Mitsugumin29, a novel synaptophysin family member from the triad junction in skeletal muscle

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In skeletal muscle, excitation–contraction (E–C) coupling requires the conversion of the depolarization signal of the invaginated surface membrane, namely the transverse (T-) tubule, to Ca^{2+} release from the sarcoplasmic reticulum (SR). Signal transduction occurs at the junctional complex between the T-tubule and SR, designated as the triad junction, which contains two components essential for E–C coupling, namely the dihydropyridine receptor as the T-tubular voltage sensor and the ryanodine receptor as the SR Ca^{2+} -release channel. However, functional expression of the two receptors seemed to constitute neither the signal-transduction system nor the junction between the surface and intracellular membranes in cultured cells, suggesting that some as-yet-unidentified molecules participate in

both the machinery. In addition, the molecular basis of the formation of the triad junction is totally unknown. It is therefore important to examine the components localized to the triad junction. Here we report the identification using monoclonal antibody and primary structure by cDNA cloning of mitsugumin29, a novel transmembrane protein from the triad junction in skeletal muscle. This protein is homologous in amino acid sequence and shares characteristic structural features with the members of the synaptophysin family. The subcellular distribution and protein structure suggest that mitsugumin29 is involved in communication between the T-tubular and junctional SR membranes.

INTRODUCTION

Excitation–contraction (E–C) coupling represents an essential feature of muscle function. In skeletal muscle, depolarization of transverse (T-) tubules of the cell-surface membrane causes Ca^{2+} to be released from the sarcoplasmic reticulum (SR) without requiring entry of extracellular Ca^{2+} , and the Ca^{2+} released from the SR causes contraction [1]. Previous studies using mutant mice have demonstrated that the two major molecules taking part in the initial step of E–C coupling are the dihydropyridine receptor (DHPR) on the T-tubule [2] and the ryanodine receptor (RyR) on the SR [3]. DHPR can function as a voltage-gated Ca^{2+} channel, but appears to act as the voltage sensor for transmitting the electrical signal to the SR during skeletal-muscle E–C coupling [4]. RyR can release Ca^{2+} from the SR by the Ca^{2+} -induced Ca^{2+} -release (CICR) mechanism, the enhancement of the channel activity by elevation of cytosolic Ca^{2+} concentration, but the channel opening appears to be regulated directly by the T-tubular voltage sensor during physiological muscle contraction [5]. Both the proteins are therefore thought to be mechanically linked to each other at the triad junction where the T-tubule is facing the SR membrane on both sides. Indeed, electron-microscopic analysis has revealed the clusters of DHPRs and RyRs grouped in orderly arrays on the T-tubule and the junctional SR, respectively [6].

Recently, expression of DHPRs and RyRs has been demonstrated using cloned cDNAs in cultured cells. Although functional expression of both the proteins was confirmed by measure-

ments of voltage-gated Ca^{2+} currents in the surface membrane and CICR activity in the endoplasmic reticulum (ER), the cells showed neither Ca^{2+} release in response to depolarization nor close association between the surface and ER membranes like the triad junction [7,8]. On the other hand, the triad junctions are still retained in skeletal muscles from mutant mice lacking either DHPR or RyR [9,10]. The results suggest that (an) unidentified component(s) other than DHPR and RyR is essential for the formation of the triad junction and may provide a foundation for the mechanical coupling between DHPR and RyR. To understand E–C coupling at the molecular level, therefore, it seems important to examine protein components of the triad junction.

Previous biochemical studies have identified the triadin and junctin as SR transmembrane proteins in the triad junction [11,12], but their functions are not fully understood. Both the molecules possess small cytoplasmic portions, but the bulk of the proteins is thought to be located in the SR lumen, and they are proposed to interact with junctional SR proteins. Hence it is unlikely that they could interact directly with T-tubular components [11,12]. Therefore, at present, no candidate for the molecular component contributing to the structural characteristics of the triad junction in skeletal muscle has been identified.

