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The androgen receptor facilitates inhibition of human *dopamine transporter* (*DAT1*) reporter gene expression by HESR1 and HESR2 via the variable number of tandem repeats

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HIGHLIGHTS

- Deletion of the VNTR in the DAT reporter gene increase its expression.
- HESR1 and HESR2 inhibit the reporter gene expression via the VNTR.
- The androgen receptor facilitates the inhibition of the DAT reporter by HESRs.

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ABSTRACT

A functional genetic polymorphism in the 3′-untranslated region (UTR) within exon 15 of the human *DAT* gene (*DAT1*) has been described. This 3′-UTR contains a variable number of tandem repeats (VNTR) 40 bp in length; many association studies of psychiatric or developmental disorders with this VNTR have been conducted. We previously demonstrated that HESR1 (the Hairy/enhancer of split related transcriptional factor 1 with YRPW motif) and HESR2 reduced *DAT* reporter gene expression via this 3′-UTR. VNTR allele-dependent altered reporter gene expression was also observed. In the present study, we wanted to clarify the molecular characterization of HESR1 and HESR2, focusing on its *cis*-element and co-factor. Deletion of the VNTR domain increased reporter gene expression both with and without transfection of HESRs, suggesting that the VNTR inhibits *DAT* expression, and is responsive to HESRs. In the presence of transfected androgen receptor (AR), activity of the luciferase reporter with the nine-repeat allele (9r) decreased, while that with the ten-repeat allele (10r), the most frequent in the population, increased significantly. Furthermore, co-expression of HESR1 or HESR2 with AR increased the inhibitory effect of the HESRs. Our data indicate that a functional modification occurs when the HESRs are coupled with AR. This HESR–AR interaction could be the molecular basis of sexual dimorphisms in DAT expression, or other dopamine-related behavioral traits.

1. Introduction

A functional genetic polymorphism in the 3′-untranslated region (UTR) within exon 15 of the human *dopamine transporter* gene (*DAT1*) has been described [19]. This 3′-UTR contains a variable number of tandem repeats (VNTR) domain, which is 40 bp in length (Fig. 1) [19,29]. Polymorphism within this region is associated with neuropsychiatric disorders, including attention deficit hyperactivity disorder (ADHD), Parkinson's disease (PD), alcoholism, and drug abuse [3,5,27,28,30], as well as genotype-dependent alteration of gene expression, both in vivo [5,13,15,20] and in mammalian cell lines [5,8–10,14,21,22,31].

We previously identified and characterized the Hairy/enhancer of split related transcriptional factor 1 with the YRPW motif (HESR1, HEY1) as a *trans*-acting repressor of gene expression that acts through the 3′-UTR of *DAT1* [7,8]. In addition, we showed that another HESR family member, HESR2, inhibited *DAT1* reporter gene expression [16].

Recently, HESR family members have been reported to interact with co-factors [6]. The candidate co-factor we want to focus on is androgen receptor (AR). In a prostate cancer study, HESR1

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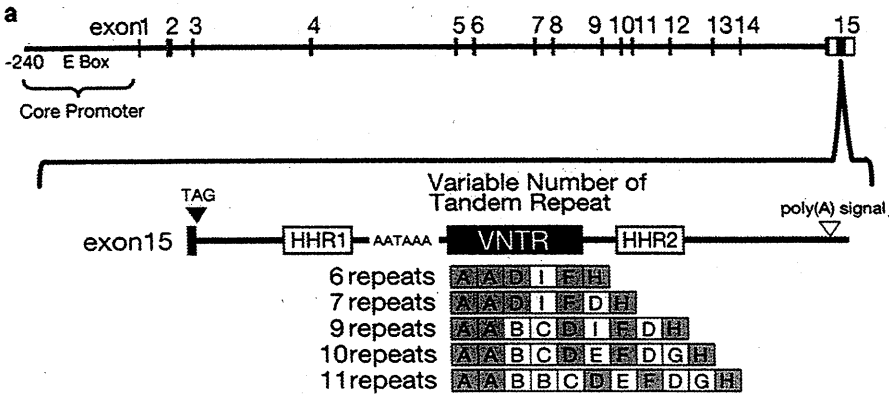

b

Repeat Units

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(A)  AGGAGCGTGTCCTATCCCCGGACGCATGCAGGGCCCCCAC
(B)  AGGAGCATGTCCTATCCCTGGACGCATGCAGGGCCCCCAC
(C)  AGGAGCGTGTACTACCCCAGAACGCATGCAGGGCCCCCAC
(D)  AGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCAC
(E)  TGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCAC
(F)  AGGAGCGTGTCCTATCCCCGGA
                          CCGGACGCATGCAGGGCCCCCAC
(G)  AGGAGCGTGTACTACCCCAGGATGCATGCAGGGCCCCCAC
(H)  AGGAGCGTGTACTACCCCAGGACGCATGCAGGGCCCCCAT
(I)  TGGAGCGTGTACTACCCCAGGATGCATGCAGGGCCCCCAC
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HHR1

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Human  AATTCATGCAAAGTCCTTTCCCGATGCGTGGCTCCCAGCAGA
Mouse  AATACATGCCAAGTCCTTTCCTGGTGCTTGGCTCCGAGCAGA
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HHR2

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Human  AATGGCTTTTTAAAATCATATTTACCTGTGAATCAAAA
Mouse  AATGGCTTTTTAAAACCATATTTATGTGTGAATCAAAA
```

c

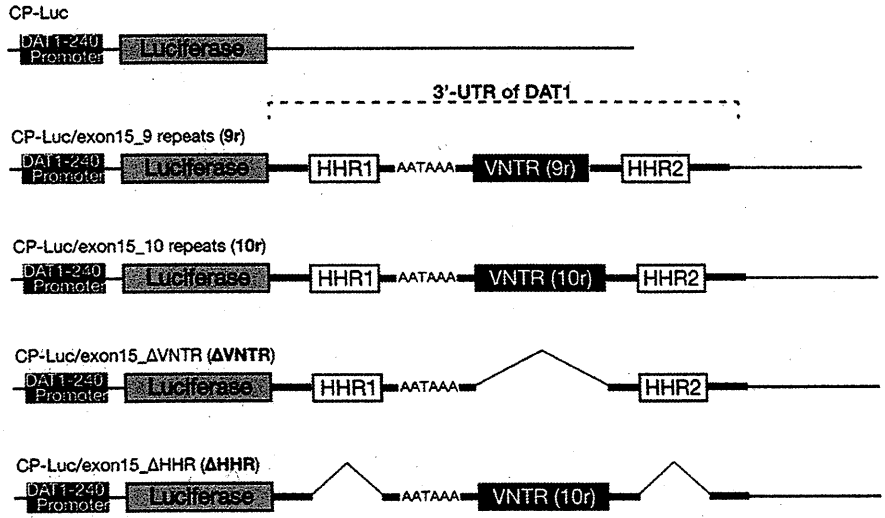

