGlnIleGlyArgSer    60

GTGCGGGAAACACTGGATACCTGGATTGGGCCAGAAACCATGCACCTGATTTCAGAGACCCTGTCTCAGGTGATGTGGGCCATCTCATCA   270
ValArgGluThrLeuAspThrTrpIleGlyProGluThrMetHisLeuIleSerGluThrLeuSerGlnValMetTrpAlaIleSerSer    90

GCCATCTCCGTGGCCTTCTTTGCCCTGTCTGGGATCGCTGCCCAGCTGCTGACTGCCCTGGGGCTCGACGGTGATCACCTCACTCAGGGC   360
AlaIleSerValAlaPhePheAlaLeuSerGlyIleAlaAlaGlnLeuLeuThrAlaLeuGlyLeuAspGlyAspHisLeuThrGlnGly   120
transmembrane segment I
CTGAAGCTCAGCCCTAGCCAGGTCCAGACCTTCCTGCTGTGGGGAGCAGGGGCCCTGGTCGTCTACTGGCTGCTGTCCCTGCTCCTCGGC   450
LeuLysLeuSerProSerGlnValGlnThrPheLeuLeuTrpGlyAlaGlyAlaLeuValValTyrTrpLeuLeuSerLeuLeuLeuGly   150
                              transmembrane segment II
TTGGTCTTGGCCGTGCTGGGGCGCATCCTGGGGGGCCTGAAGCTTGTCATCTTCCTGGCCGGCTTTGTGGCCCTGGTGAGGTCAGTGCCC   540
LeuValLeuAlaValLeuGlyArgIleLeuGlyGlyLeuLysLeuValIlePheLeuAlaGlyPheValAlaLeuValArgSerValPro   180

GATCCTTCCACCCGGGCCTTGCTCCTCCTGGCCTTGCTGACCCTGTACGCCTTGCTAAGCCGGCTCACCGGCAGCCGGGCTTCGGGGGCC   630
AspProSerThrArgAlaLeuLeuLeuLeuAlaLeuLeuThrLeuTyrAlaLeuLeuSerArgLeuThrGlySerArgAlaSerGlyAla   210
                transmembrane segment III
CAACTGGAGGCCAAGGTGCGAGGGCTGGAGCGCCAGGTGGACGAGCTGCGCTGGAGGCAGCGGCGAGCTGCCAAGGGTGCCCGGAGCGTG   720
GlnLeuGluAlaLysValArgGlyLeuGluArgGlnValAspGluLeuArgTrpArgGlnArgArgAlaAlaLysGlyAlaArgSerVal   240

GAGGAGGAATGAGACGGATGCCTCACAGCGCCACCTTCGTCATACCAAAGAGCAGAGCTGCTCCTGGCTCGCCTGCCTGCCCTCCGCCCT   810
GluGluGlu ***                                                                               243

CTGCCCCATTCTGTCTCTGAGCCCCCTGAGAAGGGAAGAGCATTCCTGTGTCTTCCGAGGTTCTCTGTCTGGGGCTGGCTCTCTCCACCC   900
CTTCTCTGCTTCCAGCCCGTCTCAGCCAGGGCAGGTTGTAGTGGCCTCCCCCTCTGGCTTCTGCATCTGCTCTCAGCTGACATCACTGCC   990
ATTAGTCTCTGGTGATTTAACCAGCTGCCCCTGCTGCTGCCTGGCTTCCTCCTTCCACTCTGGCTCCCGGGCCTTCTGCAGCTCCTCCTC  1080
TGCCAAGCCCCAGCCTGTTCCCATTGTTCTCTTCCTTCCAGCCCTGAACGTCCTGAAGGCCTGCTCCCGTCCTCCTCATTGCCCAGCTGT  1170
GGGGGAGAGGCAGAGGCCATGGGTAGAGGAAGGGGGAAACAGGCCCCCCGAGGCCTGTGGGACTCTGGGAGGACGCACAGGGAGTCTGTT  1260
ACAAGTGCCTTAATCCGAGCCAGCAGGGCCCTCTTCTGCTTGCCTGCTCCATGCTGAGTGCAGGAGAGTGCACGGGGCTGCTCTGTGCTG  1350
AGAAGAGAGCAGCCCCTCAGCGTCGCGACTCCCCCTCAGCTGCTTCCCAGGCCTGTGTGCAGGAAGGAGGCCTGAGCTCCTGCTCCGAGG  1440
GCAGTCGCTGATCAGTGCCCACCCCAGCAGCCCGGGAAGAGCAGCAGGGCCAGCCTGCTGGCTGACACCTCTCCACCAGGGCCTTGCTGT  1530
CGTTAGAGCCCACTGCTTCCTTTGAGGCTCCTGTGGAGGGGGGTGTCCTCTGCCCAGCCTGGCACGCAGAGGGAAGAAGAAATGCCCCGT  1620
TCTGACTGTTAGGGTTTGATGCGGAATCACAGCTGCAGTGATGCATATTTTTTTCAGCGCTTGGTTGGTTTTAAATAAAGTGCACGCTAT  1710
TTTATTATCTTGTTCTGAATAAAATGTATTTACTC-----3'
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Fig. 2. Nucleotide sequence of cloned cDNA encoding rabbit mitsugumin23 and the deduced amino acid sequence. Nucleotide residues are numbered in the 5′ to 3′ direction from the first residue of the ATG initiation triplet, and the preceding residues are indicated by negative numbers. Amino acid residues are numbered from the initiating methionine. Numbers of the nucleotide and amino acid residues are given at the right-hand end of the individual lines. Nucleotide 1789 is followed by a poly(dA) tract. The amino-terminal signal sequence and putative transmembrane segments are indicated by underlines. The sequence data will appear in DDBJ, EMBL and GenBank databases under accession number AB013721.