In the present study, using the monoclonal antibody (mAb) technique, we have identified a protein with the relative molecular mass of 29 kDa, named mitsugumin29, which is localized to the triad junction. The primary structure deduced by cDNA cloning revealed that mitsugumin29 is a novel member of the synaptophysin family, and a cDNA expression study suggested that

Abbreviations used: CHO, Chinese-hamster ovary; CICR, Ca^{2+} -induced Ca^{2+} release; DHPR, dihydropyridine receptor; E–C coupling, excitation–contraction coupling; ER, endoplasmic reticulum; mAb, monoclonal antibody; poly(A)⁺, polyadenylated; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

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The sequence data reported have been submitted to the EMBL, GenBank[®] and DDBJ Nucleotide Sequence Databases under the accession number AB004816.

mitsugumin29 is a junctional SR transmembrane protein of the triad junction. We also discuss the possibility that mitsugumin29 may be involved in specific communication between the T-tubular and junctional SR membranes.

EXPERIMENTAL

Monoclonal antibody production and immunochemical analysis

Membranes of total microsome, T-tubule, light SR and heavy SR were prepared from adult rabbit skeletal muscle as reported previously [13]. BALB/c mice were immunized with the heavy SR preparations containing enriched triads, and hybridoma cells were produced by fusion of the spleen cells with NS-1 cells [14]. The hybridoma supernatants obtained were allowed to react with transverse cryosections of rabbit skeletal muscle fixed with acetone and then the labelled antibodies were detected with the affinity-purified secondary antibody conjugated with FITC (Dako). Fluorescence-microscopic observation was performed using a photo microscope (IX-FLA; Olympus) and hybridomas were selected according to their staining patterns. For ultrastructural analysis by an electron microscopy (JEM-200CX; JEOL), after reaction with mAb the cryosections were labelled with anti-mouse immunoglobulins/colloidal gold (Zymed Laboratories Inc., San Francisco, CA, U.S.A.) and then fixed and stained as described previously [3]. Immunoblot analysis was performed as described previously [15] using prestained markers (Gibco-BRL) as size standards. For large-scale preparation of mAb1007, 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).

Purification and amino acid sequence analysis of mitsugumin29

Isolated microsomes were solubilized at the protein concentration of 0.5 mg/ml with 1% Triton X-100 in a buffer containing 50 mM Tris/HCl, pH 7.4, and proteinase inhibitors. The solubilized proteins were allowed to react with mAb1007 coupled to *N*-hydroxysuccinimide-activated Sepharose (Pharmacia Biotech) and, after extensive washing, 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/PAGE and transferred to a Problot membrane (Applied Biosystems). Fragments of the membrane containing mitsugumin29 were treated with lysyl endopeptidase and trypsin, and the digested peptides were separated by reverse-phase HPLC and sequenced [16]. The amino acid sequences of the peptides were identical with sequences deduced from the nucleotide sequence of the cDNA (see Figure 3 below for amino acid numbers and sequences); peptide T1A corresponds to residues 22–27, T1B to residues 236–240, T5 to residues 72–85 and T13 to residues 86–99.

cDNA cloning and RNA blot hybridization

Double-stranded cDNAs were prepared with rabbit skeletal-muscle polyadenylated [poly(A)⁺] RNA as template and were cloned into λ Zap II cloning vector (Stratagene) as described previously [16]. The resulting cDNA library was screened with the 5'-³²P-labelled probe (24 species-mixed 17-mer: 5'-AT(AC)-GA(AG)TA(CT)GA(AG)ATGCC-3') synthesized on the basis of the sequence from the peptide T5. Of several clones hybridized with the probe, the following three clones carried the full-length protein coding sequence and were used for sequence analysis [17] by an automatic sequencer (ALF Express; Pharmacia Biotech); λ MG29-1 (carrying nucleotides: -207 to 3282); λ MG29-2

(-202 to 3277); λ MG29-5 (-202 to 1893). RNA blot hybridization analysis was performed as described previously [15] using the cDNA insert of λ MG29-2 as a probe and an RNA ladder (Gibco-BRL) as size marker.

cDNA expression in cultured cells

The cDNA fragment corresponding to the residues -153 to 3277 derived from λ MG29-2 was attached with the *Hind*III linkers and cloned into an expression vector pKCRH2 in which the inserted cDNA is transcribed under the control of the simian-virus-40 promoter [18]. Chinese-hamster ovary (CHO) cells were transfected with the expression plasmid by the calcium phosphate method [19], and after 3–4 days were fixed with acetone and then allowed to react with antibody for immunofluorescence staining.

