tively, are reported to have the A allele. In our control population, the A allele frequency was 34.0%, indicating that this allele is relatively common in Mongoloids and Hispanics.

The mechanism by which SNP rs1076560 affects alcoholic susceptibility has not yet been clarified, but alternative splicing of *DRD2* may play a role. SNP rs1076560 is located in intron 6, 1.4 kb downstream from alternative exon 6 and 83 bp upstream from exon 7. The *DRD2* transcript region including SNP rs1076560 is purine-rich (5′-AGAGG G(**G/U**)GAA AGGGA GGGG-3′), and a purine-rich region in an intron has been reported to promote splicing of its upstream exon [2].

To determine whether rs1076560 affects the alternative splicing of DRD2, we are currently trying to carry out a splicing assay using minigenes that contain allele A or allele C. The studies of D2L knockout mice [11] and cultured cells [7] show distinct functions of D2L and D2S, so the difference in the alternative splicing of DRD2 may affect the dopamine system, which has an important role in alcoholism. Additional evidence for a relationship between *DRD2* and alcohol-drinking behavior was provided by an animal study in which ethanol treatment of primary cells was found to alter *DRD2* splicing [4]. In conclusion, SNP rs1076560 may affect alternative splicing of the *DRD2* gene and the balance of DRD2 isoforms, causing different individuals to have different reactions to alcohol.

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Expression of MBNL and CELF mRNA transcripts in muscles with myotonic dystrophy

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Abstract

Myotonic dystrophy type 1 (DM1) is an autosomal dominant disorder that causes muscle wasting, myotonia, cardiac conduction abnormalities, and other multi-systemic symptoms. Current evidence supports a pathogenic mechanism involving aberrantly expanded CTG repeats in the 3′-untranslated region of the DM protein kinase (*DMPK*) gene. The repeats are thought to recruit various RNA-binding proteins such as muscleblind-like (MBNL) proteins into foci in the nuclei of DM cells, resulting in loss of function. However, aberrant regulation of transcription or subsequent RNA processing of MBNL-family mRNAs might also be part of the pathogenic mechanism of DM. We used real-time RT-PCR analysis to examine the possibility that MBNL mRNA expression is altered in DM1 patients. We also examined mRNA expression for members of the CUG-BP and ETR-3-like factor (CELF) family of RNA-binding proteins given that CELF proteins regulate alternative splicing and are also implicated in DM. We found that DM1 muscles displayed aberrant regulation of alternative splicing as reported previously; however, the levels of MBNL and CELF mRNA expression did not show any significant changes. Our results suggest that the expression and stability of the mRNA for these RNA-binding proteins are unaffected in DM1.

Keywords: Alternative splicing; CUG-BP- and ETR-3-like family; Muscleblind; Myotonic dystrophy

1. Introduction

Myotonic dystrophy (DM) is an autosomal dominant disorder and the most common form of muscular dystrophy affecting adults [1]. Multiple systems are affected in DM patients, and characteristic symptoms include muscle hyperexcitability (myotonia), cataracts, defects in cardiac conduction, mental retardation, and insulin resistance [1].

Two forms of DM have been identified thus far, DM1 and DM2. The gene affected in DM1 is *DM protein kinase* (*DMPK*) on chromosome 19q. This gene contains CTG trinucleotide repeats within its 3′-untranslated region (UTR) [2,3]. The expansion of this repeat has been known to trigger the pathogenesis of DM1 and interestingly, the number of repeats is thought to be correlated with symptom severity [3].

The gene affected in DM2 is *zinc-finger protein 9* (*ZNF9*). This gene contains CCTG tetranucleotide repeats in intron 1, and as in DM1, expansion of this repeat is believed to be the cause of this disease [4]. The most strongly supported pathogenic hallmark is that the expanded repeat-containing mRNA transcribed from the altered *DMPK* and *ZNF9* genes forms foci that are retained within the nuclei of DM cells [4–6]. Given that DM1 and DM2 have phenotypic overlap in spite

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of the different loci of *DMPK* and *ZNF9*, this finding suggests that the expanded repeats themselves cause DM. Indeed, transgenic mice expressing human skeletal actin containing expanded CUG repeats manifest some DM symptoms, including myotonia [6].

The nuclear foci found in DM cells appear to recruit certain RNA-binding proteins, thus disrupting the proper functions of these proteins (loss of function) [7,8]. One family of RNA-binding proteins thus affected is the muscleblind (MBNL) family, consisting of MBNL1, MBNL2, and MBNL3 in humans [8]. Each of these proteins has been shown to co-localize with RNA foci in both DM1 and DM2 cells [8]. The MBNL proteins are zinc-finger proteins that bind to both CUG and CCUG repeats [9,10], and act as regulators of alternative splicing of certain genes that are strongly implicated in some of the symptoms of DM1. In particular, the myotonia and insulin resistance of DM1 are caused by defects in chloride channel and insulin receptor (IR) proteins, respectively, that arise because the MBNL protein is trapped in the nuclear foci and thus suffers loss of function [11–13]. Importantly, MBNL1-knockout mice exhibit some DM symptoms such as myotonia and cataracts [11], which strongly supports the involvement of MBNL proteins in the pathogenesis of DM1.

Another group of proteins strongly implicated in DM pathogenesis is the CUG-BP and ETR-3-like factor (CELF) family, which regulates alternative splicing [14–19], translation [20,21], and deadenylation [22]. Upregulation of CUG-BP protein expression has been implicated in the DM1 mechanism [14,23], suggesting that abnormal activation of CUG-BP might be involved in DM1 pathogenesis. In addition, some studies have suggested a strong involvement of CELF in DM pathogenesis, indicating that CUG-BP transgenic mice exhibit DM-like symptoms [23,24]. CUG-BP and other CELF members can regulate alternative splicing of various pre-mRNAs that are important in DM1 pathogenesis, including pre-mRNAs for IR and cardiac troponin T (cTNT) [16,24–28].

Loss of function of MBNL proteins [11,13] and altered activity [23,24,29] or localization of CELF proteins [30] have been suggested as possible pathogenic mechanisms in DM. However, aberrant regulation of transcription or subsequent RNA processing of MBNL- and CELF-family mRNAs might also be part of the DM mechanism. To our knowledge, however, this has not been previously addressed.

The functions of non-coding RNA have been the subject of increased attention. These functions include roles in DNA replication, chromosome maintenance, and regulation of transcription, as well as RNA processing, translation, and mRNA stability. One study reported that expanded CUG repeats in *DMPK* tend to form a double-helical RNA hairpin, which could be a source of microRNA and/or small interfering RNA. It also noted that MBNL contains a sequence that is almost complementary to the CUG repeats of *DMPK* [31]. These findings suggest that the CUG repeats in *DMPK* mRNA may silence expression of MBNL1, although this mechanism has not been confirmed experimentally.

In the present study, we examined whether altered expression of MBNL and CELF proteins has any relevance for understanding DM pathogenesis. Using RT-PCR, we compared the expression of MBNL and CELF mRNA in DM1 patients and non-DM individuals to determine whether expression is indeed altered in DM1.

2. Materials and methods

2.1. Tissue samples

Biopsy materials were obtained from the biceps (brachii muscle) or quadriceps (femoris muscle) of 20 DM1 patients (11 males and 9 females, 11–68 years old) and 12 confirmed non-DM individuals with no histological abnormality. All samples were stored at −80 °C. Clinically, all DM1 samples had muscle weakness with myotonia. Myotonic discharge that was detectable by EMG. Onset was during childhood or adolescence except for a 21-year-old patient who had congenital onset. Pathologically, all DM1 samples showed myopathic change with variation in fiber size. Some displayed fibers with internalized nuclei (>5%; 16/20 patients, 90%), type 1 fiber predominance (10/20, 50%), endomysial fibrosis (16/20, 80%), adipose tissue replacement (6/20, 30%), sarcoplasmic mass (7/20, 35%), and pyknotic nuclear clumps (17/20, 85%). Relatively extensive fibrosis was seen in three patients and mild adipose tissue replacement in two.

All biopsy materials used in this study were acquired with informed consent.

2.2. Real-time RT-PCR

Total RNA was isolated from the biopsy samples using Concert™ Cytoplasmic RNA Reagent (Invitrogen, Carlsbad, CA), treated with DNase, and purified by standard phenol–chloroform extraction and isopropanol precipitation. cDNA synthesis was performed using a template consisting of 100 ng total RNA and the ThermoScript™ RT-PCR System (Invitrogen) with a mixture of oligo$(dT)_{20}$ and random hexamers on a 10-μl scale. The cDNA was then diluted 50-fold with sterile water, and 10 μl of diluted cDNA was used for the real-time RT-PCR measurements.

