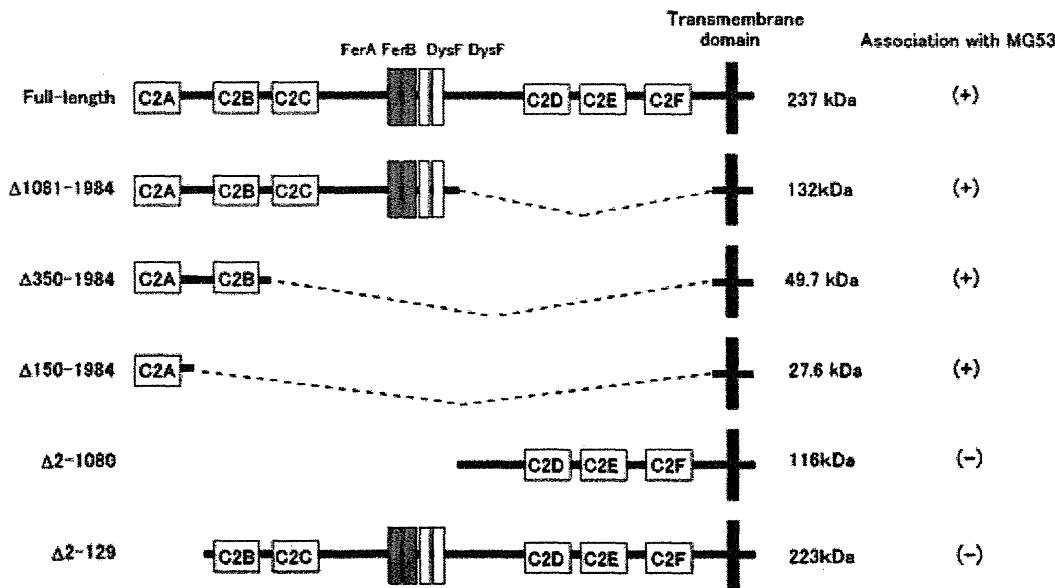


Fig. 2: Identification of MG53-binding region of dysferlin.

The dysferlin C2A domain associates with MG53. Constructs encoding dysferlin deletion mutants were used for co-IP assays, and the results of these experiments are shown on the right. Deletion mutants encoding c-myc-tagged dysferlin mutants and FLAG-tagged full-length MG53 were co-expressed in COS-7 cells. IP and immunoblotting were performed using antibodies against the c-myc and FLAG tags. MG53 was co-immunoprecipitated with full-length dysferlin and the dysferlin mutants that lacked the C-terminus, but not with the dysferlin mutants that lacked the N-terminus.

Characterization of the association of dysferlin C2A domain with MG53 monomers and MG53 oligomer

C2 domains are known to bind to phospholipids and/or proteins in a Ca^{2+}-dependent or Ca^{2+}-independent manner [15]. Therefore, we used a pull-down assay to examine whether the Ca^{2+} concentration affected the association between MG53 and the dysferlin C2A domain. We used lysis buffer containing 75 mM Tris to reduce the change in pH that can result from the addition of $CaCl_2$, to examine the calcium-dependency of the association between dysferlin and MG53. Reportedly, MG53 can exist as a monomer or an oligomer, depending on the redox state [4]. We used DTT for monomerization of MG53 by reducing sulfhydryl groups. Addition of 5 mM DTT resulted in complete dissociation of all MG53 oligomers (Fig. 3). To conduct a pull-down assay for MG53 oligomers, we treated cell lysates with an alkylating reagent, NEM, which reacts with sulfhydryl groups to form stable thioether bonds [6]. Multimers of MG53 were stabilized by chemical cross-linking with GA. Addition of 5 mM NEM to cell lysates resulted in oligomerization of MG53 (Fig. 3). In the presence or absence of Ca^{2+}, MG53 oligomers associated with wild-type C2A-GST, whereas MG53 monomers did not associate with wild-type C2A-GST. In the absence of DTT or NEM, MG53 existed as oligomers including dimers, which associated with WT C2A-GST only in 10 mM free Ca^{2+} (Fig. 3, top).