RESULTS AND DISCUSSION

Identification of mitsugumin29 unique to the triad junction

To identify novel proteins unique to the triad junction, mice were immunized with heavy-SR vesicles containing enriched junctional membranes from rabbit skeletal muscle, and mAbs were prepared. Of approx. 500 hybridoma supernatants screened by immunofluorescence observation, mAb1007 labelled rows oriented transversely in the longitudinal cryosections of skeletal muscle

Figure 1 Immunocytochemical analysis, with mAb1007, of rabbit skeletal muscle.

A longitudinal cryosection of rabbit skeletal muscle labelled by immunofluorescence using mAb1007 was imaged by fluorescence microscopy (A) and corresponding Nomarski differential-interference-contrast microscopy (B). (C) An electron micrograph of the longitudinal section labelled with mAb1007 by the immuno-colloidal-gold procedure. T, T-tubule; Tc, terminal cisternae of the SR; Z, Z-line. Scale bars represent 5 μ m in (A) and (B) and 0.5 μ m in (C).

Figure 2 Immunoblot staining of membrane preparations from rabbit skeletal muscle with mAb1007

(A) Preparations of total microsomes (lane 1), T-tubules (lane 2), light SR (lane 3) and heavy SR containing enriched junctional SR (lane 4) were analysed (5 μ g protein each) on a 4–20% polyacrylamide gel. (B) Heavy-SR preparation (5 μ g protein) was denatured in the presence of β -mercaptoethanol (lane 1) or *N*-ethylmaleimide (lane 2) and was analysed on a 12.5% polyacrylamide gel. Size markers are indicated in kDa.

fibres. The location of the stained rows is most likely to be the interphases between the A- and I-bands as observed by viewing the same field by differential-interference-contrast microscopy (Figures 1A and 1B). Since the triad junction is localized at the A/I junction, the antigen protein is thought to be localized to the triad junction. Moreover, the staining pattern with mAb1007 was indistinguishable from those with antibodies to the RyRs and DHPRs (results not shown). To confirm the subcellular distribution of the antigen molecule, we then performed electron-microscopic analysis using the immuno-colloidal-gold procedure (Figure 1C). The gold particles decorated the triad junction specifically in frequency (Figure 1C); on examination of 26 sections, the 82, 86 and 87 gold particles out of a total 245 particles associated with the junctional gaps, T-tubular and SR membranes adjacent to the junctional gaps respectively, and negligible numbers of gold particles labelled other parts randomly. The results obtained clearly indicate that the antigen for mAb1007 is a molecule unique to the triad junction.

Immunoblotting of microsomal proteins separated by PAGE showed that mAb1007 specifically reacts with a single band with an apparent molecular mass of 29 kDa (Figure 2A). The content of the antigen protein was roughly estimated from the Coomassie Blue-staining intensity to be 20–100 pmol/mg of protein in microsomes, and this value is comparable with that of monomeric RyR and is higher than that of DHPR. Microsomes prepared from skeletal muscle can be compartmentalized to specific membrane vesicles by the sucrose-density-gradient centrifugation [13]; DHPRs and RyRs were enriched in the T-tubular and heavy SR fractions respectively (results not shown). The antigen proteins were barely enriched in T-tubular preparations, but were distributed through the membrane preparations. Since this result suggests that the protein is not localized to a specific membrane structure, experimental data from immunomicroscopy and immunoblotting may be conflicting. Although the reason has not been clarified yet, we speculate that the antigen proteins

are localized specifically on the junctional SR membrane in intact muscle but are distributed between T-tubular and SR membranes during the process of homogenization of muscle for the membrane preparations due to their unique structural characteristics (see below). The mobility of the protein under reducing conditions was lower than that of the non-reducing form on SDS/PAGE (Figure 2B), suggesting the presence of intramolecular disulphide bridge(s). On the other hand, the absence of notable effects of N-glycosidase F treatment on migration (results not shown) suggested that the protein is not modified by N-glycosylation.