All primers used for generation of a standard curve template and for the quantification of IR-A, IR-B, GAPDH, β-actin, HPRT, MBNL and CELF mRNAs were designed using Primer 3 software (Whitehead Institute for Biomedical Research, Totowa, NJ). The primer sequences (shown $5' \rightarrow 3'$) were as follows. IR-A:

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TGCTG CTCCT GTCCA AAGAC and GAGAT GGACT GGGGA CGAAA; IR-B: TTCGT CCCCA GAAAA ACCTC and CACCG TCACA TTCCC AACAT; GAPDH: GAGTC AACGG ATTTG GTCGT and AATGA AGGGG TCATT GATGG; β-actin: GACAG GATGC AGAAG GAGAT TACT and TGATC CACAT CTGCT GGAAG GT; HPRT: TGAGG ATTTG GAAAG GGTGT and CATCT CGAGC AAGAC GTTCA; MBNL1: GGGTT TGTTG GTTTC ACTG and TGTCC CGAAT TGGTG TGA; MBNL2: CCACC ACGCC TGTTA TTGTT and CCCTG CATAC CTCCA GTTTG; CUG-BP: CTGGA AGCCA GAAGG AAGGT and GCAGG TCCTG ATCAC CAAAC; ETR-3: CAGGG TGATG TTCTC TCCAT TT and GCCTC GACTC AGCCC ATC.

Total RNA from skeletal muscle (BD Biosciences, Franklin Lakes, NJ) was reverse-transcribed to synthesize the cDNA, which was then used as the template for the standards. Three housekeeping genes were chosen as calibration standards: β-actin, HPRT, and GAPDH.

cDNA calibrators were prepared by PCR amplification of HEK293 cell cDNA with the primers listed above. The resulting PCR products yielded unique bands by agarose gel electrophoresis and were purified by gel extraction using GeneEluteTM Agarose Spin Columns (Sigma, St. Louis, MO) followed by standard phenol–chloroform extraction. The concentrations of these calibrators were determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE). Concentrations were calibrated from 1.0×10^{-5} to 1.0×10^{-9} μg/μl by serial 10-fold dilutions. The PCR products above were cloned into pGEM®-T-Easy vector (Promega, Madison, WI) and their sequences were confirmed with the CEQTM 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA).

Real-time PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Premix Ex Taq™ (TaKaRa Bio, Tokyo, Japan). The thermal profile consisted of an initial incubation at 95 °C for 10 s followed by 40 cycles at 95 °C for 5 s and 60 °C for 34 s. To assure specific amplification, dissociation temperatures were measured after each run. The 7300 Real-Time PCR System software was used to determine the crossing points for the individual samples, including those for the calibration standards. The expression levels of the target genes were normalized relative to expression of the β-actin gene.

For each run, data acquisition and analysis was performed using the 7300 Real-Time PCR System software. As all samples were available only in a limited amount, the mean values and *p*-values were determined by Student's *t*-test, but not the variance.

2.3. Splicing assays

cDNAs produced as described above were used as templates for the RNA splicing assay. PCR was performed using the primers and thermal conditions described below. For the cTNT splicing assay, the forward and reverse primers (shown 5′ → 3′) were ATAGA AGAGG TGGTG GAAGA GTAC and GTCTC AGCCT CTGCT TCAGC ATCC, respectively; 35 cycles of amplification were performed, each consisting of 30 s at 96 °C, 30 s at 63 °C, and 30 s at 72 °C, followed by a final 5 min extension at 72 °C. For the IR splicing assay, the forward and reverse primers were CCAAA GACAG ACTCT CAGAT and AACAT CGCCA AGGGA CCTGC, respectively; 35 cycles of amplification were performed, each consisting of 30 s at 96 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by a final 5 min extension at 72 °C. The PCR products were resolved on a 10% polyacrylamide gel that was stained with ethidium bromide and analyzed using an LAS-3000 luminescence image analyzer (Fujifilm, Tokyo, Japan).

3. Results

3.1. Aberrant splicing in DM1

We used RT-PCR to examine several genes already known to undergo abnormal alternative splicing to determine whether aberrant regulation of alternative splicing was evident in the study samples. Cardiac troponin T (cTNT) is known to shift from an immature isoform to a mature isoform during heart development and it has been reported that cardiac tissues and skeletal muscle from DM1 patients display an inappropriate retention of fetal exon 5 of cTNT [32]. Our RT-PCR analysis using RNA isolated from 20 DM patients and 12 non-DM individuals showed significant promotion of exon 5 inclusion in DM skeletal muscle (Fig. 1a), thus supporting the previous findings. Furthermore, as was also reported previously, inclusion of exon 5 was notably promoted in DM tissues, with a 1.8-fold increase in the average ratio of exon 5 inclusion (Fig. 1b).

Alternative splicing of the 36-nucleotide exon 11 of the insulin receptor (IR) gene yields two isoforms, IR-A (exon 11 removed) and IR-B (exon 11 retained). The IR-B isoform is predominant in the insulin-responsive tissues that are responsible for glucose homeostasis, such as adipose tissues, liver, and skeletal muscle whereas in skeletal muscle from DM1 patients, the IR-A isoform is most common [14]. This switching of IR isoforms has not been observed in other myopathies [14]. We found that retention of IR exon 11 was strongly suppressed in DM1 patients, whereas IR-B was predominant in non-DM individuals. The average ratio of exon 11 retention decreased from 0.56 (non-DM) to 0.26

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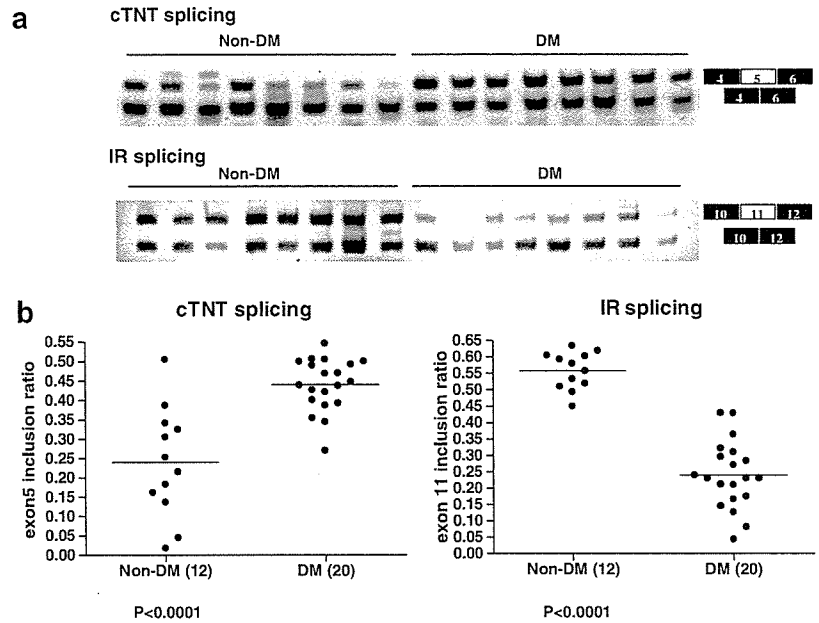

Fig. 1. Alternative splicing of cardiac troponin T (cTNT) and insulin receptor (IR) RNA. (a) Splicing products obtained by RT-PCR amplification of RNA isolated from non-DM control ($n = 8$) and DM1 ($n = 8$) biopsy samples. The exon utilization for each splicing product is shown. (b) Quantification of the RT-PCR products was performed among 20 DM1 samples and 12 non-DM individuals by using LAS-3000 luminescence image analyzer (Fujifilm). The graphs show the ratio of the alternative exon inclusion of cTNT and IR.

(DM; Fig. 1b). Therefore, the biopsy tissues used in this study manifested aberrant regulation of alternative splicing – a characteristic feature of DM pathogenesis.

3.2. Expression of mRNAs for MBNL1, MBNL2, CUG-BP, and ETR-3

An important consideration in interpreting the observed changes in splicing patterns is that both MBNL loss of function and CELF activation could explain our results [12]. For example, the retention of cTNT exon 5 is promoted by CELF but repressed by MBNL proteins. Conversely, the retention of IR exon 11 is promoted by MBNL but repressed by CELF proteins. Therefore, we sought to determine whether the observed altered splicing patterns resulted from loss of MBNL function and/or activation of CELF function.