Next, we generated two mutant versions of C2A-GST (W52R or V67D) to further characterize the association between MG53 and the C2A domain. A V67D missense mutation in the human dysferlin gene has been found in patients with MM and patients with LGMD2B [16]; similarly, the W52R dysferlin missense mutation has been found in patients with LGMD2B [17]. Each mutant C2A-GST, like the wild-type C2A, associated with MG53

oligomers when conditions included NEM in the presence or absence of Ca^{2+} (Fig. 3). However, the V67D mutation altered the calcium sensitivity of the association between C2A-GST and MG53 dimers; specifically, V67D-C2A-GST could associate with MG53 when conditions did not include NEM in the absence of Ca^{2+}. In contrast, W52R-C2A-GST did not associate with MG53 when conditions did not include NEM in the presence or absence of Ca^{2+}. These results revealed that the V67D mutation in the dysferlin C2A domain altered the Ca^{2+}-dependence of the association between dysferlin and MG53 dimers.

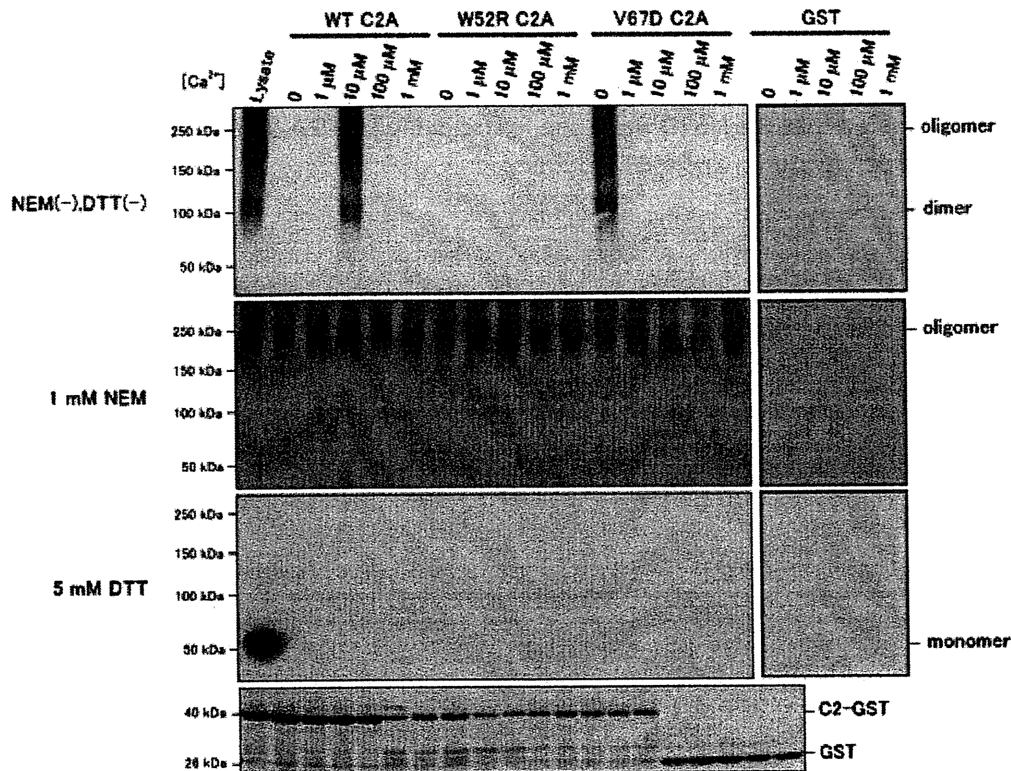
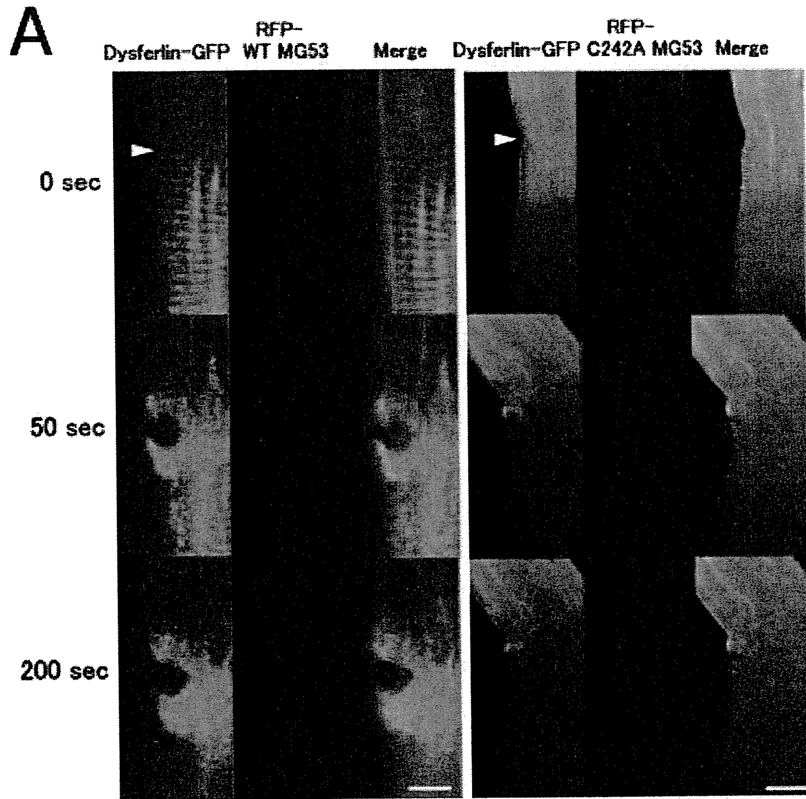