We have named the antigen protein mitsugumin29; mitsugumi means triad junction in Japanese. A T-tubular protein called TS28, with a molecular mass similar to that of mitsugumin29, has been identified previously [20]. Although mitsugumin29 and TS28 are thought to differ in localization, as determined by the immunoblot analysis using the membrane preparations, we cannot exclude the possibility that they are in fact identical.

cDNA cloning and primary structure of mitsugumin29

After solubilization of microsomes prepared from rabbit skeletal muscle using Triton X-100 as a detergent, mitsugumin29 was purified to essential homogeneity by immunoaffinity chromatography using a resin coupled with mAb1007, followed by SDS/PAGE. We could not obtain any information by N-terminal sequence analysis of the purified material, an observation indicating that the N-terminus of mitsugumin29 is modified covalently. The preparation was therefore subjected to proteinase digestion, and the resulting peptides were then separated by reverse-phase HPLC and sequenced. A synthetic oligodeoxy-ribonucleotide probe, prepared on the basis of the partial amino acid sequence (see the Experimental section), was used to screen a cDNA library derived from rabbit skeletal-muscle poly(A)⁺ RNA, thus allowing several cDNA clones for mitsugumin29. Sequence analysis of three independent clones showed 3489 nucleotides [excluding the poly(dA) tract] of the cDNA encoding mitsugumin29 (Figure 3). Taking into account the poly(A) tail and the mRNA size (see below), the cloned cDNA is thought to carry the full-length sequence corresponding to mitsugumin29 mRNA. The sequence surrounding the translation initiation codon (CCAGCATGT), which is assigned by comparing the cDNA sequences from rabbit and mouse skeletal muscle (H. Takeshima, M. Shimuta and M. Nishi, unpublished work), agrees reasonably well with the eukaryotic consensus sequence [21]. It is noteworthy that the 5'-non-coding sequence contains a putative enhancer motif CCCCAGCC found in the 5'-flanking or 5'-non-coding regions of several muscle-specific transcripts [16]. There is an open reading frame that encodes a protein of 264 amino acid residues and the partial amino acid sequences determined are encoded by the reading frame. The calculated molecular mass of mitsugumin29 from rabbit skeletal muscle was 29 311 Da (including the initiating methionine) and the theoretical pI was 5.72.

Hydropathy analysis predicts four hydrophobic putative trans-membrane segments and no N-terminal signal sequence in the mitsugumin29 molecule. A BLAST search of the database revealed remarkable amino acid sequence similarities between mitsugumin29 and the proteins of the synaptophysin family (Figure 4), and mitsugumin29 appears to be a novel member of the family. Sequence comparison reveals 45, 46, 38 or 22% identities between mitsugumin29 and synaptophysin, synaptoporin, pantophysin or synaptogyrin pairs, respectively. Previous topological studies indicate that synaptophysin spans the membrane four times and that both its N- and C-termini are located cytoplasmically [22]. The remarkable similarities in the structural