To determine the degree of change detectable by this method, we measured the expression of insulin receptor isoform B (IR-B), which has been shown by gel electrophoresis to be downregulated in DM patients (Fig. 1). The results indicated that, as predicted, the DM patients expressed significantly less IR-B compared to the non-DM group (Fig. 2a). However, nine DM samples (out of 20) showed particularly high levels of downregulation such that their expression levels did not reach the range in which the accuracy of this method is assured by the standard curve. However, when these data were omitted from the analysis, this quantification method still successfully showed a 2- to 3-fold difference in expression levels, thus testifying to the suitability of this method for our experiment. Expression of IR-A was also measured and no significant differences were observed between non-DM and DM patients (Fig. 2a), as was expected from the results shown in Fig. 1.

We also performed real-time PCR analysis to measure the expression of MBNL and CELF mRNA. Total RNA was extracted from 20 DM and 12 non-DM biopsy samples, and the RNA was reverse-transcribed using a mixture of oligo(dT) and random hexamers. To allow for rigorous calibration of the data, we also examined mRNA expression for three commonly used housekeeping proteins, β-actin, GAPDH, and hypoxanthine–guanine phosphoribosyl transferase (HPRT). Comparing the relative mRNA level of each protein to that of the other two mRNAs allowed us to identify the gene with the most stable expression. We found that while the HPRT/GAPDH and β-actin/GAPDH ratios exhibited significant variation, the HPRT/β-actin ratio remained relatively constant among all smples. This indicated that HPRT and β-actin were reliable housekeeping genes; therefore, we selected the β-actin gene for normalization of the real-time PCR data.

We found no significant differences between DM and non-DM individuals with respect to mRNA levels for MBNL1, MBNL2, CUG-BP, and ETR-3 using real-time PCR (Fig. 2b), suggesting that the expression or stability of these mRNAs is not affected in DM. However, as shown in Fig. 2b, substantial individual variation

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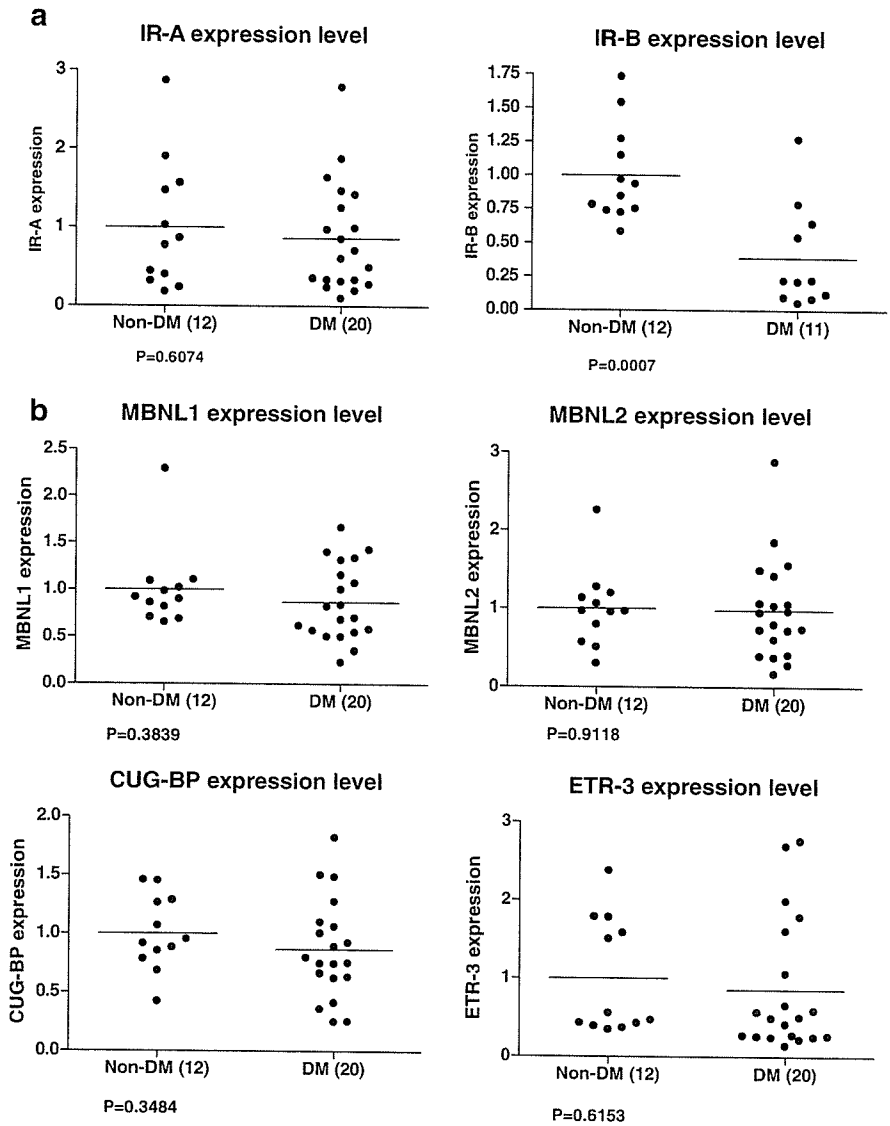

Fig. 2. (a) Expression of isoform A and B of insulin receptor in DM and non-DM samples determined by Real-time PCR. (b) Real-time RT-PCR analysis of relative expression levels of MBNL1, MBNL2, CUG-BP, and ETR-3 mRNAs in 21 DM-patients and 12 non-DM individuals. All data were normalized relative to β-actin mRNA expression. The values shown in the graphs have been divided by the average value for non-DM individuals.

in levels of these mRNAs was observed. We had predicted that the CELF and MBNL mRNA levels would be correlated with the extent of altered splicing seen in DM patients; however, our results did not support this hypothesis. Instead, DM individuals exhibiting significantly increased retention of cTNT exon 5 did not necessarily display significant abnormalities in IR splicing. Furthermore, mRNA expression of the four RNA-binding proteins examined in this experiment did not result in a significant change in IR splicing (data not shown).

The mRNA expression levels for the remaining MBNL and CELF proteins were so low that quantifying them was technically difficult in both DM and non-DM individuals. Previous reports have also described low expression levels for these proteins in muscles of normal individuals [33,34].

4. Discussion

The objective of this study was to determine whether a difference exists in mRNA expression levels of MBNL and CELF proteins between DM and non-DM muscles. Measurement by real-time RT-PCR showed that mRNA expression levels for these proteins were not significantly altered in DM patients. However, splice variants of MBNL proteins were not distinguished in this experiment because the primers for the real-time PCR assay were designed to amplify a fragment common to

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all the known variants. MBNL1 has at least nine variants [10], some of which lack zinc-finger motifs and therefore probably do not interact with mRNA. The relevance of the other variants remains largely unknown. The observation that the MBNL1 isoform with exon 5 retained localizes in nuclei, where alternative splicing takes place, and therefore it has the largest potential (i.e., the most opportunity) to influence alternative splicing is noteworthy (Kino et al., unpublished data). Thus, the abundance ratio of these variants may be altered in DM1, which may influence MBNL1 function in cells.

We also found that levels of CUG-BP and ETR-3 mRNA were not significantly different for DM and non-DM individuals. During heart development, CUG-BP and ETR-3 undergo strong downregulation, whereas MBNL1 and MBNL2 expression is maintained [35]. Our findings suggest that expression of CUG-BP and ETR-3 mRNA is regulated normally throughout muscle development in DM patients. Whether the expression of CUG-BP and ETR-3 is altered at the protein level in DM patients is thus an important question. While increased levels of CUG-BP protein have been observed in DM1 tissues [14,23], this increase could be a result of altered posttranscriptional processing affecting translation, phosphorylation, or proteolytic degradation rather than altered regulation of transcription or mRNA stability.

In summary, our data confirmed the occurrence of aberrant splicing regulation in DM1 patients and demonstrated that these abnormalities are not associated with any altered expression of MBNL or CELF mRNA.

Acknowledgments

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Neuroligins 3 and 4X interact with syntrophin-γ2, and the interactions are affected by autism-related mutations

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Abstract

Recently, neuroligins (NLs)3 and 4X have received much attention as autism-related genes. Here, we identified syntrophin-γ2 (SNTG2) as a *de novo* binding partner of NL3. SNTG2 also bound to NL4X and NL4Y. Interestingly, the binding was influenced by autism-related mutations, implying that the impaired interaction between NLs and SNTG2 contributes to the etiology of autism.