Fig. 1. Structure of the 3′-UTR in *DAT1* and its reporters. (a) The genomic structure of the human *dopamine transporter* (*DAT1*) gene. The coding region (closed box), non-coding region (open box), VNTR domain, and constant parts of the repeat units (gray box) are shown. Exon 15 of *DAT1* contains a stop codon (arrowhead) and polyadenylation signal (open arrowhead). Upstream of the VNTR domain are six nucleotides (AATAAA) that resemble a polyadenylation signal. The allelic variants of the VNTR indicate the repeat unit type (A–I) for each allele. High homology regions (HHR1 and HHR2) between human and mouse are also illustrated. (b) Nucleotide sequence of each unit of the VNTR polymorphism and HHRs in the 3′-UTR of *DAT1*. Bases that differ between human and mouse are underlined. (c) Schematic diagrams of the luciferase reporter vectors used in this study, CP-Luc and four types of CP-Luc/exon15. CP-Luc contains only the *DAT1* core promoter, while each CP-Luc/exon15 contains both the core promoter and 3′-UTR of *DAT1*. CP-Luc/exon15_ΔVNTR and CP-Luc/exon15_ΔHHR are deletion constructs made from CP-Luc/exon15_10 repeats by PCR. VNTR, variable number of tandem repeats and HHR, high homology region between human and mouse 3′-UTR in the DAT gene.

was shown to act as a co-repressor of AR [2]. AR and dopamine are associated with sexual motivation [25]. A sex difference in the disease rate was reported in Parkinson's disease, of which one of the main pathological features is the apoptosis of DA neurons [12]. In addition, DAT expression is lower in males than in females, that is believed to be one reason for the sex difference in the incidence of this disease [23]. Thus, taken together, AR appears to be involved in the dopaminergic system or function of DAT; however, the direct molecular pathway remains unknown. Therefore, the direct pathway of androgen signaling in dopaminergic neurons may be revealed if HESRs are capable of interacting with AR.

While elucidating the HERSs–AR interaction, we wanted to obtain further information regarding the *cis*-element in the 3′-UTR of DAT. There are predicted binding sequences in the 3′-UTR of DAT. Two areas of high homology between humans and mice are located in the non-coding regions. Here, we designate the regions upstream and downstream of the VNTR high homology region 1 (HHR1) and high homology region 2 (HHR2), respectively (Fig. 1). The VNTR domain does not exist in mice, but in our previous study, down-regulation of DAT was observed in Hesr1 knockout (KO) mice at postnatal day zero [7]. Therefore, the elements that the HESRs interact within the 3′-UTR of DAT may or may not be different. To identify these elements, we conducted a reporter assay and observed the effects of HESRs and AR using several DAT luciferase reporter genes.

2. Materials and methods

2.1. Constructs

Four luciferase reporter vectors were prepared (Fig. 1c): CP-Luc/exon15 contained the human *DAT* core promoter and 3′-UTR. CP-Luc, CP-Luc/exon15_10repeats (10r), and CP-Luc/exon15_9repeats (9r) reporter vectors were the same constructs used in our previous studies [8,16].

In the present study, several deletion mutants were prepared. New luciferase reporter gene constructs CP-Luc/exon15_ΔVNTR (ΔVNTR) and CP-Luc/exon15_ΔHHR (ΔHHR) were generated by inverse PCR from the 10r luciferase reporter using primers flanking the deleted regions (Fig. 1c).

The HESR vectors and myc-tagged vector (also used for the empty vector control, VEC) were used in our previous report [19]. The AR expression vector was a gift from Dr. G. Sobue (Nagoya University). Information on the AR vector is included in his work [33].

2.2. Immunocytochemistry

Human neuroblastoma SH-SY5Y cells were plated on collagen-coated cover glasses in 12-well plates, and were transfected with HESR1 or HESR2 and AR ($n=3$). Cells were fixed with 4% paraformaldehyde in PBS for 15 min and washed three times 24 h after transfection.

Cells were then stained with antibodies by standard immunocytochemical process. Images were captured with an All-in-one fluorescence microscope BZ-9000 (Keyence, Osaka, Japan).

2.3. Luciferase reporter assay

Methods used for luciferase activity measurements followed the standard methods of the Dual-Luciferase Reporter Assay System (Promega). The SH-SY5Y cells were transfected with the luciferase reporter gene and each HESR and/or AR or empty vector. Plasmid pRL (Promega) containing the sea pansy (renilla) luciferase gene was co-transfected as a control to normalize for transfection efficiency in all experiments. In the present study, luciferase activities (relative light unit = [values of firefly luc]/[values of renilla]) were standardized to the average of the 10r reporter with VEC group.

2.4. Statistics

All values are reported as the means ± SEM. Student's *t*-test or Tukey–Kramer's honestly significant difference (HSD) test were used as a *post hoc* test after two-way analysis of variance (ANOVA). Differences were considered significant at values of $P<0.05$.

Further information is described in online supplementary information.

3. Results

3.1. Localization of HESR1, HESR2 and AR in SH-SY5Y cells

As shown in Fig. 2, immunoreactivity was observed mainly in the nucleus of cells transfected with HESR1 and HESR2. AR immunoreactivity was also observed in the nucleus.