5'-----GCGGGGAGAGCAGTGGCCGAGCCCG -181
 GCGGAGCGGAGCAGCAGCGCCCCCGGACCCATCCAGACAGCCTGGATCCCGGCCCGGACGGCCACCCAGCTCGCTTCCCCAGCCG -91
 CCGGCCAGCAGCCCCAACCCACCCGCGGCCGAGCCCCGCGGAGGGAGCCGAGCCCTACTCTGTGCCACACCCACGGCTCCAGC -1
 ATGTCTCGACGGAGAGCCCCAGTCGCGCGGCGGACAAGTCGCGCGCCAGCAGGTGGACCGCTGTCTGAGGGGTTCGCTGGCGGCGG 90
 MetSerSerThrGluSerProSerArgAlaAlaAspLysSerProArgGlnGlnValAspArgLeuLeuGluGlyLeuArgTrpArgArg 30
 CTGGAGGAGCCGCTGGGCTTCATCAAAGTCTCCAGTGGCTCTTTGCTATTTTCGCTTCCGGTCTCGGTTCTACAGCGGGGAGACA 180
 LeuGluGluProLeuGlyPheIleLysValLeuGlnTrpLeuPheAlaIlePheAlaPheGlySerCysGlySerTyrSerGlyGluThr 60
 GGAGCAATGGTTCGCTGCAACAACGAAGCAAGGATGTGAGCTCCATCATTTGTTTGTTCGGCTATCCCTTCAGGTTCACCGGATTGAG 270
 GlyAlaMetValArgCysAsnAsnGluAlaLysAspValSerSerIleIleValLeuPheGlyTyrProPheArgLeuHisArgIleGlu 90
 TATGAGATGCCTCTCTGTGACGACGACTCGTCTCCAGACCATGCACCTATGGGGGACTTCTCCGCCCGGCTGAGTTCTTCGTGACC 360
 TyrGluMetProLeuCysAspAspSerSerSerLysThrMetHisLeuMetGlyAspPheSerAlaProAlaGluPheValThr 120
 CTCGGCATCTTCTCTCTTCTATACCATGGCTGCACTCGTCTGTCTACCTGCGTTTCCACAAGCTCTACACAGAGAACAAGCGCTTCCCG 450
 LeuGlyIlePheSerPhePheTyrThrMetAlaAlaLeuValValTyrLeuArgPheHisLysLeuTyrThrGluAsnLysArgPhePro 150
 CTGGTGGACTTCTGTGTGACTGTCTCTTACCTTCTTCTGGCTGGTAGCTGCAGCTGCCTGGGGCAAAGGCTGACCGACGTCAAGGGG 540
 LeuValAspPheCysValThrValSerPheThrPhePheTrpLeuValAlaAlaAlaAlaTrpGlyLysGlyLeuThrAspValLysGly 180
 GCCACGCGCCATCCAGCCTGACGGCAGCCATGTCCGTGTGCCATGGAGAGGAAGCTGTGTGCAGTCCCGGGGCCACACCTTCCATGGGA 630
 AlaThrArgProSerSerLeuThrAlaAlaMetSerValCysHisGlyGluGluAlaValCysSerAlaGlyAlaThrProSerMetGly 210
 CTGGCAACATCTCTGTGCTCTTCGGCTTTATCAACTTCTTCTGTGGGCGGGAAGTGTGGTTTGTGTTCAAGGAGACCCATGGCAT 720
 LeuAlaAsnIleSerValLeuPheGlyPheIleAsnPhePheLeuTrpAlaGlyAsnCysTrpPheValPheLysGluThrProTrpHis 240
 GGACAGGGCCAGGACCAGGGCCAGGGCCCCAGCCAGGAAAGCGCAGCCGAGCAGGGAGCAGTGGAGAAGCAGTAAGCAACCCCCACCTGG 810
 GlyGlnGlyGlnAspGlnGlyGlnGlyProSerGlnGluSerAlaAlaGluGlnGlyAlaValGluLysGln*** 264
 CTACTCCGAACCTGGACAGCACCTCTTACCACCTCCGGCTTCCAGGACCTCCCTCTTCTCTCTCCACTTCCCTTCCCCATCAGTC 900
 TGGGATGGGCAGGCATCAGCTGGTGAAACCTGAGCAGCCTTCCCATGGCTTCCATATCCCTTCTCTCGCTGGAGCCCCAGATGGCAGGAG 990
 CTCAGTGCTTCTTGTGAATGGCCTGGATCCAGGTATCTTACCTGGGTCTCACCTGGACCTCATAGCCTCAGTCTACCAGCTACTGTCTG 1080
 AGGTGAGGGTTGGGGCAGGAAACAGAGGCCCGGAGACTGGCTCAGCAGCTCTTCTGTCTTTTCAAATGTAGTGGGGGAGGGGAGCAG 1170