Keywords: Autism; Neuroligin; Scaffolding protein; Syntrophin

Neuroligin (NL) is a neural cell adhesion molecule, which was identified as a ligand for β-neurexin [1]. Rodents possess three NLs, all of which interact with β-neurexin, while humans have five NLs. The trans-synaptic interaction between NL and β-neurexin strongly induces both pre- and postsynaptic maturation [2–4]. Interestingly, mutations of human *NL3* and *NL4X* are implicated in autism and mental retardation [5–9]. Autism is characterized by impaired reciprocal social interaction and communication and restricted, stereotyped patterns of interests and activities. Here, we investigated the *de novo* binding partners of NL3 using yeast two-hybrid screening, and found that syntrophin-γ2 (SNTG2) interacted with NL3, NL4X, and NL4Y, which are autism-related NL isoforms. Interestingly, the interactions between the NLs and SNTG2 are very noteworthy in terms of the etiology of autism because they are influenced by autism-related mutations.

Materials and methods

Plasmid construction. Tag sequences and cDNA fragments were cloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) or in pECFP vector (Clontech, Palo Alto, CA) using conventional molecular biological techniques. The nucleotide sequences of the DNA inserts were confirmed using sequence analysis. The following NLin (intracellular region of human neuroligin) constructs were made: NL3in (731–848 aa), NL4Xin (698–816 aa), and NL4Yin (698–816 aa). For ΔPBM mutants, the C-terminus of NLs (six amino acids: HSTTRV) was deleted. For ΔTMD32 mutants, TMD32, the N-terminus 32 amino acids of NL3in, was deleted (see Fig. 1). The YFP-NLs contained each NL signal sequence followed by the enhanced yellow fluorescent protein (EYFP) and the mature N-terminus of each of the NLs inserted into pcDNA3.1 expression vector. The Myc × 6 tagged NLs also contained each NL signal sequence followed by Myc × 6 tags and the mature N-terminus of each of the NLs inserted into pcDNA3.1 expression vector. The full-length human syntrophin-γ2 (NCBI Accession No. NP_061841, hSNTG2) was inserted into the revised pACT2 vector to express the GAL4AD-fusion proteins in the yeast two-hybrid system. The FLAG-tagged SNTG2 contained three FLAG tags followed by the N-terminus of SNTG2. The Myc-tagged SNTG2 contained SNTG2 followed by six Myc tags.

Antibodies. We purchased the following rabbit polyclonal antibodies: anti-GFP (Molecular Probes, Eugene, OR) and anti-FLAG (Sigma, St. Louis, MO). In addition, we obtained the following mouse monoclonal antibodies: anti-synaptophysin (Chemicon), anti-MAP2, anti-FLAG M2 (Sigma), anti-Myc, and anti-V5 (Invitrogen).

Abbreviations: NL, neuroligin; PDZ, PSD/Dlg/ZO-1 homology; PSD, postsynaptic density; DIV, days *in vitro*; CNS, central nervous system.

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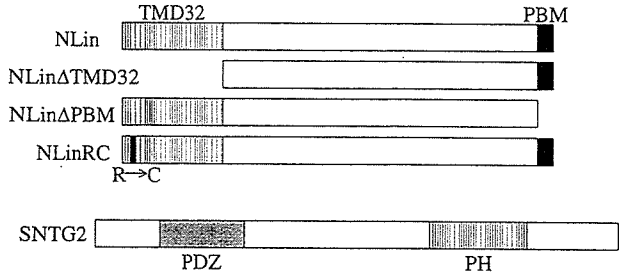

Fig. 1. Schematic depiction of the constructs of NLs and SNTG2. Vertical-striped, black, gray, and horizontal-striped rectangles show the TMD32 domain, PDZ binding motif (PBM), PDZ domain, and PH domain, respectively.

Western blotting. Samples were run on 7.5% SDS–polyacrylamide gels and transferred to PVDF membranes (Immobilon-P; Millipore). The membranes were blocked with 5% skim milk in TPBS (PBS with 0.05% Tween®20) for 1 h at room temperature and then incubated for 1–2 h with primary antibodies in TPBS. After washing, the membranes were incubated for 1 h with HRP-conjugated secondary antibodies. The immunoreactive bands were visualized using ECL.

Yeast two-hybrid screening. Yeast two-hybrid assays were performed using the MATCHMAKER GAL4 Two-Hybrid System (Clontech). The cDNA fragments encoding the intracellular region of human NL3 (711–828 aa, NCBI Accession No. NP_061850, hNL3in) were amplified by PCR and cloned into the *Eco*RI and *Bam*HI restriction sites of the pAS2-1C vector to serve as bait in the yeast two-hybrid screening. The pAS2-1C vector is the pAS2-1 vector (Clontech) revised as a single-copy plasmid in yeasts. The nucleotide sequences of the DNA inserts were confirmed by sequence analysis to verify that the inserts did not contain mutations. The human fetal brain MATCHMAKER cDNA library in the pACT2 vector (Clontech) served as prey in the yeast two-hybrid screening. The inserts were expressed as fusion proteins with the DNA-binding and DNA-activating domains of GAL4. Details of the other constructs used in the yeast two-hybrid assays are presented in Fig. 1.

Yeast was transformed using the lithium acetate method with 30% polyethylene glycol 4000. In screening the NL3in-binding partners, 5 μg cDNA library per plate were introduced into transformants of NL3in in pAS2-1C. Transformants were selected on −LWHA and −LWH + 3-AT plates, and then the surviving transformants were selected using the β-galactosidase assay. We screened more than 1×10^6 cells. The intensity of the interactions between NLs and SNTG2 was examined using the 3-AT assay.

Immunoprecipitation. COS-7 cells were transfected with Myc-tagged constructs of NL and FLAG-tagged constructs of SNTG2 using FuGENE 6 (Roche, Basel, Switzerland). Cells from two 10-cm plates were homogenized in 500 μl of Lysis Buffer (50 mM Tris/HCl [pH 8.0] containing 200 mM NaCl, 5 mM DTT, 1 mM EDTA, 1% (w/v) Triton X-100, and protease inhibitor cocktail). Then, the lysates were centrifuged at 100,000*g* for 15 min at 4 °C. The supernatant was precleared with protein G Sepharose 4 fast flow beads (Amersham Biosciences, Piscataway, NJ) for 1 h and then incubated with 1 μl of anti-FLAG M2 antibody fixed on 15 μl beads. After the beads were washed five times with homogenization buffer, the precipitates were analyzed using SDS–PAGE and immunoblotted with either anti-Myc or anti-FLAG antibody.

Immunocytochemistry and image analysis. Cortical neurons and COS-7 cells were fixed with PBS containing 2% or 4% (w/v) paraformaldehyde for 15 min, and permeabilized with 0.1% (w/v) Triton X-100 in PBS for 15 min. After the buffer was exchanged for 3% (w/v) BSA in PBS, the cells were incubated with the first antibody in 3% BSA in PBS for 1 h, washed with PBS, and then incubated with the second antibody in 3% BSA in PBS for 1 h. After washing with PBS, the samples were embedded in Mowiol (Calbiochem, La Jolla, CA). When only the cell surface was stained, the cells were incubated with the first antibody in PBS for 20 min, washed with PBS, and incubated with the second antibody for 20 min before washing and fixing. Z-stacks of four to 10 images were acquired on a Zeiss LSM510 meta laser scanning confocal microscope (Carl Zeiss, Jena, Germany). Brightest point projections of the Z-stacks were used for image analysis.

Neuronal cultures and transfection. Neurons were dissociated from the cerebral cortices of E18 rats and cultured at a density of 5×10^4 cells/cm^2 in Neurobasal medium with 2% B27 supplement (Invitrogen), 200 mM L-glutamine, and 10 mM L-glutamate at 37 °C under a controlled atmosphere containing 10% CO_2. After 7 or 11 days *in vitro*, the neurons were transfected with 1 μg DNA per well in 12-well plates using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's directions.

Results

Library screening identified syntrophin-γ2 as an NL3in-binding protein

While some reports have implied that GABA synapses are somehow involved in the pathology of autism [10,11], no scaffolding protein which exclusively localizes in the inhibitory synapse has been identified as a binding partner of NL3, which is thought to be strongly tied to autism [5,6]. NLs have been reported to be associated with both excitatory and inhibitory synapses. Therefore, we hypothesized that some unknown scaffolding proteins were associated with NL in inhibitory synapses, and that the interactions were involved in the pathology of autism. To identify these unknown scaffolding proteins, we performed yeast two-hybrid screening using the intracellular region of NL3 (NL3in) as bait to screen a human fetal brain cDNA library. Syntrophin-γ2 (SNTG2) was identified as a putative candidate. We confirmed the authenticity of the clone by transforming it back into yeast with NL3in.