Fig. 3: Pull-down assay of dysferlin C2A-GST and MG53.

COS-7 cells overexpressing FLAG-tagged MG53 were lysed and supplemented with DTT or NEM, and proteins in these lysates were cross-linked with GA. Cross-linked proteins were incubated with glutathione Sepharose 4B beads bound to wild-type C2A-GST, V67D C2A-GST, or GST. GST fusion proteins bound to beads were separated by SDS-PAGE, followed by Coomassie Brilliant Blue R-250-staining. Precipitated MG53 oligomers/monomers were detected on immunoblots using an anti-FLAG antibody. Mutations in the C2A domain affect the association of between dysferlin and MG53.

MG53 with a C242A missense mutation shows impaired accumulation at wound sites and attenuates the formation of dysferlin patches

To examine the biological role of the association between dysferlin and MG53 in sarcolemmal repair, we used mouse skeletal muscle co-transfected with dysferlin-EGFP and RFP-tagged wild-type MG53 or RFP-tagged mutant MG53 to perform a membrane repair assay. The mutant MG53 carried a C242A missense mutation and is designated RFP-C242A-MG53 here. MG53 with a C242A missense mutation reportedly exists as a monomer or dimer when expressed in mammalian cells, but does not form oligomers via disulfide bonding [4,6]. RFP-C242A-MG53 did not accumulate at wound sites as reported previously, and it was associated with defective sarcolemmal repair [4]. Co-expression of RFP-C242A-MG53 did not affect the subcellular localization of dysferlin in myofibers, and dysferlin was localized in a striated pattern (Fig. 4A). However, RFP-C242A-MG53 compromised the accumulation of dysferlin at injury sites (Fig. 4A, B). When the movement of dysferlin and wild-type MG53 were observed simultaneously in mouse skeletal muscle, RFP-wild-type MG53 accumulated more slowly at injury sites than dysferlin-EGFP (Fig. 4A). Accumulation of dysferlin-EGFP at wound sites stops within 5 seconds of injury and disperses gradually, while wild-type MG53 continues to accumulate for 200 seconds after injury (Fig. 4A and 4B).


A
Dysferlin-GFP
RFP-
WT MG53
Merge
Dysferlin-GFP
RFP-
C242A MG53
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0 sec
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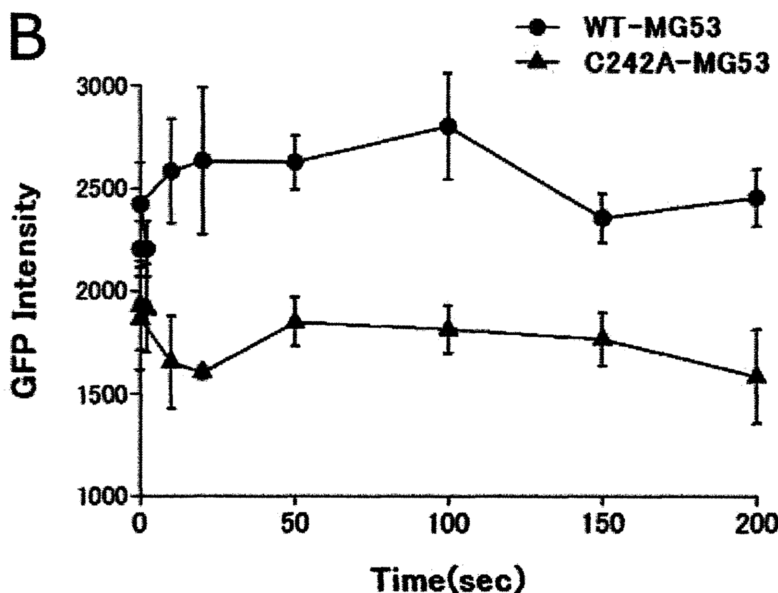

Fig. 4: Membrane repair assay of myofiber transfected with dysferlin-GFP and RFP-MG53.

