Mitsugumin 53 attenuates the activity of sarcoplasmic reticulum Ca^{2+}-ATPase 1a (SERCA1a) in skeletal muscle

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ABSTRACT

Mitsugumin 53 (MG53) is a member of the membrane repair system in skeletal muscle. However, the roles of MG53 in the unique functions of skeletal muscle have not been addressed, although it is known that MG53 is expressed only in skeletal and cardiac muscle. In the present study, MG53-binding proteins were examined along with proteins that mediate skeletal muscle contraction and relaxation using the binding assays of various MG53 domains and quadrupole time-of-flight mass spectrometry. MG53 binds to sarcoplasmic reticulum Ca^{2+}-ATPase 1a (SERCA1a) via its tripartite motif (TRIM) and PRY domains. The binding was confirmed in rabbit skeletal muscle and mouse primary skeletal myotubes by co-immuno-precipitation and immunocytochemistry. MG53 knockdown in mouse primary skeletal myotubes increased Ca^{2+}-uptake through SERCA1a (more than 35%) at micromolar Ca^{2+} but not at nanomolar Ca^{2+}, suggesting that MG53 attenuates SERCA1a activity possibly during skeletal muscle contraction or relaxation but not during the resting state of skeletal muscle. Therefore MG53 could be a new candidate for the diagnosis and treatment of patients with Brody syndrome, which is not related to the mutations in the gene coding for SERCA1a, but still accompanies exercise-induced muscle stiffness and delayed muscle relaxation due to a reduction in SERCA1a activity.

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1. Introduction

The sarcoplasmic reticulum (SR) acts as an internal Ca^{2+}-store and the Ca^{2+} in the SR is the major Ca^{2+} source for the contraction of skeletal muscle [1,2]. During skeletal muscle contraction, ryanodine receptor 1 is responsible for the channel that releases Ca^{2+} from the SR to the myoplasm [1,2]. To reduce myoplasmic Ca^{2+} level to the resting state (nano-molar range) during muscle relaxation and, at the same time, to re-fill the SR with Ca^{2+}, SR Ca^{2+}-ATPase (SERCA) uptakes Ca^{2+} from the myoplasm to the SR [3]. SERCA1a is the main isoform of adult skeletal muscle (more than 70% in all types of skeletal muscle fibers) [4,5].

Mitsugumin 53 (MG53, also called TRIM72), a muscle-specific tripartite motif (TRIM) family protein, contains a TRIM domain at the N-terminus and a PRY domain followed by a SPRY domain at the C-terminus [6–10]. The TRIM domain consists of a Ring domain harboring ubiquitin E3 ligase activity, a B-box harboring a zinc-binding moiety and two coiled-coil domains [6,7]. MG53 is a member of the membrane repair system [8,9], and an injury of the sarcolemmal membrane in mouse skeletal muscle fibers leads to a resealing of the injured membrane by gathering the intracellu-

lar vesicles coated with MG53 [8]. During this process, the oligo-merization of MG53 through the oxidation of the thiol group of cysteine at 242 and a leucine zipper motif between the two coiled-coil domains are needed for the intracellular vesicle trafficking to the injury site [11]. Dysferlin, polymerase I and transcript release factor, and non-muscle myosin type IIA interact with MG53 to facilitate the vesicle trafficking to the injury site [12–14]. The binding of caveolin 3 to MG53 moderates the robust vesicle trafficking to the injury site [9,14].

MG53 knockout mice show progressive skeletal myopathy associated with defective membrane repair [8]. Subcutaneous injection of purified MG53 to a mouse model of Duchenne muscular dystrophy alleviates the muscle pathology by promoting membrane repair [15]. Muscle-specific overexpression of MG53 in a hamster model of muscular dystrophy also ameliorates the pathology by enhancing membrane repair [16]. Interestingly, defective membrane repair by altered subcellular localization of MG53 is thought to be the cause of human muscular dystrophy in association with mutations in caveolin 3 [14].