3.2. Luciferase reporter assay

3.2.1. Effects of putative elements in the 3′-UTR of DAT1

A reporter assay was conducted to identify the effects of each sequence on gene expression with or without transiently expressed HESRs in cultured SH-SY5Y cells using the luciferase reporters illustrated in Fig. 1c.

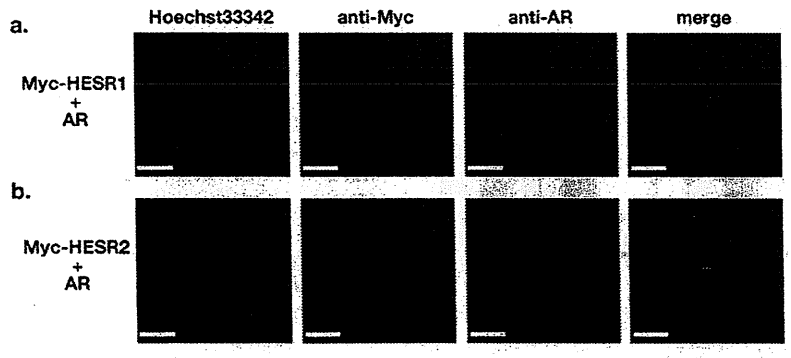

Fig. 2. Cellular localization of transfected HESRs and AR in SH-SY5Y cells. (a) Blue, nucleus; green, Myc-tagged HESR1; and red, androgen receptor (AR). (b) Blue, nucleus; green, Myc-tagged HESR2; and red, AR. Scale bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

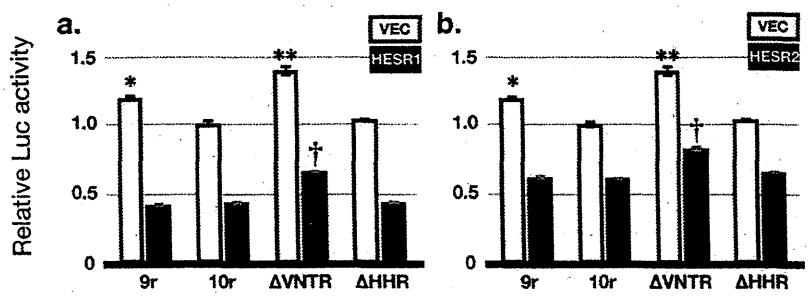

Fig. 3. Regional effects of the *DAT1* 3′-UTR on luciferase reporter activity. Relative luciferase activity of the reporter genes expressed with the empty control vector (a and b), HESR1 (a) or HESR2 (b) in the SH-SY5Y cells are shown. CP-Luc/exon15_9 repeats (9r), CP-Luc/exon15_10 repeats (10r), CP-Luc/exon15_ΔVNTR (ΔVNTR) and CP-Luc/exon15_ΔHHR (ΔHHR) were used. Values represent the means ± SEM. All values were standardized to the 10r with empty vector (VEC) group. *$P<0.05$ vs. the other values of VEC group; **$P<0.01$ vs. the other values of VEC group; and †$P<0.0001$ vs. the other values of HESR1 (a) or HESR2 (b) groups (Tukey–Kramer's HSD test after two-way ANOVA). Comparing the values of HESR1 or HESR2 groups with that of VEC group under each condition of luciferase reporter (9r, 10r, ΔVNTR and ΔHHR), each value of HESR1 or HESR2 groups was significantly lower than that of VEC (Student's *t*-test).

As shown in Fig. 3a, two-way ANOVA indicated a significant effect of transfected factors (VEC and HESR1; $F_{[1,31]}=1855.0$, $P<0.0001$), reporters (9r, 10r, ΔVNTR and ΔHHR; $F_{[3,31]}=82.5$, $P<0.0001$) and interaction of them ($F_{[3,31]}=10.3$, $P=0.0002$). In Fig. 3b, two-way ANOVA also indicated a significant effect of transfected factors (VEC and HESR2; $F_{[1,31]}=792.0$, $P<0.0001$), reporters ($F_{[3,31]}=62.1$, $P<0.0001$) and interaction of them ($F_{[3,31]}=9.5$, $P=0.0003$).

When VEC was co-expressed, the value of 9r was significantly different from the others ($P<0.05$, Fig. 3a and b). Under all conditions, values of ΔVNTR were significantly higher compared to the others in each group (VEC, $P<0.01$, Fig. 3a and b; HESR1 and HESR2, $P<0.0001$, Fig. 3a and 3b, respectively).

3.2.2. Effects of HESRs and AR on luciferase activity with 9r or 10r

The effect of AR both with and without HESRs on luciferase activity was examined with either 9r or 10r (Fig. 4). Values of VEC, HESR1 and HESR2 were the same as those in Fig. 3.

Two-way ANOVA indicated significant effect of transfected factors (VEC, AR, HESR1, HESR1 + AR, HESR2 and HESR2 + AR; $F_{[5,47]}=1098.6$, $P<0.0001$). The effect of reporters (9r and 10r) was not significant. However, two-way ANOVA indicated significant interaction of transfected factors and reporters ($F_{[5,47]}=27.5$, $P<0.0001$).

The value of 9r was significantly higher than that of 10r with transfection of VEC while significantly lower with transfection of AR (Student's *t*-test, $P<0.0001$). There was no other significant difference of 9r vs. 10r.

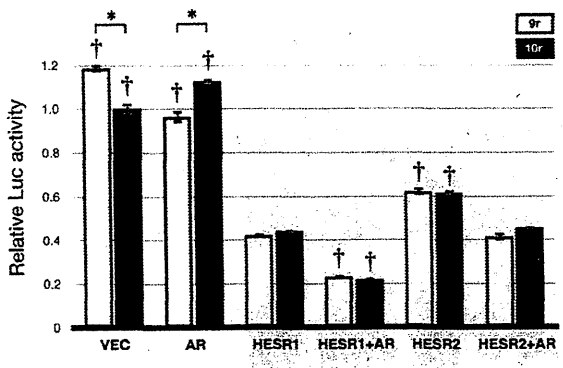

Fig. 4. AR facilitates the inhibitory effect of the HESRs. Relative luciferase activities of the reporter genes were measured when CP-Luc/exon15_9repeats (9r) and CP-Luc/exon15_10repeats (10r) with HESR1 or HESR2, and/or AR were co-expressed in SH-SY5Y cells. Values represent the means ± SEM. *$P<0.0001$, 9r vs. 10r (Student's *t*-test) and †$P<0.0001$ vs. the other values of the same repeats (9r or 10r) group (Tukey–Kramer's HSD test after two-way ANOVA).