Moreover, to determine the SNTG2-binding regions in NLs, we performed the HIS3 assay using various constructs (Fig. 1) that express mutant NL-fusion proteins in yeasts (Table 1). We examined the binding of NL3, 4X, and 4Y to SNTG2 in yeasts. All of the autism-related

Table 1
Results of the HIS3 assay of the NL mutants

Construct	HIS3 assay
Vector (DBD)	+
NL3in	++++++
NL3inΔPBM	+
NL3inR737C	+++
NL3inR737CΔPBM	+
NL3inΔTMD32	+++
NL4Xin	++++++
NL4XinΔPBM	+
NL4XinR704C	+++
NL4XinR704CΔPBM	+
NL4Yin	++++
NL4YinR704C	+++

We examined the strength of the interactions between NL or NL mutants and SNTG2 using the HIS3 assay. Since the HIS3 gene product, which is required for cell growth on plates lacking histidine, is competitively inhibited by 3-AT, the concentration of 3-AT at which yeast transformants can grow represents the activity of HIS3. Therefore, it reflects the strength of the interaction. Six, five, four, three, two, and one plus (+) indicate 3-AT concentrations of 20, 15, 10, 5, 1, and 0 mM, respectively.

NLs bound to SNTG2. The deletion of the PDZ binding motif (PBM) from NL3, 4X, and 4Y disrupted this binding to SNTG2. Notably, the introduction of an R→C point mutation, an autism-related mutation [9], weakened the binding. Similarly, the deletion of TMD32, the region including the autism-related mutation, also weakened the binding of NL3 to SNTG2. These results suggest that the binding of NLs to SNTG2 depends mainly on PBM and is stabilized by TMD32.

NLs bound to SNTG2 in COS-7 cells

Next, we confirmed that NLs would bind to SNTG2 in COS-7 cells using immunoprecipitation. Myc-NL3 and Myc-NL4X were co-immunoprecipitated with SNTG2 when FLAG-SNTG2 was immunoprecipitated using anti-FLAG antibody, whereas Myc-NL3 and Myc-NL4X were not immunoprecipitated by normal IgG, suggesting that NL3 and NL4X specifically interacted with SNTG2 (Fig. 2). In addition, Myc-NL4Y bound to FLAG-SNTG2 in COS-7 cells (data not shown).

We also examined the binding of NL mutants to SNTG2 in COS-7 cells. As with the HIS3 assay, the deletion of PBM in NL drastically weakened the binding to SNTG2 (Fig. 3A), indicating that the interaction between SNTG2 and NL in mammalian cells is mostly dependent on PBM. In contrast, the autism-related R704C mutation in NL4X did not seem to alter the binding (Fig. 3B). This result seems to be inconsistent with HIS3 assay. However, we suppose that we couldn't detect the effect of the autism-related R704C on the interaction between NL4X and SNTG2 because immunoprecipitation is not sensitive enough to detect minute differences of affinity. We also examined the regions of SNTG2 involved in its binding to NLs in COS-7 cells. The deletion of either the N- or C-terminal half of SNTG2 dramatically weakened the binding to NL3 and NL4X (Fig. 3C), suggesting that both the N-terminal part, including the PDZ domain, and the C-terminal half of SNTG2 are involved in the binding to NL.

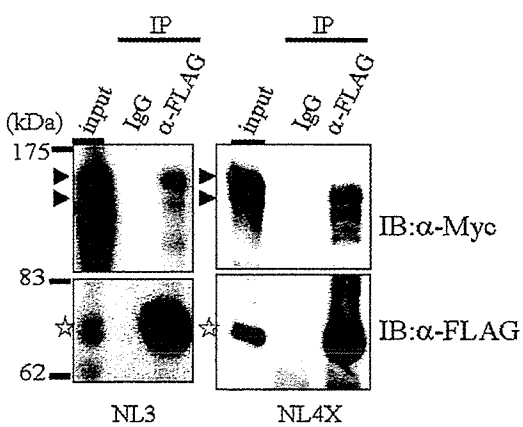

Fig. 2. The binding between SNTG2 and NLs in COS-7 cells. Myc-NLs were co-immunoprecipitated with FLAG-SNTG2 using anti-FLAG M2 antibody in Lysis buffer. Immunoprecipitation using normal mouse IgG was the negative control. Arrowheads and stars indicate Myc-NLs and FLAG-SNTG2, respectively. Both arrowheads may denote the posttranslational modification of NLs.

NLs partially colocalize with SNTG2 in both COS-7 cells and rat cortical neurons

To determine whether NL and SNTG2 colocalize because of their interaction, we immunostained COS-7 cells expressing YFP-NLs and SNTG2-myc. When YFP-NL3 or YFP-NL4X was expressed after transfecting COS-7 cells, it formed a small cluster on the cell surface (Fig. 4A). When SNTG2-myc was also expressed, it partially colocalized with YFP-NL (Fig. 4B); however, SNTG2-myc did not alter the localization of YFP-NL on the cell surface substantially, and vice versa.

Next, we examined the localization of exogenous NL3 and SNTG2 in rat cortical neurons at DIV9. When YFP-NL3 was expressed by transfection, YFP-NL3 formed small clusters on the surface of soma and dendrites, just like endogenous NL3 (Fig. 4C). Clusters of YFP-NL3 often colocalized with SNTG2-myc when neurons were doubly transfected, supporting the idea that NL3 is associated with SNTG2 in neurons and that SNTG2 functions as a scaffolding protein.

Discussion

This study revealed functional differences among NLs, which were related to binding to syntrophin-γ2 (SNTG2). Here, SNTG2 was identified as a *de novo* binding partner of autism-related NLs using yeast two-hybrid screening. SNTG2 is a member of the syntrophin family, whose members have a PDZ domain near the N-terminus and a PH domain near the C-terminus. Syntrophins play a key role as scaffolding proteins in both neuromuscular junctions and the CNS, where they interact with dystrophin and dystrobrevin [12]. Interestingly, the CNS syntrophins have been reported to localize exclusively in the inhibitory postsynaptic regions by binding to dystrophin and dystrobrevin [13,14]. Therefore, SNTG2 may be an unknown NL-binding scaffolding protein in inhibitory synapses.

The interaction between SNTG2 and NLs was mainly dependent on the PBM-PDZ interaction because it was disrupted by the deletion of PBM in NLs (Table 1 and Fig. 3A). Meanwhile, the binding of NLs to SNTG2 was influenced by factors other than the PBM-PDZ interaction, involving TMD32 in NLs and the region including PH in SNTG2 (Table 1 and Fig. 3B and C). Since deletion of the PBM disrupted the binding, we postulated that these non-PBM-PDZ factors somehow stabilize the PBM-PDZ core interaction. In particular, the effect of TMD32 on binding is interesting because it was previously reported to be necessary for the proper localization of NL1 [15]. In addition, TMD32 is variable

