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Mitsugumin 56 (hedgehog acyltransferase-like) is a sarcoplasmic reticulum-resident protein essential for postnatal muscle maturation

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ABSTRACT

Mitsugumin 56 (MG56), also known as the membrane-bound O-acyl-transferase family member hedgehog acyltransferase-like, was identified as a new sarcoplasmic reticulum component in striated muscle. *Mg56*-knockout mice grew normally for a week after birth, but shortly thereafter exhibited a suckling defect and died under starvation conditions. In the knockout skeletal muscle, regular contractile features were largely preserved, but sarcoplasmic reticulum elements swelled and further developed enormous vacuoles. In parallel, the unfolded protein response was severely activated in the knockout muscle, and presumably disrupted muscle development leading to the suckling failure. Therefore, MG56 seems essential for postnatal skeletal muscle maturation.

1. Introduction

The sarcoplasmic reticulum (SR) is the powerful Ca^{2+}-handling organelle of muscle cells, and evolutionarily represents a highly specialized form of the endoplasmic reticulum (ER). During contractions of striated muscle, the activation of dihydropyridine receptors/L-type Ca^{2+} channels (DHPRs) in the transverse (T-) tubule opens ryanodine receptor channels (RyRs) to trigger SR Ca^{2+} release [1]. Such functional coupling between the T-tubular and SR Ca^{2+} channels takes place in junctional membrane complexes formed by junctophilins (JPs) *i.e.* the triad in skeletal muscle and the diad in cardiac muscle [2–5]. The SR region closely associated with the T-tubule is called the junctional SR or the terminal cisternae, and contains abundant JPs and RyRs to control Ca^{2+} release. The rest of the SR portion, called the longitudinal SR, is responsible for Ca^{2+} uptake mediated by enriched SR/ER Ca^{2+}-ATPase (SERCA). The major SR Ca^{2+}-handling proteins, including RyRs, SERCA and luminal Ca^{2+}-binding proteins, have been extensively characterized, and such studies have deepened our understanding of intracellular Ca^{2+} stores. However, there are still many SR components with no functional annotation, and it is also important to examine such as-yet-unknown proteins in striated

Abbreviations: EDL, extensor digitorum longus; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; DHPR, dihydropyridine receptor; HHATL, hedgehog acyltransferase-like; IP_3R, inositol trisphosphate receptor; JP, junctophilin; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MBOAT, membrane-bound O-acyltransferase; MG56, mitsugumin 56; P, postnatal day; RyR, ryanodine receptor; SERCA, SR/ER Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TA, tibialis anterior; T-tubule, transverse tubule; UPR, unfolded protein response

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muscle. In this paper, we report the identification of a new SR protein, designated mitugumin 56 (MG56)/hedgehog acyltransferase-like protein (HHATL), and describe its essential role in the integrity of the skeletal muscle SR.

2. Materials and methods

2.1. Biochemical and immunochemical analyses

All animal experiments were conducted with the approval of the Animal Research Committee at Kyoto University according to the regulations for animal experimentation. In the course of screening new proteins in rabbit muscle microsomes [4–8], we identified MG56 as a transmembrane protein localized in the SR. Rabbit MG56 was enriched in the heavy SR fraction from skeletal muscle, and further purified with SDS–PAGE as described previously [4]. Purified MG56 transferred onto nylon membranes (ProBlot, Applied Biosystems) was analyzed using an automated Edman sequencer (PPSQ-31, Shimadzu, Japan). A cDNA fragment was amplified with PCR primers designed based on the mouse *Hhatl* sequence data (Accession number, NM_029095). It was used as a hybridization probe for Northern blotting in adult C57BL mouse tissues and for library screening to clone the full-length mouse *Mg56*/*Hhatl* cDNA.

Immunochemical analyses were performed essentially as described previously [4], except that ×2 sampling buffer for SDS–PAGE contained 2% SDS, 8 M urea, 10% 2-mercaptoethanol, 0.01% bromphenol blue and 40 mM Tris–HCl (pH 6.8) in this study. To prepare monoclonal antibody against mouse MG56, a synthetic peptide containing the C-terminal 21 residues was conjugated with keyhole limpet hemocyanin and injected with adjuvant into the foot pads of Wister rats. Popliteal lymph node cells were prepared from the rats and fused with NS-1 cells to yield hybridoma clones producing antibody specific to MG56. Hybridoma supernatant was used for immunochemical analysis. Several commercial and in-house antibodies were also used in this study; anti-RyR (Thermo Scientific, MA3-925), DHPR (Sigma–Aldrich, D218), MG53 [7], JPs [4], SERCA (Thermo Scientific, MA3-911), triadin (Thermo Scientific, MA3-927), TRIC channels [8], Bip/GRP78 (BD Transduction Laboratories, 610979), GRP94 (Medical & Biological Laboratories, M181-3), eukaryotic initiation factor 2 (Cell Signaling, 9721 and Santa Cruz, sc-11386), calumin [6], calsequestrin (Thermo Scientific, PA1-913) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma–Aldrich, G9545).