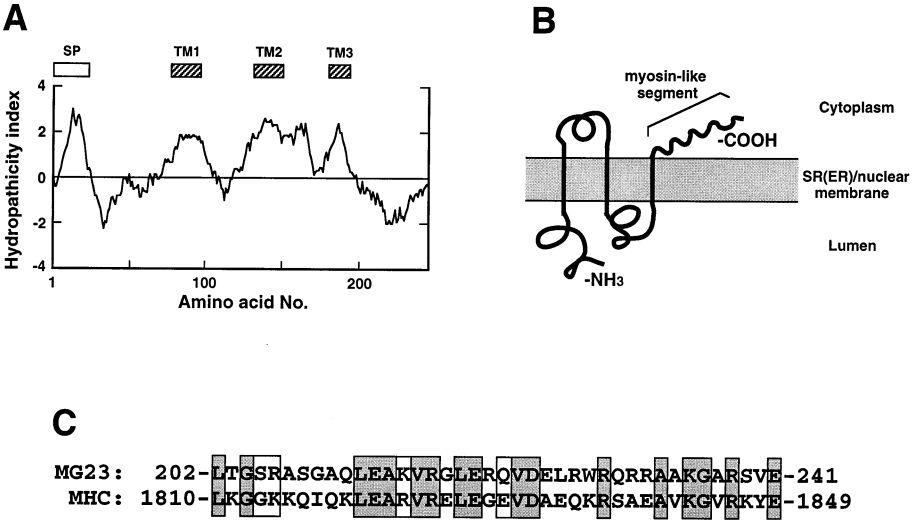

Fig. 3. Structural analysis of mitsugumin23. A: Hydropathicity profile of mitsugumin23. The averaged hydropathicity index of a nonadecapeptide is plotted against the amino acid number [17]. The signal peptide (SP) and putative transmembrane segments (TM1–TM3) are indicated. B: Proposed transmembrane topology of mitsugumin23 on the endoplasmic reticulum and outer nuclear membranes. C: Amino acid sequence similarity between the carboxy-terminal region of mitsugumin23 (MG23) and the tail region of chicken skeletal muscle myosin heavy chain (MHC). The sequence data for avian myosin heavy chain were taken from [18]. Numbers of amino acid residues (in one-letter code) at both ends are given, and identical residues between them are shadowed.

were separated by reverse-phase HPLC and sequenced. A synthetic oligodeoxyribonucleotide probe, prepared on the basis of the partial amino acid sequence, was used to screen a cDNA library derived from rabbit skeletal muscle poly(A)$^+$ RNA. To ascertain successful cloning, CHO cells were transfected with the plasmid for transient expression of the isolated cDNA. Immunofluorescence microscopic observations revealed that the transfected cells produced antigen specific to mAb2595 in the nuclear membrane and cytoplasm (Fig. 1C), whereas no fluorescence signals were detected in control CHO cells. The results demonstrate that the isolated cDNA indeed encodes the antigen protein recognized by mAb2595. Sequence analysis of the three independent clones thus isolated showed 1789 nucleotides (excluding the poly(dA) tract) of the cDNA encoding mitsugumin23 (Fig. 2). There was an open reading frame that encoded a protein of 243 amino acid residues, and all of the partial amino acid sequences determined were encoded by the reading frame. The translational initiation site was assigned to the first in-frame ATG triplet that appeared downstream of the nonsense codon TGA at residues −33 to −31. The validity of this assignment is supported by the fact that the sequence surrounding the ATG triplet agrees reasonably well with the consensus sequence for functional initiation codons in eukaryotic mRNAs [14].