Activities of the 9r reporter significantly decreased to 35.5%, 52.3%, and 81.3% upon expression of HESR1, HESR2, or AR, respectively, compared to the empty vector (VEC). Furthermore, luciferase activity with 9r significantly decreased to 19.5% upon co-expression of HESR1 and AR (HESR1 + AR) and to 34.9% with HESR2 + AR. With 10r allele, a similar tendency was observed, but in the presence of AR alone, activity of the 10r reporter significantly increased to 112.6%.

4. Discussion

4.1. Regional effects of the 3′-UTR in DAT1: an inhibitory role for the VNTR

It is possible that the 3′-UTR has an inhibitory role in gene expression by comparing the activity of CP-Luc and CP-Luc/exon15, as demonstrated in our previous studies [8,16], but it is unclear whether the VNTR domain itself inhibits DAT expression. To exclude this possibility, we demonstrated that the luciferase activity of ΔVNTR increased compared to that of VNTR-containing reporter genes, suggesting that the VNTR domain itself has a partial inhibitory role and may be a strong candidate *cis*-element for HESRs, even though luciferase activity was reduced in the presence of both HESR1 and HESR2 compared to the VEC control (Fig. 3).

4.2. Androgen receptor facilitates HESR-mediated inhibition of DAT1 expression

When designing these experiments, we were inspired by a report on prostate cancer and a VNTR association study. The research of prostate cancer reported that HESR1 is an androgen receptor-interacting factor [2]. It has also been shown that HESR1 is excluded from the nucleus in most human prostate cancers, raising the possibility that abnormal HESR1 subcellular distribution plays a role in the aberrant hormonal responses observed in prostate cancer. However, in the present study, co-localization of HESR1 or HESR2 and AR in the nucleus was observed without any abnormal cellular localization (Fig. 2). Protein expression of HESRs in the nucleus is consistent with data from our previous reports in SH-SY5Y cells and in mouse brain [8,16]. AR expression does not appear to affect HESR localization; however, data of a previous study [2] and ours suggest that HESRs interact with AR in the nucleus via formation of a protein complex, which then down-regulates DAT.

In the present study, we aimed to demonstrate the molecular biological associations among DAT, HESRs and AR. The large decrease in the presence of both HESRs and AR (Fig. 4) appears to be an additive effect, because both HESRs and AR alone exerted an inhibitory effect on the 9r reporter. With 10r

reporter a similar tendency is shown; however, in the presence of AR alone, the activity of the 10r reporter increased to 112.6%. Thus, the coupling of both an inhibitory and facilitative factor resulted in greater inhibition. Therefore, rather than a simple additive effect, a functional modification is believed to occur when the HESRs and AR are coupled, as reported in prostate cancer [2,32].

However, further element analysis of this gene is needed due to the presence of E-box sites in both the mouse and human 3′-UTRs of *DAT*, and because HESR1 and HESR2 may act through the core promoter in the CP-Luc, as shown in our previous report [16]. In any case, since the effect of AR differed depending on the VNTR allele (9r or 10r), the VNTR may be one of the most important elements in this region.

4.3. *A functional consideration: implication for the biological significance of HESRs and AR*

Here, we demonstrated that the interaction between HESRs and AR strongly inhibits the *DAT* reporter gene. Expression of AR in the midbrain dopaminergic regions is detected in the ventral tegmental area (VTA) rather than the substantia nigra (SN) [4]. Additionally, DAT expression is lower in the VTA than in the SN, together with regional differences in electrophysiological properties [18]. The distribution of Hesr1 or Hesr2 did not differ between the regions [16], but Hesrs may contribute to the regional difference in DAT expression by interacting with AR, which localizes specifically in the midbrain. Furthermore, this interaction may be associated with the fact that DAT expression is lower in males than in females [23].

A previous study indicated that the number of sexual partners of the 9r/9r genotype of *DAT1* is fewer than that of 9r/10r or 10r/10r carriers [11]. In Fig. 4, 9r and 10r displayed different responsiveness to AR. This could be the molecular basis for the different number of sexual partners, depending on the VNTR alleles (9r/9r or any 10r) [11] via synaptic tuning of dopamine by DAT, since both dopamine and androgen are strongly involved in sexual motivation [25].

Mutations of the *HESR* genes have not been reported in clinical studies of psychiatric or developmental disorders, but a recent study reported that HESR1 was up-regulated in cell lines derived from patients with an autism spectrum disorder whose disease rate is higher in males [26]. In our study, we demonstrated that the HESRs–AR interaction strongly inhibited *DAT* reporter gene expression. We previously demonstrated that HESR1 having a naturally occurring nonsynonymous SNP at codon 94 (Lue94Met, SNP ID rs11553421) in the HLH domain did not have an ability to repress the DAT reporter gene expression [8]. In addition, this SNP converts HESR1 from an androgen receptor corepressor to co-activator and abolishes HESR1-mediated activation of p53 [32], which has been reported as a schizophrenia susceptibility gene [1]. The VNTR of *DAT1* [3] and DAT expression level [17] are associated to ADHD, of which features are shared with autism spectrum disorder to a certain degree [24]. Taken together, we believe that HESRs together with the VNTR of *DAT1* and AR have important roles in psychiatric disorders, developmental delay and behavioral traits that should be further investigated.

5. Conclusion

We demonstrated that AR facilitates an inhibitory effect of HESR1 and HESR2, which act through the VNTR, on *DAT1* reporter gene expression. Based on these findings, functional analysis of this interaction should be conducted.

Acknowledgements

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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.neulet.2012.07.021.