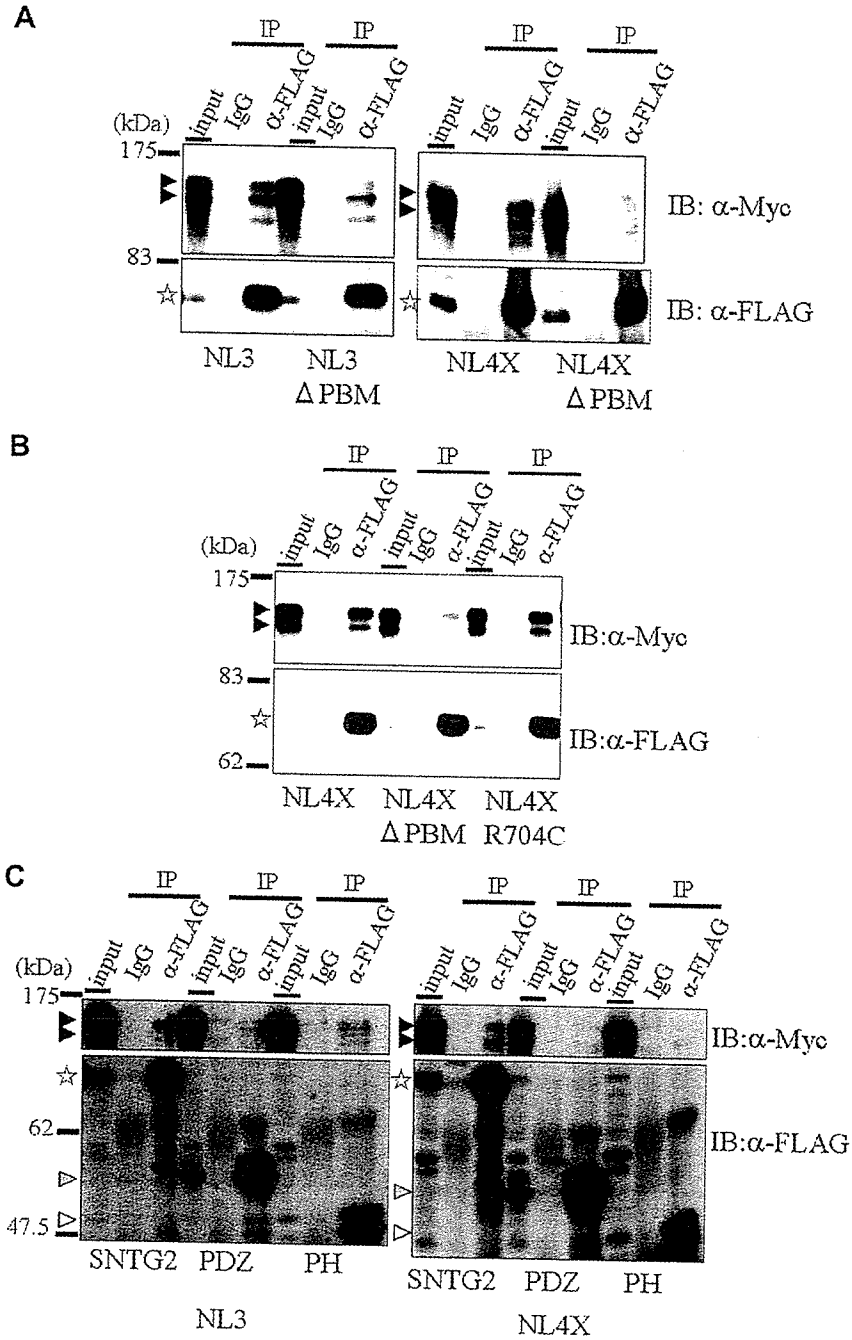

Fig. 3. (A) Effects of the deletion of PBM in NLs on the binding between SNTG2 and NLs in COS-7 cells. Various Myc-tagged NLs were co-immunoprecipitated with FLAG-SNTG2 in Lysis buffer. The deletion of PBM in NLs weakened the binding of NLs to SNTG2. Arrowheads and stars indicate Myc-NLs and FLAG-SNTG2, respectively. (B) The effect of R704C in NL4X, the autism-related mutation, on the binding to SNTG2 in COS-7 cells. Various Myc-tagged NL4Xs were co-immunoprecipitated with FLAG-SNTG2 in Lysis buffer. Under these conditions, the effect of the R704C mutation on the binding to SNTG2 was not detected. (C) The role of PDZ or the PH domain of SNTG2 in binding to NL. Whenever the regions containing the PDZ or PH domain were deleted, binding to NL was weakened. Therefore, both these regions might be involved in the interaction between NL and SNTG2. Black, gray, and white arrowheads and the star indicate Myc-NLs, the FLAG-tagged region containing the PDZ domain, the FLAG-tagged region containing the PH domain of SNT2G2, and the FLAG-tagged full-length SNTG, respectively.

in comparison with other parts of NL. These findings led us to hypothesize that due to the diversity of TMD32, NLs are functionally different from each other and the sequence of TMD32 for each NL determines whether the NL localizes in the inhibitory synapse. In this experiment, we could not examine the interaction between SNTG2 and NL2, which has been reported to localize in the inhibitory synapse exclusively. However, NL2 is

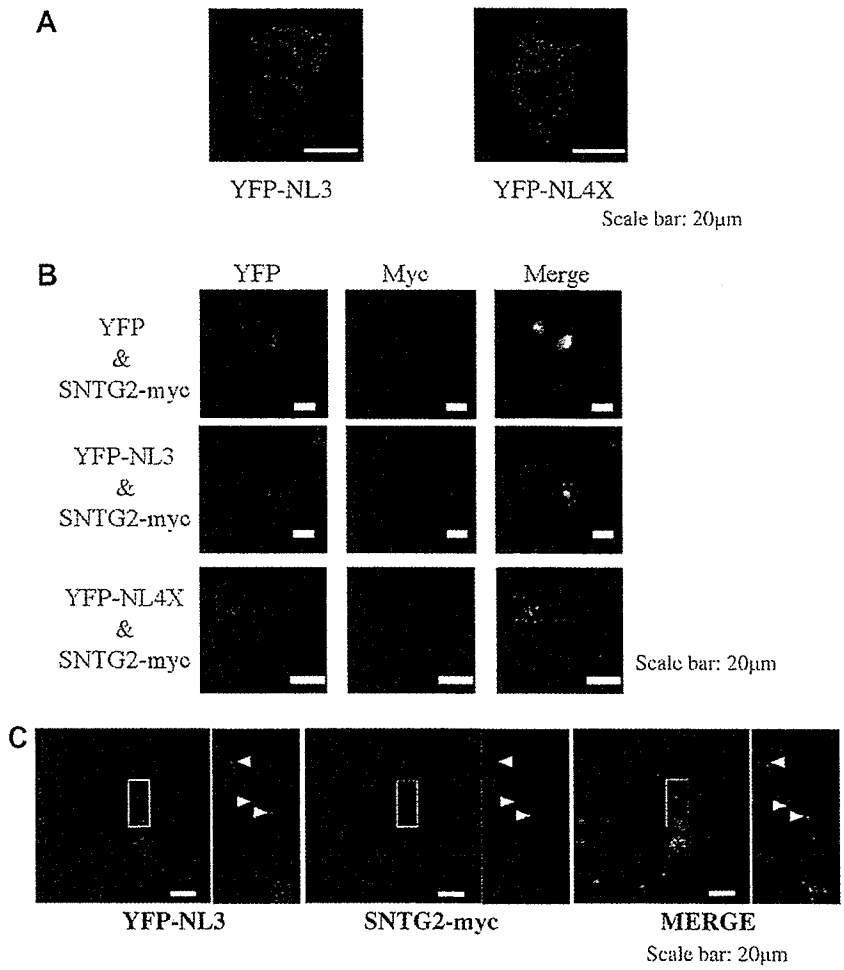

Fig. 4. (A) Localization of NL3 and NL4X on the surface of COS-7 cells. Only YFP-NL3 and YFP-NL4X on the cell-surface were immunostained using anti-GFP antibody and the secondary antibody conjugated with Oregon Green 488 without membrane permeabilization. They were clustered on the cell surface. (B) Localization of NLs and SNTG2 in COS-7 cells. The upper panels are negative controls. YFP or YFP-NL of cell-surface, SNTG2-Myc and merged images are shown on the left, center, and right, respectively. Co-transfected YFP-NL and SNTG2-Myc partially colocalized. Scale bar, 20 μm. (C) Localization of NL3, NL4X, and SNTG2 in rat cortical neurons. Neurons were transfected with YFP-NL3 and SNTG2-Myc at DIV7 using Lipofectamine 2000. After a 48-h incubation, the neurons were fixed, immunostained, and observed under confocal microscopy. The boxed area is enlarged in the bottom row. The clusters of YFP-NL3 of cell-surface sometimes colocalized with the cluster of SNTG2-Myc (arrowhead). Scale bar, 20 μm.

expected to bind to SNTG2 much stronger than any other NL, considering that both NL2 and SNTG2 dominantly localize in the inhibitory synapse [13,14,16]. The interaction will be determined in future.

Recently, some reports have suggested that the balance between excitatory and inhibitory synapses is disrupted in patients with autism [17,18]. A postmortem analysis of the brains of subjects with autism indicated that glutamic acid decarboxylase (GAD) was reduced in the autistic parietal and cerebellar cortices [11], suggesting that the impairment of GABAergic synaptic transmission causes autism. Meanwhile, the binding of NLs to SNTG2, a putative inhibitory-synaptic scaffolding protein, were affected by autism-related mutations in *NLs* in our experiments. Totally, the interactions between NLs and SNTG2 at the inhibitory synapse may be deeply involved in the etiology of autism. In future, screening autistic individuals for *SNTG2* mutations may elucidate the association between autism and the binding of SNTG2 to NLs.