Hydropathicity analysis (Fig. 3A) predicts a hydrophobic amino-terminal sequence indicative of a signal sequence [15] and three transmembrane segments in the defined primary structure of mitsugumin23. Because purified mitsugumin23 is thought to be resistant to Edman degradation (see above), a pyroglutamine residue is suggested as the amino-terminal residue of the mature molecule after cleavage by signal peptidase. Thus, a signal sequence of 33 amino acids is indicated in the primary translated molecule. The molecular masses of mitsugumin23 and its precursor were calculated to be 22.8 kDa and 26.1 kDa, respectively. In our predicted transmembrane topology of mitsugumin23 (Fig. 3B), the amino-terminal region composed of 57 amino acid residues and the loop region of 28 residues between the second and third transmembrane segments are located on the lumenal side of the SR/nuclear membranes, and the loop region composed of 18 residues between

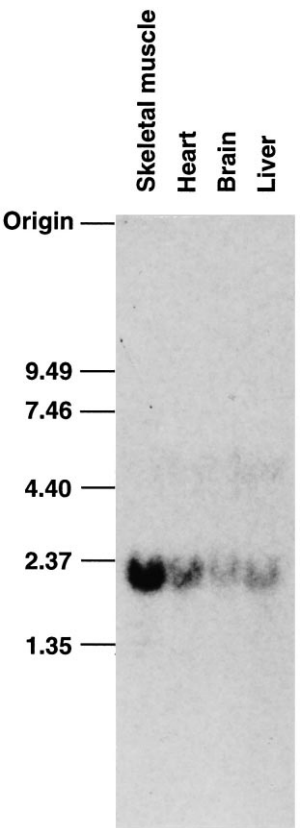

Fig. 4. Blot hybridization analysis of RNA from rabbit tissues with a probe specific for mitsugumin23 mRNA. Total RNAs (15 μg each) from skeletal muscle, heart, brain and liver were analyzed. The size markers are given in kilobases.