RFP-C242A MG53 perturbed the accumulation of dysferlin at wound sites in the sarcolemma. A. Dysferlin-GFP was simultaneously expressed with RFP-tagged wild-type MG53 or the RFP-C242A-MG53 mutant in mouse skeletal muscle. Arrowheads indicate sites of membrane injury, which were induced with a two-photon laser microscope. Dysferlin-GFP accumulated at the injury site in the presence of RFP-wild-type MG53, but no obvious accumulation of dysferlin-GFP was observed in the presence of the RFP-C242A-MG53 mutant. Scale bar, 10 mm. B.Time course fluorescence intensity (n=3) at wounded sites versus time. For every image taken, the fluorescence intensity of dysferlin-GFP at the site of the damage (circle of 5 mm in diameter) was measured with Zeiss LSM5 Image Examinar software. Data are means ± standard deviation.

MG53 accumulates normally at injury site of sarcolemma in dysferlin-deficient mice.

A previous study revealed that exogenous expression of MG53 in undifferentiated C2C12 cells was necessary for recruitment of GFP-dysferlin to sites of injury [5]. Conversely, to examine whether the recruitment of MG53 requires dysferlin, and to elucidate the molecular pathology of dysferlinopathy, we used skeletal muscle from dysferlin-deficient A/J mice transfected with EGFP-MG53 to perform a membrane repair assay. We confirmed that EGFP-MG53 accumulated at sites of injury (Fig. 5). Sarcolemmal repair was observed and confirmed by FM4-46-loading in A/J mice (data not shown). The accumulation of MG53 at the sarcolemmal wound was observed in A/L mice, similar to wild-type mice. Similar results were obtained from the membrane repair assay using dysferlin-deficient SJL mice.

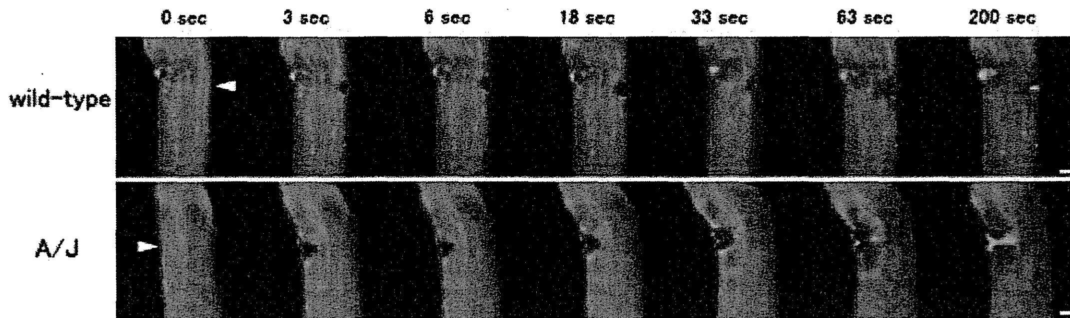

Fig. 5: Membrane repair assay of myofiber using dysferlin-deficient myofiber transfected with GFP-MG53.

GFP-MG53 accumulated at sites of injury in the sarcolemma in dysferlin-deficient A/J mice, similar to wild-type mice. GFP-MG53 was expressed in wild-type or dysferlin-deficient A/J mice, and a membrane repair assay was performed using transfected myofibers. Subcellular localization of GFP-MG53 was similar between wild-type and A/J mice. Arrowheads indicate membrane injury sites, which were induced with a two-photon laser microscope. Scale bar, 10 μm.