Until now, studies on MG53 have been focused on MG53-mediated membrane repair, as mentioned above. There have been only a few reports of other roles of MG53 in skeletal muscle. In an MG53 knockdown study of the C2C12 cell line, MG53 facilitates the differentiation of C2C12 cells to myotubes by enhancing vesicle

trafficking and membrane fusion [9]; and, another study shows that MG53 attenuates the insulin-like growth factor (IGF)-induced differentiation of C2C12 [17]. Considering that MG53 is expressed only in skeletal and cardiac muscle [8], that MG53 is related to skeletal muscle diseases [8,14–16], and that MG53 plays a certain role in muscle cell differentiation [9,17], it is possible that MG53 also participates in unique functions of skeletal muscle such as muscle contraction and relaxation. Therefore, in the present study, we searched MG53-binding proteins among proteins mediating skeletal muscle contraction and relaxation using biochemical and cellular approaches. We found that MG53 directly binds to SER-CA1a via its TRIM and PRY domains and attenuates SERCA1a activity in skeletal muscle.

2. Materials and methods

2.1. cDNA construction and protein expression of GST-fused MG53 proteins

Using the full-length mouse MG53 cDNA (GenBank accession number: NM_001079932) as a template, GST-fused full-length MG53 or MG53 domains were synthesized and expressed in *Escherichia coli* (DH5α), as previously described [18].

2.2. Preparation of triad vesicles and binding assay of GST-fused MG53 proteins with triad proteins

The triad vesicles were prepared, as previously described [19,20]. All surgical interventions and pre- and post-surgical animal care were provided, as previously described [21,22]. Binding assays were performed as previously described [18] and presented in Supplementary Material 2.

2.3. In-gel digestion, protein identification by quadrupole time-of-flight mass spectrometry (qTOF MS), and database searches

Protein bands obtained from the binding assay were subjected to in-gel digestion with trypsin, as previously described [20]. The digested peptide solution was subjected to qTOF MS and database searches for protein identification, as described in Supplementary Material 2.

2.4. Cell culture, co-immunoprecipitation, immunoblot assay, and immunocytochemistry

Mouse primary skeletal myoblasts were derived from mouse skeletal muscle, then proliferated and differentiated to myotubes, as previously described [21–23]. The myotubes were solubilized and subjected to co-immunoprecipitation, as previously described [20] and presented in Supplementary Material 2.

2.5. MG53 knockdown by small interference RNA (siRNA) and quantitative real-time PCR (qPCR)

Sequences of two different siRNAs for mouse MG53 (Supplementary Material 3) were selected using siRNA design software (Dharmacon RNAi Technologies, Lafayette, CO). Transfection of the siRNAs to mouse primary skeletal myotubes and qPCR were performed as previously described [21] and presented in Supplementary Material 3.

2.6. Preparation of myotube homogenate and the oxalate-supported $^{45}Ca^{2+}$-uptake experiment

The MG53 knockdown myotubes were homogenated, as described in Supplementary Material 2. The myotube homogenate was subjected to the oxalate-supported $^{45}Ca^{2+}$-uptake experiment, as previously described [24] and presented in Supplementary Material 2.

2.7. Statistical analysis

The results are presented as the mean ± S.E. The significant differences were analyzed using a paired *t*-test (GraphPad InStat, v2.04, GraphPad Software, La Jolla, CA). The differences were considered to be significant at $p < 0.05$.

3. Results and discussion

3.1. MG53 binds to SERCA1a via its TRIM and PRY domains

To investigate the MG53-binding proteins among proteins mediating the contraction and relaxation of skeletal muscle, first, cDNAs for five GST-fused MG53 proteins were constructed (Fig. 1A and Supplementary Material 1): GST-TRIM, GST-PRY, GST-SPRY, GST-PRY–SPRY, and GST-MG53 (full-length). Each GST-fused MG53 protein was expressed in *E. coli* and the bacterial cell lysate was separated on a SDS–PAGE gel and stained with Coomassie Brilliant Blue (Fig. 1B). The GST-fused MG53 proteins were successfully expressed. For binding assays, affinity beads were prepared by immobilizing each GST-fused MG53 protein on GST beads and the affinity beads were incubated with the solubilized triad vesicle sample from 'rabbit' skeletal muscle. The triad vesicles are composed of junctional SRs and *t*-tubules that are enriched portions with triad proteins that mediate the contraction and relaxation of skeletal muscle [1,2,20]. The proteins that were bound to the affinity beads were separated at three different percentages of SDS–PAGE gels (7, 10 and 12% for a clear view of proteins with different molecular weights) and were stained with Coomassie Brilliant Blue in order to evaluate the proteins that were specifically bound to the GST-fused MG53 proteins (Fig. 1C). The bands for the proteins bound to GST itself were excluded from consideration. Nine bands appeared as proteins that were bound to the GST-fused MG53 proteins, and the GST-fused MG53 proteins showing the nine bands are summarized in Fig. 1D.