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筋強直性ジストロフィーの分子病態

Molecular pathomechanism of myotonic dystrophy

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◎筋強直性ジストロフィーの原因がCTGまたはCCTGリピートの伸長であることがわかって以来，この病気の全身症状を説明するいくつかの説が提出されたが，現在では長いリピートをもったRNAの機能異常という考えが有力になっている．この小論では選択的スプライシングと症状との関係，およびリピートRNAに結合する蛋白質の生理機能に的を絞って最近の研究の動向をまとめてみたい．

Key word：筋強直性ジストロフィー，トリプレットリピート，RNA結合蛋白質，スプライシング異常，MBNL

筋強直性ジストロフィー(dystrophia myotonica：DM)は筋強直だけではなく，耐糖能異常(インスリン抵抗性)，性腺異常，禿頭，白内障，精神遅滞などが特徴の全身性の疾患である．DMには2種類の原因が知られており，DM1は第19染色体の*DMPK*遺伝子の3′非翻訳領域のCTG三塩基の伸長，DM2は第3染色体の*ZNF9*遺伝子の第1イントロン中にあるCCTG四塩基の伸長によって起こる[1-4]．DM1とDM2の症状は似ているが，DM1では筋萎縮がひどく，中枢神経症状も認められるのに対し，DM2では新生児からの症状はないことが特徴である．また，症状が強く出るのが遠位筋(DM1)と近位筋(DM2)という差もある(図1)．

DMの発症原因と考えられている諸説

DM1の発症に関して現在までいろいろな説が提唱されてきた．たとえば，①DMPKの発現量が少ないために病気となるというハプロ不全説，②長いCTGリピートが近接遺伝子*Six5*の発現を変えるという説，そして③RNA機能獲得説がある．①と②については，*DMPK*およびDM1座位から1kb下流にある転写因子*Six5*ノックアウトマウスがDM1と同じ症状を呈さないという反論があり，いまのところ③が有力視されている．

その理由はいくつかある．まず，CTGおよびCCTGという翻訳領域以外の部分の繰返し配列の伸長で同じような症状が出る点，また染色体の座が離れていて近接遺伝子がまったく異なるにもかかわらず症状が似ていること，骨格筋アクチンプロモーターの下流にCUGリピートだけをつないで発現させたHSA^{LR}マウスがDMと同じ筋強直症状を呈したこと[5]，が考えられる．③では核内でたしかに伸長したRNAリピートが転写されており，そこにRNA結合蛋白質が結合して核内凝集体をつくり，この蛋白質群の本来もつスプライシング調節などの機能が果たせないため，と説明されている．また，核外では長いmRNAからの翻訳抑制も示唆されている．

RNAリピート結合蛋白質と機能

DMの表現型にはいろいろあるが，現在までに知られているDMでの13種類のmRNAのスプライシング異常によってほとんどの症状が説明できる(表1)[6]．塩素チャネル(ClC-1)は筋強直，インス

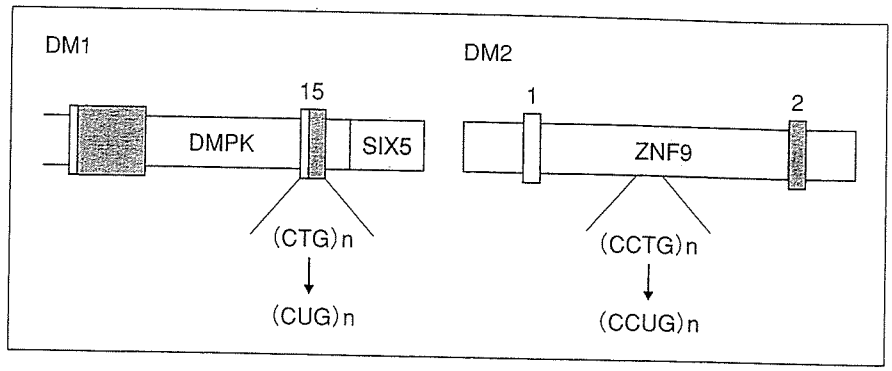

図 1　DMの原因とリピート長との関係

表 1　DMでスプライシング異常が報告されている遺伝子

遺伝子	DM での異常	推定されている症状との関連
塩素チャネル(Clc-1)	エクソン 7a の挿入 イントロン 2 の保持	筋強直(ミオトニア)
インスリン受容体(IR)	エクソン 11 の除外	耐糖能の異常
心筋トロポニン T(Tnnt 2)	エクソン 5 の挿入	心異常？
タウ(MAPT)	エクソン 2 と 3 の除外 エクソン 10 の除外	精神遅滞 精神遅滞
マイオチューブラリン関連蛋白質 1(MTMR1)	エクソン 2.1, 2.3 の除外	筋萎縮？
速筋トロポニン T(Tnnt 3)	胎児型エクソンの挿入	心異常
NMDA 受容体(NMDAR1)	エクソン 5 の挿入	精神遅滞
アミロイド前駆体(APP)	エクソン 7 の除外	精神遅滞
リアノジン受容体(RyR)	エクソン 70 の除外	筋萎縮？
小胞体 ATP アーゼ 1(SERCA1)	エクソン 22 の除外	筋萎縮？
小胞体 ATP アーゼ 2(SERCA2)	イントロン 19 の保持	筋萎縮？

リン受容体は耐糖能異常，心筋型トロポニン T は心臓異常，マイオチューブラリン関連蛋白質 1 (MTMR1)や小胞体の CaATP アーゼ(SERCA)は筋萎縮，NMDA 受容体や APP は精神遅滞，といった具合である．たとえば，心筋型トロポニン T はスプライシングによって胎児型が出たりするが，これが DM でみられる心筋の異常にかかわる可能性がある．なぜスプライシングに異常がくるかというと，異常に伸長した CUG リピートや CCUG リピートに RNA 結合蛋白質が捕捉され，そのために正常スプライシングに働いていた RNA 結合蛋白質の機能が変化し，スプライシングのパターン変化が生じるというものである．

ヒトではリピート RNA に結合する蛋白質として報告されているものに，CELF(CUG-BP and ETR-3-like factors)ファミリーや MBNL(muscleblind-like)ファミリーがある[7]．前者には 6 種類(CELF1-6)，後者には 3 種類(MBNL1-3)の分子が知られている．これらの分子はスプライシングに関して協調して働く場合と拮抗して働く場合がある．

MBNL はショウジョウバエの muscleblind のホモログとして単離されたもので，MBNL1 は筋肉の分化促進に，MBNL2 はインテグリン mRNA の局在に関与し，MBNL3 は MBNL1 の機能を抑制すると報告されている．すくなくともハエの変異体にヒトの MBNL1 を導入すると欠陥がレスキューされることから，MBNL1 と muscleblind の機能の普遍性が推測されている．また著者らは，MBNL 群の RNA リピート結合特異性を調べた結果，MBNL どうしでは特異性は類似しており，MBNL1 は CUG, CCUG 両リピートに強く結合することが明らかとなった[8]．また，MBNL 蛋白質はどれも DM 患者の核内にある CUG または CCUG リピート含有 RNA 凝集体と共局在することも知られている．DM への MBNL1 の関与を決定的にしたのは *MBNL1* ノックアウトマウスである[9]．このマウスは筋強直症状を呈したのであった．また，siRNA

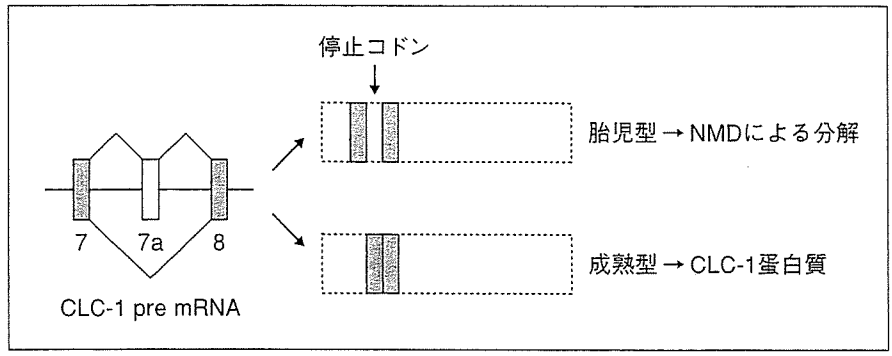

図 2　塩素チャネル(CLC-1)のスプライシングと誤った転写産物の行方

による機能消失実験において，DM にみられるようなミススプライシングを誘導したことも，MBNL が DM の症状発現に強く関与することを示唆するものであった．

一方，CELF のなかで一番先に発見された CUG-BP(CELF1)の機能についてはいろいろの見解に分かれている．名前のとおり，発見されたときには CUG 含有 RNA に結合して局在したようなデータが得られたために DM 症状との関連も示唆された[10]が，著者らがこの蛋白質の結合特異性を明らかにし，CUG リピート RNA に結合するのではなく，UG ジヌクレオチドリピートのほうに強く結合することを発表[11]して以来，主役の座を MBNL に追われたような形になっている．その後，CELF ファミリーは CUG や CCUG リピート RNA を含む凝集体とは共局在しないようだと結論づけられた．しかし，後述するスプライシング活性を強力に変えることもわかり，DM において何らかの機能を果たしていることは間違いない．

DMにおける選択的スプライシング

ヒトゲノムには 22,000～24,000 の遺伝子が存在するといわれている．そのうちの 3/4 の遺伝子に選択的スプライシングが認められている．またそのうち 8 割は翻訳領域で起こり，これによって 1 つの遺伝子から機能の異なる蛋白質をつくりだすことができる．選択的スプライシングは私たち生物の多機能性を生み出す大切な機構である．