2.2. Generation of knockout mice

The generation of *Mg56*-knockout mice was carried out as previously described [9]. For the construction of the targeting vector (Fig. 3A), *Hhatl* genomic DNA fragments were amplified from C57BL/6 mouse genetic DNA and used for the preparation of the short and long-arm regions. The linearized vector was transfected into embryonic stem RENKA cells derived from C57BL/6 mice [10] and several clones carrying the expected homologous mutation were selected by PCR and Southern blot analysis. Chimeric mice generated with the positive clone were crossed with C57BL/6 mice and transmitted the mutant gene to their pups. To determine the mouse genotypes, PCR analysis was conducted using Hhatl-1 (GAGTGGACCAGTCTCCTCAGAG) and Hhatl-2 (CTGTCACCGAGGCA GCTGGCAC) primers.

Histological and ultrastructural analyses were carried out as described previously [11]. Briefly, mouse tissues were fixed in 3% paraformaldehyde, 2.5% glutaraldehyde and 0.1 M sodium cacodylate (pH 7.4). After the tissues were dehydrated and embedded in Epon, ultrathin sections (~80-nm thickness) were prepared and stained with toluidine blue for histological observation or uranyl acetate and lead citrate for ultrastructural analysis (JEM-200CX, JEOL). Mouse tissues were also fixed with phosphate buffered saline containing 3% paraformaldehyde and embedded in Tissue-Tek OCT compound (Sakura Finetek, Japan) for histological section preparations.

2.3. Muscle contraction measurements

Extensor digitorum longus (EDL) muscle bundles were dissected from mouse hindlimbs and subjected to isometric tension measurements as described previously [12]. The muscle preparation was mounted on a force transducer in a chamber containing modified Krebs–Ringer solution (121.9 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 15.5 mM $NaHCO_3$ and 11.5 mM glucose) constantly bubbled with 95% O_2 and 5% CO_2 at 24 °C. To induce contraction, field stimulation (10 ms duration) with supramaximal voltage was given at various frequencies for 10 s, and the developed force was recorded online using LabChart 7 software (AD Instruments). After each experiment, the muscle bundle was fixed with a 4% paraformaldehyde-containing saline for microscopic observation; cross sectional area was determined by confocal microscopy using the accompanying imaging software (FV-1000, Olympus).

2.4. Cardiac function measurements

The echocardiogram was performed using a 30-MHz microprobe (Vevo 2100, Visual Sonics) as described previously [13]. M-mode images of the interventricular septum were recorded to measure left ventricular chamber dimensions. The electrocardiogram was performed using a radiofrequency transmitter device (ETA-F20, Data Science International) as essentially described previously [14]. Transmitter leads were attached to mouse body surfaces at clavicular and pelvic regions, and signals were recorded at 4 kHz and analyzed using the LabChart 7 software.

2.5. Gene expression analysis

Total RNA samples were prepared from mouse tissues using a commercial kit (Isogen, Nippon Gene, Japan). The RNA preparations from tibialis anterior (TA) muscle and hearts were subjected to in vitro transcription and analyzed using the GeneChip Mouse Genome 430 2.0 (Affymetrix) according to the manufacture's instructions; the data obtained have been deposited in the NCBI-GEO database under accession number GSE64868. To analyze the detailed expression of ER stress-related genes, mRNA contents were examined by quantitative RT-PCR as described previously [14]. To analyze the tissue contents of ER stress-related proteins, mouse TA muscle was homogenized with a Physcotron (Microtec, Japan) in a buffer (150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 8.0) containing phosphatase inhibitors (10 mM Na pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 1 mM Na orthovanadate and 1 mM EDTA) and a proteinase inhibitor cocktail. The homogenate was centrifuged at 8000×*g* for 10 min, and the resulting supernatant was analyzed by Western blotting.

2.6. Membrane lipid analysis

Membrane phospholipids were analyzed by the liquid chromatography–tandem mass spectrometry (LC–MS/MS) system consisted with a NANOSPACE SI-2 HPLC (Shiseido, Japan) and a TSQ Quantum Ultra (Thermo Fisher Scientific) triple quadropole mass spectrometer equipped with a heated electrospray ionization source as described previously [15]. To examine lysophospholipids,