The nine bands were subjected to in-gel digestion and to qTOF MS for protein identification. Supplementary Material 4 and Table 1 show the results of q-TOF MS and database searches. Band 1 was identified as a mouse MG53 fragment that would bind only to PRY–SPRY (Fig. 1C and D), suggesting that MG53 could homooligomerize through an inter-domain formed by PRY and SPRY domains but not by each PRY or SPRY domain. Bands 2, 3, 6, and 9 were identified as non-specifically bound proteins that originated from the *E. coli* lysate during the binding assay. Band 4 was identified as a protein complex composed of SERCA1a that originated from 'rabbit' skeletal muscle and two other non-specifically bound proteins that originated from either the *E. coli* lysate or the *Pasteurella*. Band 5 was also identified as SERCA1a like band 4, suggesting that SERCA1a could be a MG53-binding protein. Considering that bands for SERCA1a would bind to TRIM, PRY, PRY–SPRY, and to a full-length MG53 but not to SPRY (Fig. 1D), the TRIM and PRY domains of MG53 were involved in binding to SERCA1a. For bands 7 and 8, there was no matching signal in the known databases.

The binding of MG53 to SERCA1a was directly accessed in rabbit skeletal muscle tissue and mouse primary skeletal myotubes. MG53 was co-immunoprecipitated with SERCA1a in the triad
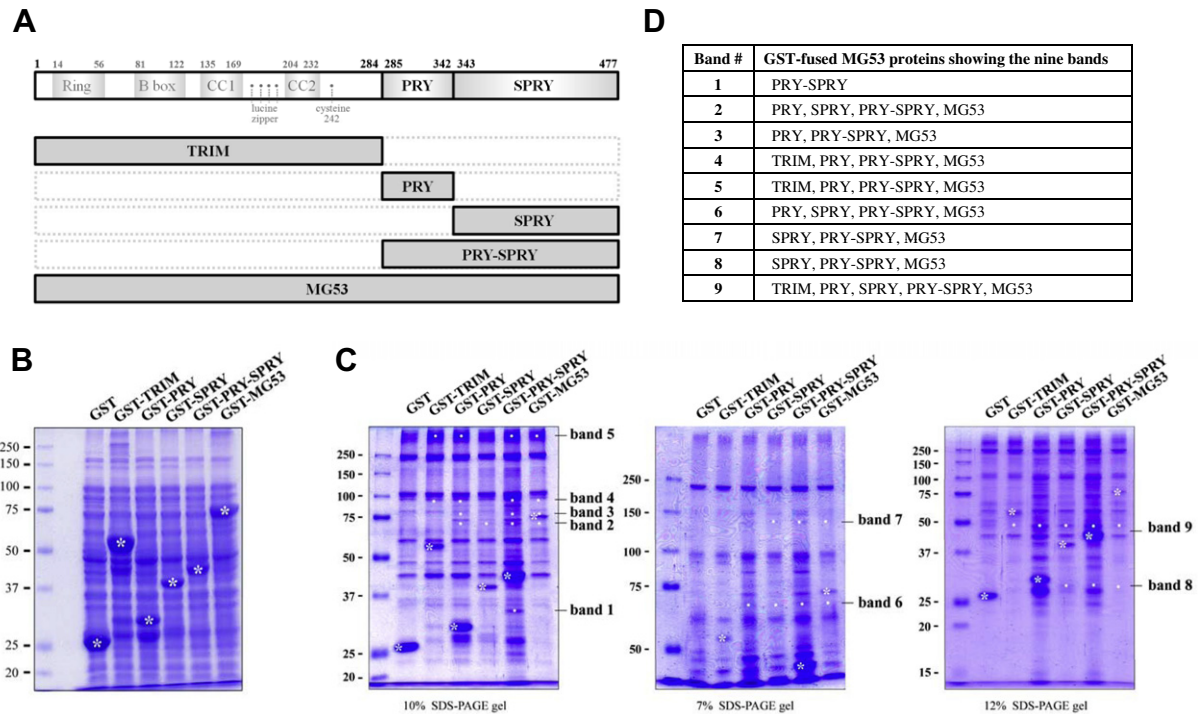
Fig. 1. Binding assays of GST-fused MG53 proteins with triad proteins. (A) Schematic diagrams of full-length mouse MG53 and domains. Numbers indicate the sequence of amino acids. (B) GST-fused MG53 proteins expressed in *E. coli* were separated on a SDS–PAGE gel (10%) and stained with Coomassie Brilliant Blue staining. GST-fused MG53 proteins are indicated by white asterisks. (C) The bound proteins obtained from the binding assays of GST-fused MG53 proteins with the triad proteins from rabbit skeletal muscle were separated on three different percentages of SDS–PAGE gels and stained with Coomassie Brilliant Blue. GST was used as a negative control. GST or GST-fused MG53 proteins are indicated by white asterisks. The specifically bound proteins to the GST-fused MG53 proteins are indicated by white dots. The newly appearing nine bands compared with the GST control are indicated on the right (bands 1–9). (D) The GST-fused MG53 proteins showing the nine bands are summarized.