 CTGGGACGCTTCCCCCTGTCCCTGTGGCCAGGTGGCCACTGTGGTTGGGTGGGCCCCCTCACAGGCAGCCATCTGGTCTCTGTGCTG 1260
 ATCCCTAGTCAGCCAGAGCTCAGCGGCTTCCACTCTGTCTCTGTCTGTGCAGAGTCCACCCATGTGGACAGGGAGGAGTCTGG 1350
 CCTAGCAAACAGCCCTTTCCAGGGTCTGATGAGGTCTCGCTCAGCTGGGATCAGGTAATTTGCAACCTGAAGTAAAGCCAAGAAAAT 1440
 GCTGGCAGAATCAGCTTCTTGGAGCCCTCAGCCCCCGCCCCCTAGGCTGGGGCACTTCCACCAACACTGTGAGCTATACTACTCTCTGG 1530
 AAATCCGTCCTTCCCCCTTACCCAGTCTTCCCTCTTGGGCTGTATCACACCTGTGACACTAGGTGAGGGGGCTGGGAGCAGCCTCACTT 1620
 CAGTGGCCCCACACCCACTACCTCCCCCTCCCCATCTTCTCCCTTCCCCAGCCTCTTGGATGTGAGGCATTTGAGATCTCTCTGAAGA 1710
 CGGTTGGGGCTTCCCTTTCATTCTCCCTGCGGGGCCAGAAGGGGGCTTTTGCAAGGCCAAAGGACCATCAGGAGGCCAAGTTGCCCCAGAGC 1800
 CCCAGGACGTGGATGTGTGGGCCACTTCTTCTCTCTCTGCCCCCTGCTCTTCCACGACTCTTCTCATCTTTACCAAAGGATGCGT 1890
 CCTGTCCCTTGGCCCTCCCATTCGCCCCCTTCTCTCTTCTTCTCCAGTCCGCTTCCCTTCCAGTCCAGGGCAGGCCGATGCTATTTGGT 1980
 GCTTCTTCACTTTCGGGACCCAGCTCCGTATTTGTCTTCTCAGTGGCTCTCTCTTCTTCCAGTCCCTTCCAGTCCCTTCTGAGCCCCCAAGCT 2070
 CTGGGAGCCGGGGCTGGCTGGGTGAGGGCCATCTGCCTCCCACCCAGCTCAAAATGCTCCATCCGACTTGGCCACAGGGCTGCTGGCAGA 2160
 AAGATGAAAAGATCCAGCCATTTTCCAATCCCATGCCAATTTGGCCATTTAGTATCCCAAAGGTGAATGTGCCCTGTGCCAGTCTTTCC 2250
 TCAGGGCGCAGCCAACCCCTTCCAGCTTCCCTCCCTCCCTGGATGCCATCCACAGTAACATGTGCATTAAGTGGGTACCCAGAGGTCTGGC 2340
 CCACCAGCTCTCTTTGGATCAGAGTTTGGGTCCGGGGGAATGTGAGATTTACACCCCAAGGCTCCGGAGTCCCTTGGTGAAGAAT 2430
 AGGATGTTGGTTTGTGTGTTTGGTGTGGGGAAGGGGTCTGAGGAGCAGAAAGGCGATGAGGTGAGAATATTTCTGAAACTCACTCTCTC 2520
 TGTCCGTCTGAGCTGTCCCATGTGTTACTCAAGGACAAGGGGGGACGGTTTGGCAACAGCTCCATAGAACAAAGGGGTGACCTTCACT 2610
 TTCTTTTCAAGCCAGCCCCGCGGGTGGTCCGTGAGCAGCTTCTGACGAATTTGTTTGGATTTCTGTCTGTATTTGTAAATTTATTTGAA 2700
 ATATGATGGATAAAGTAGTCTCAATCGGGGGCCACGGTTAGCAGCTATAGTCCCTTCAACTAGGGAAGTGGGGGGCGGGGATCTGCTCC 2790
 TGCCGAGGACCCAGCCAGGCCCTCTCGCCGAGCACAGAAGCCTCTCTGCTTCTGCGCGTGTCTCAGCTGGGAGCCCTCCATTCTGGCT 2880
 GTCTCCCTCTCCACAGCACCTGGGAGGAAAGGCCACTTACCTCATACCTCCAGAGGGCTGGGCTGTCTCACAGGACTTCTGTCCCC 2970
 TCCAACAGGGCTGGCATTTGCCATTTTGCCTAGTGGGGCTGAGGTAGGAGACGGTGTCTGGTTTCTCCAGTGTCTGAGGAGGCCAGGC 3060
 AGGGCACTTGTCTGTTTGCCTAGTGAACCCTGACCCCTGCCAGGAGCCACCCAGGACCTCTCTGGCCACAGATTTCCCTCCAACGTCATG 3150
 GTCATCGGTCTCTACGTGAGGATGTCAGATGGGGTGGGGGGAGAATGTGCATGTGCTTTTCTGGTGCTTGTATTTACACATTTGAAT 3240
AAACAGTGTCTGCGAGCATTTGCCACAGAGGAGCCGAGACTGC-----3' 3282