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PLOS | CURRENTS MUSCULAR DYSTROPHY

The C2A domain in dysferlin is important for association with MG53 (TRIM72)

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Abstract

In skeletal muscle, Mitsugumin 53 (MG53), also known as muscle-specific tripartite motif 72, reportedly interacts with dysferlin to regulate membrane repair. To better understand the interactions between dysferlin and MG53, we conducted immunoprecipitation (IP) and pull-down assays. Based on IP assays, the C2A domain in dysferlin associated with MG53. MG53 reportedly exists as a monomer, a homodimer, or an oligomer, depending on the redox state. Based on pull-down assays, wild-type dysferlin associated with MG53 dimers in a Ca2+-dependent manner, but MG53 oligomers associated with both wild-type and C2A-mutant dysferlin in a Ca2+-independent manner. In pull-down assays, a pathogenic missense mutation in the C2A domain (W52R-C2A) inhibited the association between dysferlin and MG53 dimers, but another missense mutation (V67D-C2A) altered the calcium sensitivity of the association between the C2A domain and MG53 dimers. In contrast to the multimers, the MG53 monomers did not interact with wild-type or C2A mutant dysferlin in pull-down assays. These results indicated that the C2A domain in dysferlin is important for the Ca2+-dependent association with MG53 dimers and that dysferlin may associate with MG53 dimers in response to the influx of Ca2+ that occurs during membrane injury.

To examine the biological role of the association between dysferlin and MG53, we co-expressed EGFP-dysferlin with RFP-tagged wild-type MG53 or RFP-tagged mutant MG53 (RFP-C242A-MG53) in mouse skeletal muscle, and observed molecular behavior during sarcolemmal repair; it has been reported that the C242A-MG53 mutant forms dimers, but not oligomers. In response to membrane wounding, dysferlin accumulated at the injury site within 1 second; this dysferlin accumulation was followed by the accumulation of wild-type MG53. However, accumulation of RFP-C242A MG53 at the wounded site was impaired relative to that of RFP-wild-type MG53. Co-transfection of RFP-C242A MG53 inhibited the recruitment of dysferlin to the sarcolemmal injury site. We also examined the molecular behavior of GFP-wild-type MG53 during sarcolemmal repair in dysferlin-deficient mice which show progressive muscular dystrophy, and found that GFP-MG53 accumulated at the wound similar to

wild-type mice. Our data indicate that the coordination between dysferlin and MG53 plays an important role in efficient sarcolemmal repair.

Funding Statement

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Introduction

Dysferlin is a sarcolemmal protein, and dysferlin deficiency causes Miyoshi myopathy (MM) and limb girdle muscular dystrophy type 2B (LGMD2B) [1,2]. Based on the observation that dysferlin accumulates at wound sites in myofibers in a Ca^{2+}-dependent manner, dysferlin is thought to mediate Ca^{2+}-dependent sarcolemmal repair [3].

Mitsugumin 53 (MG53), also known as muscle-specific tripartite motif 72, is a recently identified protein involved in membrane repair in skeletal muscle [4]. Mice lacking MG53 suffer progressive myopathy [4], similar to dysferlin-null mice [3]. MG53 is localized in intracellular vesicles and plasma membranes in skeletal muscle, and it accumulates at injury sites in an oxidation-dependent, but not Ca^{2+}-dependent, manner [4].

MG53 interacts with dysferlin and caveolin-3 to regulate sarcolemmal repair [5]. When expressed in C2C12 myoblasts that lack endogenous MG53, damaged membrane sites cannot be repaired in the presence of GFP-dysferlin, however, co-transfection of MG53 and GFP-dysferlin in these myoblasts results in GFP-dysferlin accumulation at injury sites [5]. These findings indicated that recruitment of dysferlin to the injury site of the membrane depends on MG53. However, it remains unclear whether the absence of dysferlin perturbs recruitment of MG53 to the injury site for membrane repair. A previous report has demonstrated the association of dysferlin with MG53 with co-immunoprecipitation (IP) assays using mouse skeletal muscle and C2C12 myoblasts transfected with dysferlin and MG53 [5]. However, which protein domains participate in this interaction between dysferlin and MG53 and whether this interaction is dependent on Ca^{2+} remain unclear. MG53 oligomerizes via disulfide bonds [4] and forms homodimers via a leucine-zipper motif in the coiled-coil domain [6]. The interaction between dysferlin proteins and MG53 monomers or oligomers has not been characterized in detail. To understand the precise role of dysferlin and MG53 in sarcolemmal repair, it would be helpful to determine whether dysferlin associates with MG53 monomers, oligomers, or both in a Ca^{2+}-dependent manner.

Thus, to examine the biological role of the association between dysferlin and MG53, we used the following strategy to examine the effect of the absence of MG53 oligomers on dysferlin. We co-transfected mouse skeletal muscle with wild-type dysferlin-EGFP and RFP-tagged wild-type MG53 or a RFP-tagged MG53 mutant (RFP-C242A -MG53), and conducted a membrane-repair assay using a two-photon laser microscope. The C242A-MG53 mutant has been reported to form dimers, but not oligomers [6]. There is no report of simultaneous observation of dysferlin and MG53 during sarcolemmal repair; however, we have successfully performed real-time imaging of dysferlin-GFP and MG53-RFP after membrane injury in mouse skeletal muscle.

Dysferlin protein is absent or severely reduced in the skeletal muscle of patients with dysferlinopathy [7] and of SJL and A/J mice with mutations in the dysferlin genes [8]. To examine whether the absence of dysferlin affects the recruitment of MG53 to injury sites, we transfected skeletal muscle from dysferlin-deficient SJL and A/J mice with EGFP-MG53 and conducted membrane repair assays. These experiments are helpful in elucidating the

molecular pathology of dysferlinopathy and revealed that MG53 accumulated in the skeletal muscles of dysferlin-deficient mice, which develop progressive muscular dystrophy.

We present evidence indicating that efficient sarcolemmal repair requires both dysferlin and MG53.

Methods

Immunoprecipitation. To examine the interaction between MG53 and dysferlin, mouse gastrocnemius muscles were lysed in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, and Complete mini EDTA-free protease inhibitor cocktail (Roche) [9] supplemented with 1 mM $CaCl_2$ or 2 mM EGTA. Lysates pre-cleared with Protein A/G agarose (Pierce) were incubated with polyclonal antibodies against mouse MG53 [4] or mouse dysferlin; the anti-dysferlin antibody was made in rabbit by injecting bacterial recombinant protein containing residues 1669 to 1790. The immunoprecipitated proteins were separated by SDS-PAGE and detected on immunoblots using the same antibodies used for IP or the anti-human dysferlin monoclonal antibody, NCL-Hamlet (Novocastra Laboratories).