Table 1
List of proteins identified by q-TOF MS.

Band #	Protein name	Mascot #	Mass (Da)	Species	Matching score	Matching peptides
1	MG53 (Trim72)	gi|45500997	41411	Mouse	65	632.3 RWALGVMAADASRR
2	Chaperone protein htpG	HTPG_ECOHS	71378	*E. coli*	46	635.3 RALSNPDLYEGDGELRVR.V
3	Chain A, Crystal Structure Of C418a, C419a Mutant Of Pf1	gi|6730181	85279	*E coli*	98	1033.2 KYGYDISGPATNAQEAIQWTYFGYLAAVKS
4	SERCA1a (Chain A of Ca^{2+}-ATPase)	gi|18159010	110787	Rabbit	114	758.9; 781.4 KVGEATETALTTLVEKM
	Pyruvate dehydrogenase subunit E1	gi|15799798	99948	*E. coli*	66	752.4 RAQYLIDQLLAEARK
	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	gi|15602069	95141	*Pasteurella*	71	841.4 KGFPLAYVGDVVGTGSSRK
5	SERCA1a (Chain A of Ca^{2+}-ATPase)	gi|18159010	110787	Rabbit	82	758.9; 781.4 KVGEATETALTTLVEKM
6	Heat shock protein 90	gi|15800202	71374	*E. coli*	69	743.4 RGLIDSSDLPLNVSRE
7	No matching signal					
8	No matching signal					
9	Tryptophanase	gi|41936	53098	*E. coli*	87	551.3; 993.5; 1103.9 RGIEEVGPNNVPYIVATITSNSAGGQPVSLANLKA

vesicle sample from rabbit skeletal muscle (Fig. 2A). Immunocytochemistry with anti-MG53 and anti-SERCA1a antibodies in mouse primary skeletal myotubes showed a co-localization of MG53 and SERCA1a near the nucleus (Fig. 2B). Therefore, based on the three different approaches (binding assay and qTOP MS, co-immunoprecipitation, and immunocytochemistry), we suggest that, with the exception of the proteins that mediate membrane repair, SERCA1a represents the first identification of a protein that binds directly to MG53 in skeletal muscle.