Figure 3 Nucleotide sequence of cloned cDNA encoding the rabbit mitsugumin29 and the deduced amino acid sequence

Nucleotide residues are numbered in the 5' → 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. Sequences of the CCCAGCC motif in the 5'-non-coding region and the polyadenylation signal in the 3'-non-coding region are underlined. Nucleotide 3283 is followed by a poly(dA) tract.

features indicate that the common transmembrane topology is shared by the family members.

Tissue distribution and cDNA expression of mitsugumin29

RNA preparations from different rabbit tissues were subjected to blot hybridization analysis with a probe specific for mitsugumin29 mRNA (Figure 5). Skeletal muscle contains a hybrid-

izable RNA species of 3.7 kb, but no signal was detected in other tissues examined. This result suggests that mitsugumin29 is a skeletal-muscle-specific product.

To analyse the isolated cDNA further, CHO cells were transfected with the plasmid for transient expression of mitsugumin29. Immunofluorescence observation revealed that the transfected cells produced specific antigen to mAb1007 and that the antigen molecules were distributed in the peripheral cyto-

channel activity upon reconstitution into planar lipid bilayers [28]. Other synaptophysin family members are also proposed to participate in the secretion system in a variety of cell types. Moreover, in conjunction with gap-junction proteins, the synaptophysin/connexin superfamily has been proposed, on the basis of similarities in the transmembrane topology, oligomeric structure and channel activity [29]. These proteins seem to share the characteristic of involvement in connecting two different membranes. It may therefore be likely that mitsugumin29 forms a homo-oligomer and serves as an essential component for communication between junctional SR and T-tubular membranes by interacting with unknown T-tubular and/or junctional SR proteins. However, the possibility that mitsugumin29 takes part in vesicle fusion to supply membrane proteins and lipids from the junctional SR to T-tubule may be remote, since recent studies have implicated caveolae as the best candidate for this role [30]. It is seems also unlikely that mitsugumin29 participates in exocytic transport, because the triad junction is thought to be a specialized structure for E-C coupling and secretion from triad junctions has not been recognized yet. A more plausible assumption is that mitsugumin29 may support the close association between the SR and T-tubular membranes. Nevertheless, to examine the role of mitsugumin29 in the triad junction, production of knockout mice will be required.

Figure 5 Blot hybridization analysis of RNA from rabbit tissues with a probe specific for the mitsugumin29 mRNA

Total RNA from skeletal muscle (2.5 µg, lane 1), heart (20 µg, lane 2), brain (20 µg, lane 3) and liver (20 µg, lane 4) were analysed. The size markers are given in kb.

Figure 6 Immunofluorescence microscopy of CHO cells expressing mitsugumin29

CHO cells transfected with an expression plasmid for mitsugumin29 were stained with mAb1007, and immunofluorescence-positive cells were photographed. No fluorescence-positive cells were detected from the parent cells and cells transfected with a control plasmid.

Of the proteins specific to the junctional SR in skeletal muscle identified so far, junctin and triadin show high sequence identities with aspartyl β -hydroxylase, which modifies secretory proteins in secretory or related intracellular vesicles [12]. Mitsugumin29 is the third member of a family of components shared by the junctional SR and secretory vesicles. These observations might

imply a possible relationship between junctional SR and secretory vesicles at a molecular level.

Note added in proof (received 12 February 1998)

Expression of mitsugumin29 in the kidney was found in our current experiments (M. Shimuta and H. Takeshima, unpublished work).

We thank Izumi Dobashi for help in some experiments. This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture, the Life Science Foundation, the TMFC and the Mitsubishi Foundation.

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