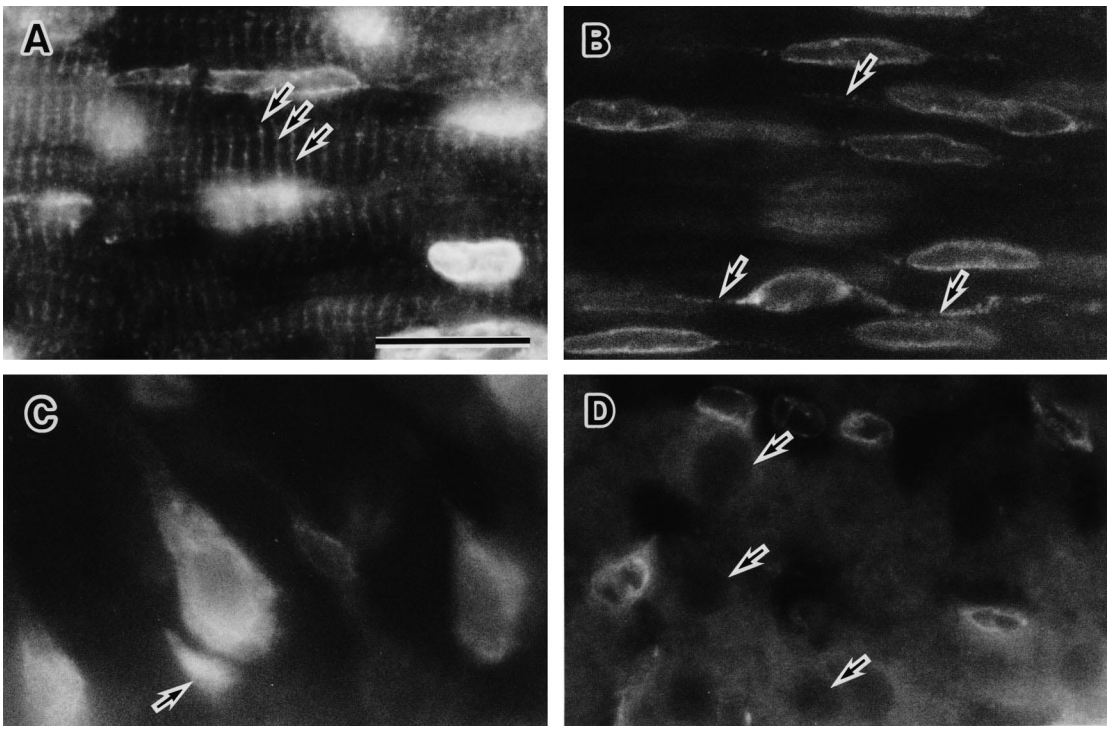

Fig. 5. Immunofluorescence analysis of rabbit tissues using mAb2595 against mitsugumin23. Cryosections of various tissues were analyzed by immunofluorescence microscopy. A: In cardiac muscle cells, fluorescence signals were observed in the nuclear membrane and cytoplasmic rows corresponding most likely to diad structures (arrows). B: In intestinal smooth muscle cells, nuclear membranes and cytoplasmic regions corresponding most likely to SR (arrows) were immunofluorescence-positive. C: In cerebral cortex, both neurons (larger cells) and glial cells (smaller cells; arrow) exhibited positive signals from the nuclei and cytoplasm. D: In liver, the nuclei of hepatic cells (arrows) were fluorescence-negative, but the smaller nuclei of endothelial cells and Kupffer cells were positive. Scale bar, 20 μm.

the first and second transmembrane segments and the carboxy-terminal tail of 43 residues are located cytoplasmically. A BLAST search of the database revealed significant sequence similarity (~43% identity) between the carboxy-terminal cytoplasmic region of mitsugumin23 and the tail portion of the myosin heavy chain (Fig. 3C).

3.3. Tissue distribution of mitsugumin23

RNA preparations from different rabbit tissues were subjected to blot hybridization analysis with a probe specific for mitsugumin23 mRNA (Fig. 4). Skeletal muscle contains a hybridizable RNA species of ~1.9 kb abundantly and hybridization signals similar in size were detected in other tissues examined. Since the results suggest ubiquitous expression of mitsugumin23, we examined various tissues with mAb2595 by immunohistochemical technique. Fluorescence microscopy demonstrated that a wide variety of cell types express mitsugumin23. The fluorescence signals were suggested to be derived from the outer nuclear and endoplasmic reticulum (ER) membrane, and we could not detect positive signals in the cell surface or inner nuclear membranes. However, no signals were detected, for example, in hepatic cells (Fig. 5D), cerebellar Purkinje cells and epithelial cells of small intestine. Furthermore, the subcellular distribution of mitsugumin23, between the nuclear and ER membranes, seems to depend on cell type. The immunofluorescence density suggests that mitsugumin23 is abundantly present in the nuclear membrane in muscle cells (Figs. 1 and 5A,B) and many other cells. By contrast, in neuronal cells from the cerebral cortex (Fig. 5C) and parotid glandular cells, the nuclear and ER membranes seem to exhibit roughly equivalent fluorescence signals. The results of our observations are summarized in Table 1.

3.4. Possible intermolecular interaction of mitsugumin23

Although the role of mitsugumin23 in the SR/ER and nuclear membranes is unknown at present, the distribution throughout various cell types suggests a housekeeping function for the protein. The carboxy-terminal cytoplasmic region of mitsugumin23 bears sequence similarity with the tail portion of the myosin heavy chain (Fig. 3). The myosin heavy chain can be divided into two functional domains: a globular head domain which provides the motor function and an elongated rod domain which constitutes the α-helically coiled-coil structure. The tail portion of myosin, showing sequence similarity with mitsugumin23, belongs to the rod domain, and previous studies have indicated that the tail portion is most likely to be responsible for myosin filament assembly [16]. It is therefore possible that the carboxy-terminal region of mitsugumin23 forms an α-helical coiled structure and is involved in intermolecular interactions or homodimer formation. As the carboxy-terminal region is assigned to the cytoplasmic side in our proposed transmembrane topology, mitsugumin23 could potentially interact with cytoplasmic protein(s).

Immunohistochemical observations indicated the existence of mitsugumin23 in the outer nuclear and ER membranes in many cell types. In CHO cells transfected with the cDNA, expressed mitsugumin23 proteins were distributed throughout the nuclear and ER membranes (Fig. 1C). However, the distribution ratio between the nuclear and ER membranes seems to depend upon the cell type (Table 1). An interesting possi-

bility is that the subcellular distribution of mitsugumin23 is switched by intermolecular interactions with cytoplasmic protein(s) expressed in a cell-type specific manner.

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