3.2. MG53 attenuates SERCA1a activity in skeletal myotubes

To examine how MG53 is functionally related to SERCA1a, we performed a knockdown of MG53 in mouse primary skeletal myotubes using siRNA transfections. Two different siRNAs were used to knockdown MG53 (Supplementary Material 3), and qPCR results showed that siRNA #2 more effectively reduced the mRNA level of MG53 (86.45 ± 6.67% reduction) compared with the untransfected control (Fig. 3A). A scrambled siRNA was used as the negative control. Immunoblot assay with anti-MG53 antibody using the lysate of the MG53 knockdown myotubes by siRNA #2 showed that MG53 expression was reduced by up to 80% (Fig. 3B), suggesting the successful knockdown of MG53 in mouse primary skeletal myotubes. On the other hand, the MG53 knockdown myotubes showed hampered differentiations to myotubes, with thinner and shorter myotube formations than those found in either the untransfected or the scrambled siRNA-transfected control (Fig. 3C). This suggests that MG53 is not indispensable but is needed to facilitate the differentiation of mouse primary skeletal myoblasts to myotubes. In accordance with this, severely ham-
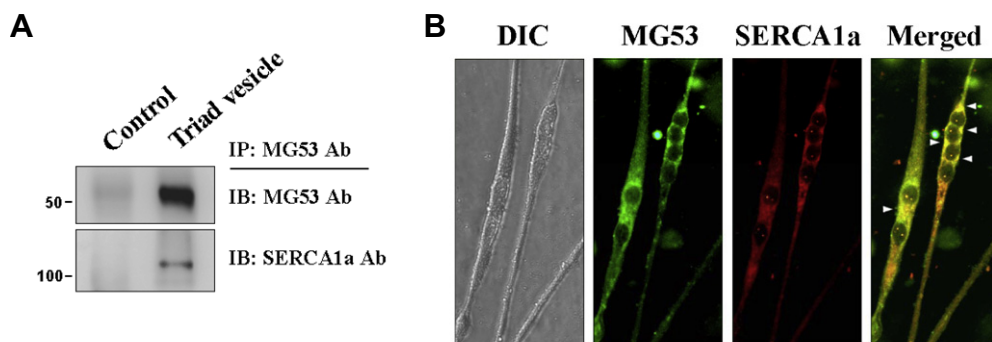
Fig. 2. Co-immunoprecipitation and co-localization of full-length MG53 with SERCA1a in rabbit skeletal muscle and in mouse primary skeletal myotubes. (A) MG53 was co-immunoprecipitated with SERCA1a in the triad vesicle sample from rabbit skeletal muscle. Control indicates a reaction without anti-MG53 antibody. (B) MG53 was co-localized with SERCA1a in the mouse primary skeletal myotubes (indicated by arrowheads). DIC, differential interference contrast microscopy. At least three independent experiments were conducted.
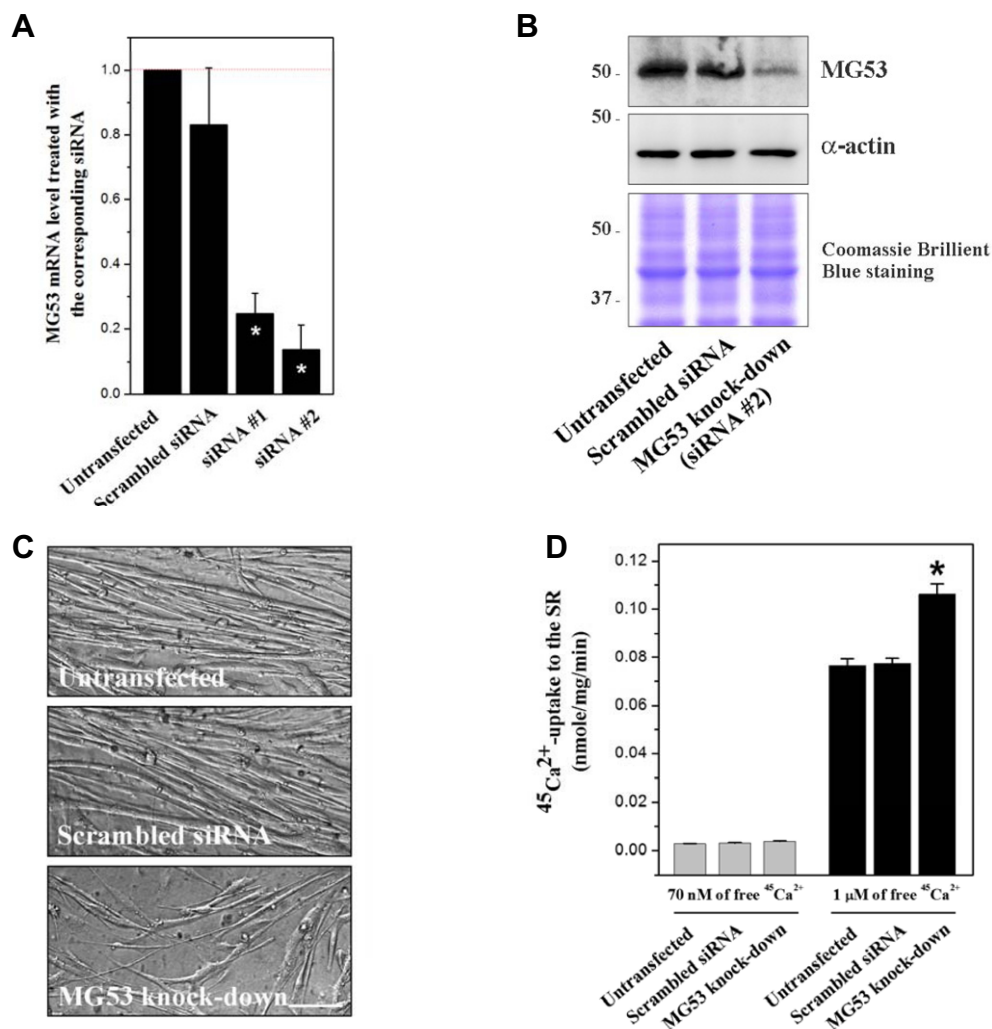
Fig. 3. Knockdown of MG53 in mouse primary skeletal myotubes and $^{45}Ca^{2+}$-uptake into the SR. (A) qPCR results show the reduced mRNA levels of MG53 by the siRNA transfections. siRNA #2 knocked down MG53 more effectively. A scrambled siRNA was used as a negative control. The values were normalized to the value from the untransfected control. The results are presented as the mean ± S.E. of duplicated experiments. (B) The lysate of the MG53 knockdown myotubes by siRNA #2 (50 μg of total protein) was subjected to immunoblot analysis with anti-MG53 antibody. α-actin and Coomassie Brilliant Blue staining were loading controls. The expression of MG53 protein was significantly reduced by up to 80%. Three independent experiments were conducted. (C) The MG53 knockdown myotubes show thinner and shorter myotube formations compared with the untransfected or the scrambled siRNA-transfected control. The bar represents 100 μm. (D) Oxalate-supported $^{45}Ca^{2+}$-uptake into the SR using the homogenate of the MG53 knockdown myotubes was measured at 70 nM or 1 μM of free $^{45}Ca^{2+}$. The MG53 knockdown myotubes showed a significantly increased $^{45}Ca^{2+}$-uptake only at 1 μM of free $^{45}Ca^{2+}$. The results are presented as the mean ± S.E. of five independent experiments. *Significant difference versus untransfected control ($p < 0.05$).

