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Functional analysis of SERCA1b, a highly expressed SERCA1 variant in myotonic dystrophy type 1 muscle

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ABSTRACT

Myotonic dystrophy type 1 (DM1) is a genetic disorder in which multiple genes are aberrantly spliced. *Sarco/endoplasmic reticulum Ca^{2+}-ATPase 1* (*SERCA1*) is one of these genes, and it encodes a P-type ATPase. SERCA1 transports Ca^{2+} from the cytosol to the lumen, and is involved in muscular relaxation. It has two splice variants (SERCA1a and SERCA1b) that differ in the last eight amino acids, and the contribution of these variants to DM1 pathology is unclear. Here, we show that SERCA1b protein is highly expressed in DM1 muscle tissue, mainly localised at fast twitch fibres. Additionally, when SERCA1a and SERCA1b were overexpressed in cells, we found that the ATPase and Ca^{2+} uptake activity of SERCA1a was almost double that of SERCA1b. Although the affinity for both ATP and Ca^{2+} was similar between the two variants, SERCA1b was more sensitive to the inner microsomal environment. Thus, we hypothesise that aberrant expression of SERCA1b in DM1 patients is the cause of abnormal intracellular Ca^{2+} homeostasis.

1. Introduction

Myotonic dystrophy type 1 (DM1) is a multisystem disorder. The symptoms of DM1 include muscle symptoms such as weakness and myotonia. Cardiac and cognitive defects, cataracts, insulin resistance, and hormone deficiency in multiple organs are also important symptoms in DM1. The genomic mutation that results in DM1 is an aberrant elongation of CTG triplet repeats located in the 3′-UTR region of the *Dystrophia myotonica protein kinase* (*DMPK*) gene [1]. When elongated CTG repeats are transcribed into mRNA, they inhibit normal function of RNA-binding splicing factors by interacting with those factors, and hence result in RNA toxicity [2]. This subsequently leads to abnormal splicing of multiple genes throughout the body.

ATP2A1 is one of the genes that is aberrantly spliced in DM1 muscle. *ATP2A1* encodes Sarco/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1). SERCA1 has two isoforms (SERCA1a, the adult form, and SERCA1b, the neonatal form) produced by the alternative splicing of exon 22, which is included in SERCA1a, but not in SERCA1b (Fig. 1A). During development, the expression of SERCA1 migrates from the SERCA1b form to the SERCA1a form. Thus, in adult skeletal muscle tissue, only SERCA1a is expressed. However, *SERCA1b* mRNA has been detected in adult skeletal muscle from DM1 patients [3].

SERCA1 protein is a representative member of P-type ATPase transporter that is exclusively located in the sarcoplasmic reticulum (SR) of fast-twitch skeletal muscle; it functions to transport Ca^{2+} from the cytosol to the lumen of the SR. Because it is the only pump that uptakes Ca^{2+} to SR in fast-twitch skeletal muscle, SERCA1 is crucial for maintaining intracellular Ca^{2+} homeostasis. Mutations in the *ATP2A1* gene cause Brody disease, which is characterised by abnormalities in muscle relaxation after exercise [4]. The SR of Brody patients has half, or less, Ca^{2+}-dependent ATPase activity compared with the control group.

Because exon 22 contains a stop codon, SERCA1a is shorter (994 amino acids) than SERCA1b (1001 amino acids), with the last eight amino acids differing between the two. The last eight amino acids (DPEDERRK) of SERCA1b are rich in hydrophilic amino acids, suggesting a specific role in the SR. In earlier studies, the two isoforms were compared to determine whether they have functional differences. The results were contrary to expectations; ATPase and Ca^{2+} uptake activities were similar between the two SERCA1 isoforms [5]. As large amounts of SERCA1a protein can be easily purified from rabbit thigh muscle, its properties have been well characterised [6–8]. SERCA1b has received less attention, most likely because it has similar activity to SERCA1a and has a limited expression period.