Discussion

Both dysferlin and MG53 are involved in membrane repair after injury in skeletal muscle. Dysferlin accumulates at wounded sarcolemmal sites, and this accumulation requires the influx of Ca^{2+} into the myofiber [3]. MG53 forms oligomers at the sarcolemmal injury site in an oxidation-dependent manner [4,6]. MG53 associates with dysferlin and facilitates vesicle trafficking to the site of membrane injury, and a recent finding suggests that MG53 and dysferlin may form a complex that participates in membrane repair in striated muscle [5]. To characterize the association between dysferlin and MG53, we used an IP assay and mouse muscle extract with or without exogenously added EGTA or $CaCl_2$ to examine the Ca^{2+} dependency of this association. Using lysis buffer that lacked EGTA and $CaCl_2$, we observed the association of dysferlin with MG53 in mouse skeletal muscle. Lysates lacking exogenously added EGTA and $CaCl_2$ contain physiological concentrations of free calcium. Hence, low concentrations of calcium are likely to be necessary for the interaction between MG53 and dysferlin.

Our results indicated that MG53 oligomers associated with the dysferlin C2A domain in the presence or absence of Ca^{2+}, whereas MG53 dimers associated with the dysferlin C2A domain in a Ca^{2+}-dependent manner. We also revealed that pathogenic mutations in the dysferlin C2A domain (W52R and V67D) alter the association between this domain and MG53 dimers in a pull-down assay. In the absence of EGTA or Ca^{2+}, dysferlin with a C2A missense mutation (W52R or V67D) did not associate with MG53 in an IP assay that used extracts from co-transfected COS-7 cells; however, full-length dysferlin with the most common pathogenic mutation found in Japan, a W999C missense mutation in the dysferlin domain, did associate with MG53 in these IP assays (data not shown). These results indicate that the dysferlin C2A domain is important for the association between dysferlin and MG53. Amino acid W52 in human dysferlin is located between the b5-sheet and the b6-sheet, and V67 is located in the b6-sheet [18]. Both residues are reportedly important for the C2 structure, particularly those of the b-sheet, and are predicted to coordinate calcium [18].

Recently, MG53 was reported to form homodimers, which are essential for MG53-mediated sarcolemmal repair [6]. We used pull-down assays to investigate associations between MG53 monomers or MG53 dimers and the

dysferlin C2A domain, and we found that MG53 dimers associated with dysferlin in a Ca^{2+}-dependent manner. An increase in the cytoplasmic Ca^{2+} level is necessary for dysferlin accumulation at wounded sarcolemmal sites [3]. The intracellular Ca^{2+} level is maintained at 50-100 nM in resting mammalian cells, but this increases to 6 μM after membrane puncture in Swiss-3T3 cells [19]. The influx of extracellular Ca^{2+} through the wound site is required for vesicle fusion with the plasma membrane and formation of a repair patch in skeletal muscle, but MG53 trafficking to the wound site does not require Ca^{2+} [4]. In pull-down assays in the present study, we demonstrated a selective association between the wild-type dysferlin C2A domain and MG53 dimers at a free Ca^{2+} concentration of 10 μM, but not at lower or higher free Ca^{2+} concentrations. These findings indicated that the concentration of free Ca^{2+} is important for association of dysferlin with MG53 dimers, and suggest that MG53 dimers not only form oligomers, but also associate with dysferlin in response to sarcolemmal injury. The altered Ca^{2+} sensitivity of the association between dysferlin with a mutation in the C2A domain and MG53 dimers in the pull-down assay also suggested that the C2A domain was important in the Ca^{2+}-dependent association between dysferlin and MG53 dimers.

We were able to analyze the movement of dysferlin and MG53 in real time during sarcolemmal repair in a membrane repair assay that employs mouse myofibers that express dysferlin-EGFP and RFP-MG53. This is the first report to demonstrate that dysferlin and MG53 have different accumulation patterns at wound sites, and this result indicated that dysferlin and MG53 have different functions in sarcolemmal repair. Our studies also revealed that MG53 carrying a C242A missense mutation can suppress the accumulation of dysferlin at the wound site; this finding, together with results from pull-down assays, suggests that MG53 dimers play an important role in sarcolemmal repair.

Our studies also revealed that MG53 accumulated at injury sites in the sarcolemma in dysferlin-deficient mice, similar to wild-type mice. However, dysferlin-deficient SJL and A/J mice have a progressive muscular dystrophy phenotype, suggesting that MG53 is necessary but not sufficient for efficient sarcolemmal repair.

Competing Interests

The authors have declared that no competing interests exist.

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