pered differentiation has been reported in an MG53 knockdown of the C2C12 cell line [9].

Ca^{2+}-uptake from the myoplasm to the SR by SERCA1a is an important event for skeletal muscle relaxation [3]. Therefore, the Ca^{2+}-uptake activity of SERCA1a was examined in MG53 knockdown myotubes using an oxalate-supported $^{45}Ca^{2+}$-uptake assay. The Ca^{2+}-uptake activity of SERCA1a was not changed at a resting myoplasmic Ca^{2+} concentration (70 nM of free $^{45}Ca^{2+}$ ([21,22]), Fig. 3D (left-hand)), suggesting that MG53 is not involved in the regulation of SERCA1a activity in the resting state of skeletal muscle. At a higher myoplasmic Ca^{2+} concentration, such as that found during skeletal muscle contraction (1 μM of free $^{45}Ca^{2+}$ [1,25]), Ca^{2+}-uptake activity of SERCA1a was significantly increased (more than 35% increase compared with the untransfected control, Fig. 3D (right-hand)), suggesting that MG53 is an attenuator of SERCA1a activity at micro-molar Ca^{2+} concentrations in skeletal muscle. There are two possible explanations for the role of MG53 at micro-molar Ca^{2+} concentration. One is that MG53 attenuates SERCA1a activity during skeletal muscle contraction in order to maximize the myoplasmic Ca^{2+} concentration for contraction. The other is that MG53 is needed to maintain a moderate Ca^{2+}-uptake to the SR by attenuating SERCA1a activity during skeletal muscle relaxation, and to subsequently moderate the rate of muscle relaxation and contraction.

3.3. MG53 shares functional and structural homologies with sarcolipin

The structure–function relationship of SERCA1 is well-understood, but the regulatory aspects are best understood for SERCA2a, which is the major isoform in cardiac and slow-twitch skeletal muscle [26]. Phospholamban (PLN) binds to SERCA2a, and the unphosphorylated form of PLN results in decreases in SERCA2a activity and in the rate of muscle relaxation and contraction [27]. However, PLN is absent from fast-twitch skeletal muscle that expresses SERCA1a as its major SERCA isoform [28], which suggests that PLN is not an in situ regulator of SERCA1.