A human MG53 cDNA was amplified by PCR and subcloned into pFLAG-CMV-4 (Sigma). Wild-type and truncated human dysferlin that were each tagged with c-myc were generated previously [10]. We also created five truncated human dysferlin constructs with the C2A domain (aa 1-149, 1-349, and 1-1080) and without the C2A domain (aa 130-2080 and 1081-2080). The sequence of each construct was verified by DNA sequencing. FuGENE 6 or E-xtremeGENE 9 (Roche) was used to transiently transfect COS-7 cells with MG53 and wild-type or mutant dysferlin constructs. Transfectants were cultured for 48 h and subsequently lysed in the same lysis buffer used to lyse mouse muscle, except that this buffer lacked $CaCl_2$ and EGTA. Lysates pre-cleared with Protein G-Sepharose (GE Healthcare) were incubated with anti-FLAG (M2, Sigma) or anti-c-myc (9E10, Santa Cruz Biotechnology) monoclonal antibodies; Protein G-Sepharose was then added. Immunoprecipitated proteins were analyzed by immunoblotting using M2 and anti-c-myc polyclonal (A14, Santa Cruz Biotechnology) antibodies.

Pull-down assay. Fragments of the dysferlin C2A domain (corresponding to aa 1-129 of human dysferlin) were amplified as cDNA by PCR and subcloned into pGEX-5X-3 (GE Healthcare). Dysferlin p.W52R (TGG to CGG at c.527-529) and p.V67D (GTG to GAT at c.572-574) mutations were introduced by PCR using appropriate primers. GST fusion proteins expressed in BL21 *E. coli* were purified using sarkosyl [11] and bound to glutathione Sepharose 4B (GE Healthcare). COS-7 cells overexpressing FLAG-tagged human MG53 were lysed in lysis buffer containing 10 mM Na_2HPO_4, 1.8 mM KH_2HPO_4, 1% NP-40 (pH 7.4), 2 mM EGTA, various concentration of $CaCl_2$, and Complete mini EDTA-free protease inhibitor cocktail. EGTA was used to chelate the free Ca^{2+} in solution and $CaCl_2$ at various concentrations. The free calcium concentration was calculated using the free software CALCON3.6. Lysates were centrifuged to remove cellular debris, supplemented with 5 mM N-methylmaleimide (NEM) or 5 mM dithiothreitol (DTT), and finally subjected to protein cross-linking by treating with 2 mM glutaraldehyde (GA) for 5 min at room temperature, which was quenched with 100 mM Tris-HCl (pH 7.5) [6]. The cross-linked lysates were diluted with 75 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 2 mM EGTA, various concentrations of $CaCl_2$, and Complete mini EDTA-free protease inhibitor cocktail. Lysates pre-cleared with GST bound to glutathione Sepharose 4B were divided into aliquots and incubated with wild-type, p.W52R, and p.V67D dysferlin C2A-GST fusion protein bound to beads for 2 hr at 4°C. After three washes in lysis buffer containing 75 mM Tris-HCl (pH 7.5), 2× sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% (v/v) glycerol, and 0.004% bromophenol blue) was added to the beads, and the mixtures were incubated for 10 min at 85°C. Bound proteins were separated by SDS-PAGE and subjected to immunoblotting with the anti-FLAG antibody M2.

In vivo transfection and membrane repair assay. Twenty micrograms of N-terminal RFP-tagged human MG53 cDNA/pcDNA3.1 and/or C-terminal GFP-tagged human dysferlin cDNA/pcDNA3.1 plasmid DNA were injected into

the flexor digitorum brevis of anesthetized, 4-week-old male C57BL6J and dysferlin-deficient SJL and A/J mice. Electroporation of plasmid DNA was performed using an electric pulse generator (CUY21SC, NEPAGENE) as described previously [12]. Seven days after electroporation, skeletal muscle myocytes (for whole-mount viewing) or individual myofibers were isolated and subjected to plasma membrane injury created by a two-photon laser microscope, LSM 710NLO with GaAsp Detectors (Zeiss) and Chameleon Vision II System (Coherent)[3]. Myofiber wounding using the 820-nm infrared laser and resealing analysis based on the kinetics and extent of FM1-43 or 4-46 dye (Molecular Probes) entry through open disruptions was carried out as previously described [3,13,14].

Ethics Statement. All experiments involving animals were performed according to the Procedure for Handling Experiments involving Animals of AIST (National Institute of Advanced Industrial Science and Technology) and approved by the Institutional Animal Care and Use Committee of AIST.

Results

Association of MG53 and dysferlin in mouse skeletal muscle

We used an IP assay with protein from mouse muscle to confirm that endogenous MG53 associates with dysferlin *in vivo*. MG53 and dysferlin associated only in the absence of EGTA and $CaCl_2$ (Fig. 1). The same result was obtained using C2C12 myotubes (data not shown). MG53 was specifically co-immunoprecipitated by the anti-dysferlin antibody, and conversely dysferlin was specifically co-immunoprecipitated by the anti-MG53 antibody. Thus, we confirmed that endogenous MG53 and endogenous dysferlin form a protein complex in mouse skeletal muscle without EGTA or $CaCl_2$ supplementation.