The discovery of *SERCA1* aberrant splicing in DM1 muscle has raised questions about the possible importance of SERCA1b. Additionally, in DM1, cytosolic Ca^{2+} concentrations were reported to be increased [9], indicating a functional difference, and a series of intracellular Ca^{2+}

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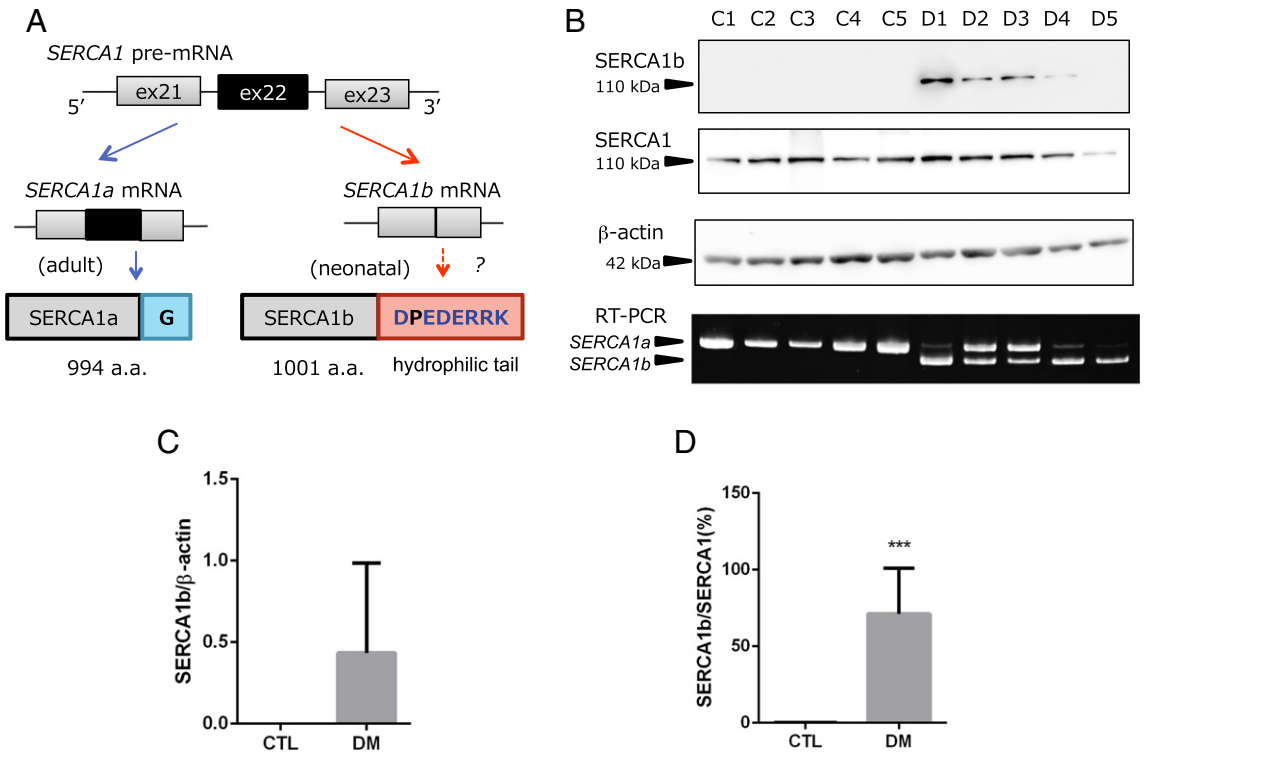