On the other hand, sarcolipin, a small protein composed of 31 amino acids, is thought to be a PLN-like protein in skeletal muscle [29]. Sarcolipin knockout mice have shown increased SERCA1a activity in skeletal muscle (in soleus and red gastrocnemius) compared with wild-type mice [30], and MG53 knockdown myotubes also showed an increase in SERCA1a activity in the present study. Therefore, MG53 seems to share a functional homology with sarcolipin. The 3D structure of sarcolipin obtained from solution and solid-state nuclear magnetic resonance spectroscopy (PDB ID: 1JDM) showed an alpha-helix (Supplementary Material 5A, upper panel) that participated in binding to SERCA1a [31], and the helix was accurately predicted by a prediction program for protein secondary structures (Jpred3 [32], GenBank accession number: NM_025540.2) (Supplementary Material 5A, bottom panel). The TRIM domain of mouse MG53 was also predicted, with a high degree of confidence, to have two alpha-helixes (Supplementary Material 5B, GenBank accession number: NM_001079932). This structural homology suggests the possibility that the helixes in the TRIM domain may play the same role in SERCA1a regulation that the alpha-helix in sarcolipin plays.

3.4. The binding of MG53 to SERCA1a could be mediated by unique ways

Considering that the regulation of SERCA2a activity by PLN depends on the phosphorylation status of PLN [27], although sarcolipin has no obvious phosphorylation site [33], we searched phosphorylation sites in the TRIM and PRY domains of mouse MG53 using in silico tools. A NetPhos program based on the primary sequences of proteins [34] suggested that 11 serines and 2 threonines in the TRIM and PRY domains were possible phosphorylation sites. Among them, serine at 307 in the PRY domain was also predicted with a high degree of confidence using a Phos3D program that is based on the spatial contexts of the known 3D protein structures [35] (emphasized in Supplementary Material 5C). Interestingly, the serine at 307 in mouse MG53 is a unique amino acid compared with other proteins that contain a PRY domain (mainly aspartic acid in other proteins, Supplementary Material 6).

The 3D structures of several proteins containing the TRIM domain have been revealed. However, these are not useful in predicting SERCA1a binding sites in the TRIM domain of mouse MG53 because they have a very low identity among amino acid sequences. The 3D structure of the PRY domain of human MG53 has been revealed as a complex with the SPRY domain (Supplementary Material 5C, PDB ID: 3kb5, GenBank accession number: NM_001008274.3, '91% identity in amino acid sequence with mouse MG53'). In the PRY and SPRY complex, the prominent binding pocket for protein–protein interactions is mainly composed of the SPRY domain ([10], indicated by a transparent red circle in Supplementary Material 5C). The predicted phosphorable serine at 307 is irrelevant to the prominent binding pocket on the SPRY domain. In addition, the structure of the PRY and SPRY domains of human MG53 differs from those of other PRY- and/or SPRY-containing proteins [10]. Therefore, based on the prediction of phosphorylation sites and the structural considerations above, the binding of MG53 to SERCA1a via its TRIM and PRY domains could be mediated by different ways from the binding by other proteins containing a TRIM and/or a PRY domain.

3.5. MG53 could be a new candidate for the diagnosis and treatment of patients with Brody syndrome

Brody disease is an inherited myopathy due to mutations in the SERCA1a gene, and to the subsequent reduction in SERCA1a activity [36]. However, some groups of patients with Brody disease show reduced SERCA1a activity 'without mutations in the SERCA1a gene,' although they suffered from exercise-induced muscle stiffness and delayed muscle relaxation (so called Brody syndrome) [37,38]. This suggests that SERCA1 is not the only candidate protein for the cause of the Brody disease and Brody syndrome. Sarcolipin was a prime candidate, but unfortunately, as with the SERCA1a gene, no sarcolipin mutation has been found in patients with Brody syndrome [33]. The present study showed that MG53 attenuates SERCA1a activity by direct binding. MG53 knockout mice have shown progressive myopathy [8], and MG53 has ameliorated the pathology of muscular dystrophy in animal models [15,16]. Therefore, it is possible that MG53 is a new candidate for the diagnosis and treatment of patients with Brody syndrome.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.063.

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