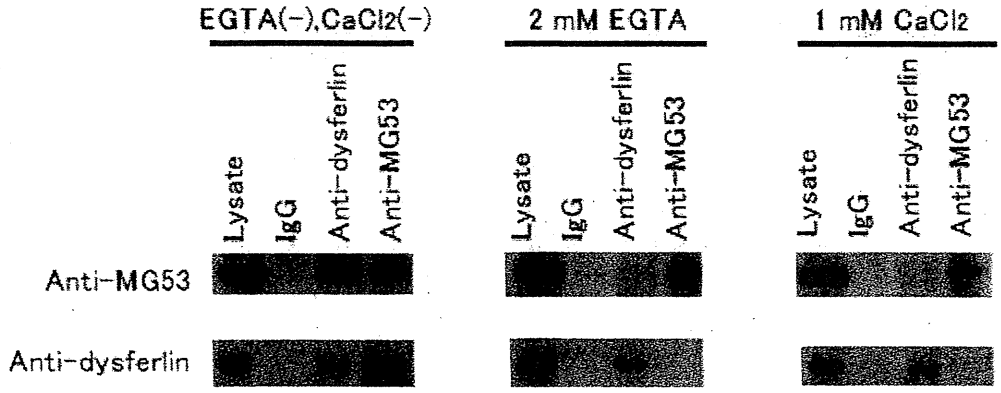


Fig. 1: IP assay of dysferlin and MG53.

MG53interacts with dysferlin in mouse skeletal muscle. Extracts from wild-type mouse skeletal muscle were subjected to IP with polyclonal anti-MG53 antibodies or polyclonal anti-dysferlin antibodies. Immunoprecipitated proteins were subjected to SDS-PAGE and visualized on immunoblots treated with the same antibodies that were used for IP.

Identification of the MG53-associating domain of dysferlin

Next, we used IP to define the region of dysferlin that associates with MG53. Specifically, we used transient co-transfection to introduce a construct encoding full-length human MG53 tagged with FLAG and a construct encoding human dysferlin tagged with c-myc into COS-7 cells; for each co-transfection, full-length dysferlin or one of five deletion mutant forms of tagged dysferlin was used (Fig. 2). For deletion mutants that lacked the C-terminal domain of dysferlin, the transmembrane domain of dysferlin was retained to increase protein stability [10]. Transfectants were lysed in the same buffer that was used for IP assays of mouse skeletal muscle extract, except that this buffer lacked EGTA and $CaCl_2$. Full-length