Fig. 1. Detection of SERCA1b in DM1. A. Schematic diagram showing two *SERCA1* splicing patterns in DM1 patients. Dotted line, unverified; solid line, verified. B. Western blot analysis of SERCA1b and SERCA1 expressed in DM1 patients' biopsies detected by specific SERCA1b antibody or SERCA1 antibody. β-actin was used as a loading control. C1-5 indicates non-DM individuals; D1-5 indicates DM1 patients. 5 μg protein per lane was loaded. RT-PCR analysis on *SERCA1* splicing patterns of each individual was shown at the lowest panel. Black arrows indicate *SERCA1a* or *SERCA1b*. C. SERCA1b specific band intensity of Western blot analysis in B. $p = 0.167$, ($n = 5$, Student's *t*-test, mean ± SD). D. *SERCA1b* specific band intensity of RT-PCR in B. $^{***}p < 0.0001$, ($n = 5$, Student's *t*-test, mean ± SD).

homeostasis disturbances in DM1 patients have been reported [10,11]. Hence, identification of the specific function of SERCA1b is needed.

Here, we re-examine the functional differences between SERCA1a and SERCA1b. We first analyse SERCA1b protein levels in muscle tissue from DM1 patients. Next, we attempt to dissect the functional differences between the two isoforms by overexpressing them in cultured cells, and we conduct further biochemical analyses on the ATPase activity of SERCA1b. Finally, we propose a new, *SRECA1* splice-dependent pathway in DM1 pathology.

2. Methods

2.1. DM1 muscles

Biopsies were obtained from the skeletal muscle of five patients with DM1 and five non-DM individuals (see Table S1). The individuals' range in age is from 15 to 55. All samples were stored at −80 °C.

2.2. Western blot analysis

Biopsied muscles were homogenised with 10 times volume of buffer A (10 mM MOPS-KOH pH 7.0, 10% sucrose, 0.1 mM EDTA, 1% NP-40, and 0.1% protease inhibitor). Microsomes expressing SERCA1a or SERCA1b were used as a protein sample directly. Proteins were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes. SERCA1 antibody (1:2500, MA3-912, Thermo Scientific, USA), SERCA1b antibody (mentioned below, 1:2500), and Actin antibody (1:2500, A2066, Sigma-Aldrich, USA) were used to detect each protein. Membranes were analysed using a LAS3000 imager (Fujifilm, Japan). The intensities of band signals were quantified using MultiGauge software (Fujifilm, Japan). The polyclonal antibody against SERCA1b was made to order by MBL, Japan, and generated by immunising rabbits (specific pathogen free) with a synthesised peptide, CLEDPEDERRK, conjugated containing Keyhole limpet hemocyanin at its N-terminus.

2.3. SERCA1 splicing assay

Total RNA was extracted from frozen sections using TRIzol reagent (Life Technologies, USA) according to the manufacturers' instructions. 0.5 μg of total RNA was reverse transcribed using a PrimeScript 1st Strand cDNA Synthesis kit (TAKARA BIO, Japan) with a 1:1 mixture of oligo dT and random hexamer as primers. Splicing assay of *SERCA1* was performed as described previously [12]. PCR cycles were modified to 24 amplification cycles. Band intensities were digitized and quantified using Multi-gauge (Fujifilm, Japan).

2.4. Immunohistochemistry

Frozen sections (10 μm) of muscle biopsy specimens were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. Slides were incubated with blocking buffer containing 3% BSA and 5% goat serum in PBS for 15 min at 37 °C. Primary antibodies were diluted in blocking buffer to an appropriate concentration and incubated for 2 h at 37 °C. Primary antibody concentrations used in immunohistochemistry are as follows: SERCA1b (the same antibody used at Western blot analysis), 1:100; SERCA1 (the same antibody used at Western blot analysis), 1:100; MHCf (NCL-MHCf, Leica Biosystems, UK), 1:50; MHCs (NCL-MHCs, Leica Biosystems, UK) 1:80; Laminin (ab11576, abcam, USA), 1:50. Slides were then washed with PBS. Secondary antibodies are Alexa-488 or Alexa-568 conjugated secondary goat antibodies against mouse, rabbit and rat. All from Invitrogen, USA were diluted in 3% BSA in PBS at 1:500 for 1 h at room temperature. Images were acquired with a confocal laser scanning microscope (FluoView FV10i; Olympus, Japan).