dysferlin and deletion mutants that retained the N-terminal C2 (C2A) domain of dysferlin were co-immunoprecipitated by anti-MG53 antibody. In contrast, dysferlin mutants that lacked this N-terminal domain, Δ2-1080 and Δ2-129, failed to interact with MG53. These results indicated that the C2A domain of dysferlin was necessary for association with MG53.

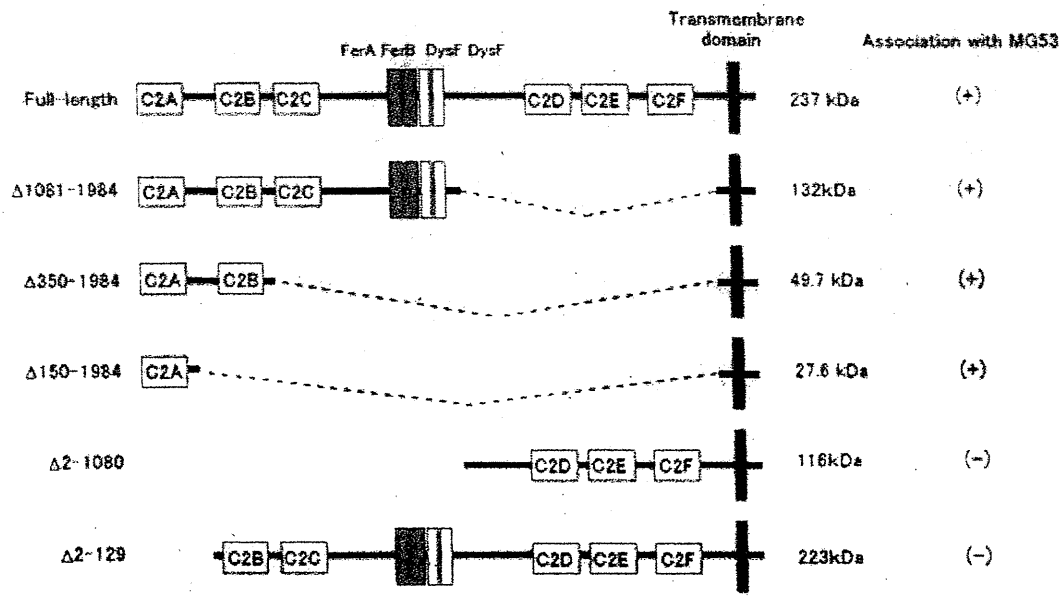


Fig. 2: Identification of MG53-binding region of dysferlin.

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.

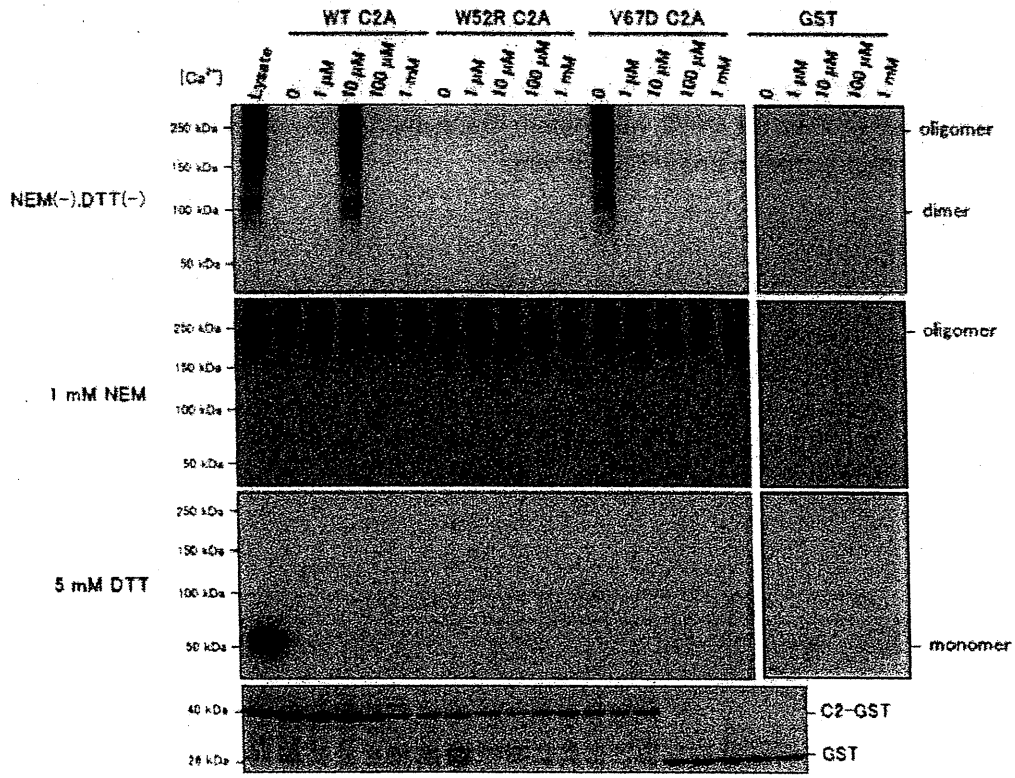
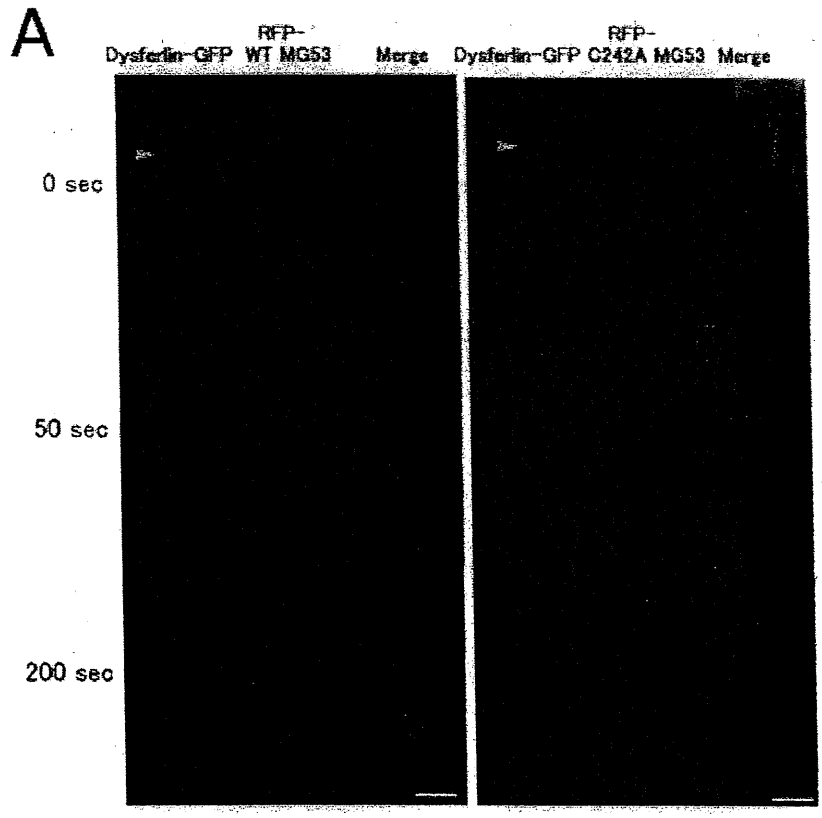


Fig. 3: Pull-down assay of dysferlin C2A-GST and MG53.

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).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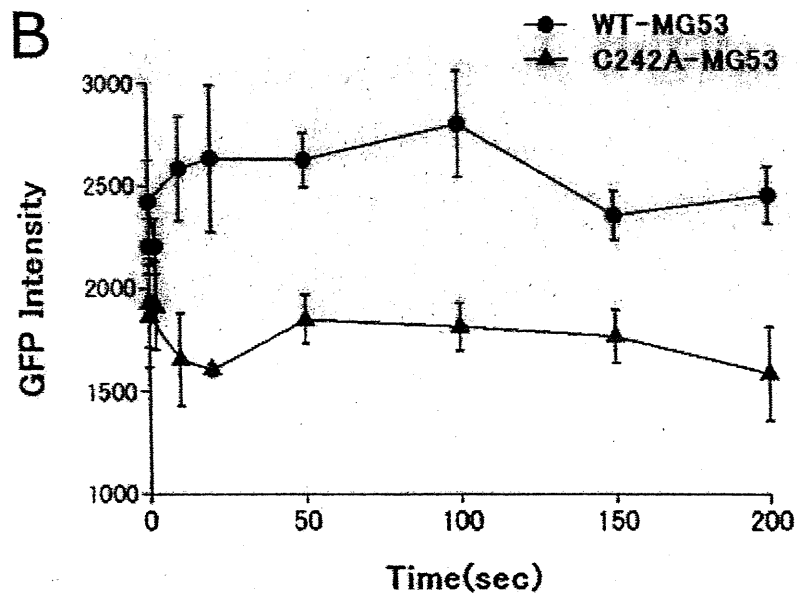

Fig. 4: Membrane repair assay of myofiber transfected with dysferlin-GFP and RFP-MG53.

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.

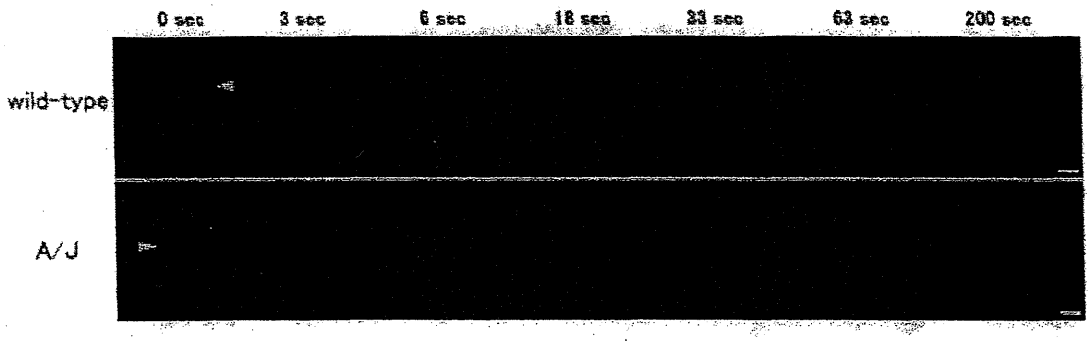

Fig. 5: Membrane repair assay of myofiber using dysferlin-deficient myofiber transfected with GFP-MG53.

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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