ところが，選択的スプライシングを起こしたものの約 3 割には停止コドン(premature termination codon：PTC)が入り，ナンセンス変異依存性 mRNA 分解(nonsense-mediated mRNA decay：NMD)が起こるといわれている．このような場合には重篤な疾患が生じることがある．

しかし DM は，ほかの遺伝性疾患のようにエクソン・イントロン連結部位あたりに変異が入って選択的スプライシング自体に異常が生じるのではない．非翻訳部位に長い挿入があるために，選択的スプライシングの時間的空間的調節ができないのである．すなわち，発生過程での胎児型から成熟型への変換がうまくいかなかったり，組織特異的なスプライシングがうまくいかなかったりするのである．たとえば，塩素チャネル遺伝子をみてみよう(図 2)[3]．ヒト DM ではエクソン 7a を飛ばすと成人型 ClC-1 蛋白質ができ，エクソン 7a を含むようにスプライシングが起こると途中に停止コドンが入った胎児型 ClC-1 蛋白質がつくられる．この場合，NMD が起こって mRNA 量が少なくなる．ClC-1 蛋白質の量が少なくなると筋強直(ミオトニア)が起こる．このようなことから，DM での ClC-1 蛋白質の発現量は正常の 10%以下といわれている．

おわりに

DM のように RNA 機能異常によって発病すると考えられる疾患の数はしだいに多くなっており，非コード領域にリピートが入った脆弱 X 震顫失調症候群(Fragile X tremor ataxia syndrome)，SCA8，SCA10，SCA12，Huntington 病様 2 型なども RNA 機能異常が推測されている．しかし，これらにも MBNL や CELF ファミリーがかかわっているという証拠は少なく，本当に DM のような RNA 機能獲得変異が起こっているのか，今後の検討が望まれる．

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Characterization of the protease activity that cleaves the extracellular domain of β-dystroglycan ☆

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Abstract

Dystroglycan (DG) complex, composed of αDG and βDG, provides a link between the extracellular matrix (ECM) and cortical cytoskeleton. Although the proteolytic processing of βDG was reported in various physiological and pathological conditions, its exact mechanism remains unknown. In this study, we addressed this issue using the cell culture system of rat schwannoma cell line RT4. We found that the culture medium of RT4 cells was enriched with the protease activity that degrades the fusion protein construct of the extracellular domain of βDG specifically. This activity was suppressed by the inhibitor of matrix metalloproteinase-2 (MMP-2) and MMP-9, but not by the inhibitors of MMP-1, MMP-3, MMP-8, and MMP-13. Zymography and RT-PCR analysis showed that RT4 cells secreted MMP-2 and MMP-9 into the culture medium. Finally, active MMP-2 and MMP-9 enzymes degraded the fusion protein construct of the extracellular domain of βDG. These results indicate (1) that RT4 cells secrete the protease activity that degrades the extracellular domain of βDG specifically and (2) that MMP-2 and MMP-9 may be involved in this process.

Keywords: Dystroglycan; Extracellular matrix; Matrix metalloproteinase; MMP-2; MMP-9

The dystroglycan (DG) complex is a cell adhesion molecular apparatus expressed in a wide variety of tissues ubiquitously [1,2]. DG is encoded by a single gene *Dag1* and cleaved into two proteins, αDG and βDG, by post-translational processing [1]. αDG is a cell surface peripheral membrane protein, which interacts with laminin in the extracellular matrix (ECM) [1–3]. βDG is a type I integral membrane protein, which anchors αDG to the cell membrane via the N-terminus of the extracellular domain and associates with dystrophin via the C-terminal cytoplasmic domain [4,5]. Thus the DG complex provides a tight link between the ECM and cortical cytoskeleton.

Because of these characteristics, the DG complex needs to be disrupted efficiently when tissue remodeling takes place in both physiological and pathological conditions. As a candidate for such a molecular mechanism, we have reported previously the proteolytic processing of βDG by the putative matrix metalloproteinase (MMP) activity [6]. Because the 30 kDa C-terminal fragment of βDG created by this processing behaves as an integral membrane protein biochemically, we predicted that the cleavage site of this processing would exist in the extracellular domain of βDG [6]. We also reported that this processing of βDG was activated in the skeletal muscle of Duchenne muscular dystrophy (DMD) and sarcoglycanopathy [7,8]. Similar phenomena were reported in other types of cells, such as cutaneous cells, carcinoma cells, and sympathetic neurons after axotomy [9–13].

☆ *Abbreviations:* DG, dystroglycan; ECM, extracellular matrix; MMP, matrix metalloproteinase; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; GST, glutathione *S*-transferase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction.

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At present, however, we do not know what type of MMP is actually responsible for the processing of βDG or if this MMP activity really cleaves the extracellular domain of βDG specifically. In this study, we addressed these issues using the cell culture system of rat schwannoma cell line RT4.

Materials and methods

Cell culture. Rat schwannoma cell line RT4 was described previously [6,7]. RT4 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 16.7 mM glucose, 2 mM glutamine, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin. Culture medium was changed every 3 days. When cells grew to near confluence, they were digested with 0.25% trypsin/0.02% EDTA and planted in 10 cm^2 plate at a density (3×10^6/dish) for 3 days in the culture medium without serum and antibiotics. Living cells were harvested by scraping the culture dishes with rubber policemen and homogenized in a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.6 μg/ml pepstatin A, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.75 mM benzamidine, and 0.1 mM PMSF, while the culture medium was gathered and concentrated 20-fold so that the final volume equals that of the total cell homogenate.

Proteolysis of fusion proteins. Glutathione *S*-transferase (GST) fusion proteins of βDG, αDG, and dystrophin were prepared as described previously (Fig. 1) [14]. βDG fusion proteins corresponding to amino acids 654–750 (βDGext) and 775–895 (βDGint) correspond to the entire extracellular and intracellular domains of βDG, respectively (Fig. 1a). αDG fusion proteins corresponding to amino acids 30–341 (αDG N-ter) and 343–652 (αDG C-ter) correspond to the N- and C-terminal domains of αDG, respectively (Fig. 1a). Dys corresponds to amino acids 3054–3271 of dystrophin. Forty nanograms/microliter of fusion proteins were incubated at 37 °C with the RT4 cell homogenate or concentrated cell culture medium. In addition, βDGext was incubated at 37 °C with the concentrated RT4 cell culture medium in the presence or absence of various inhibitors of MMPs. These included (2R)-[(4-biphenylylsulfonyl)amino]-*N*-hydroxy-3-phenylpropionamide (Merck Biosciences), doxycycline hydrochloride (Calbiochem), Ac-Arg-Cys-Gly-Val-Pro-Asp-NH_2 (Calbiochem), and CL-82198 (Calbiochem), which are commercially available inhibitors of MMP-2/MMP-9, MMP-1/MMP-8, MMP-3, and MMP-13, respectively [15–18]. Forty nanograms/microliter of βDGext was also incubated with 2 ng/μl of active human MMP-2 and recombinant human MMP-9 (Calbiochem) at 37 °C. Samples were analyzed by SDS–PAGE and immunoblotting using anti-GST-HRP conjugate (Amersham Biosciences). Molecular mass standards were obtained from BIO-RAD (precision plus protein standards).

Zymography. Zymography of RT4 cell homogenate and culture medium were performed according to von Moers et al. [19]. RT4 cells were harvested and homogenized in 1% SDS, while the cell culture medium was gathered and concentrated to the same volume as the cell homogenate. Samples as well as 1 ng of active human MMP-2 and recombinant human MMP-9 were loaded on a 10% SDS–polyacrylamide gel containing 1 mg/ml gelatin. Electrophoresis was carried out at 100 V for 2.5 h. The gel was then washed twice for 10 min and once for 1 h with 2.5% Triton X-100, 50 mM Tris–HCl, pH 7.5 at 20 °C, to remove SDS and to re-nature the gelatinases. For activation of the enzymes, the gel was incubated in 5 mM $CaCl_2$, 50 mM Tris–HCl, pH 7.5, for 18 h at 37 °C under continuous shaking. After fixation in 40% methanol, 10% acetic acid for 30 min at 20 °C, the gel was stained with 0.5% Coomassie brilliant blue G-250 for 1 h, rinsed in distilled water for 2 h, and destained in 7% acetic acid.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from RT4 cells using Isogen reagent (Wako). First strand cDNA was synthesized using SuperScript™ III kit (Invitrogen), and served as a template for RT-PCR. Primers used to amplify MMP-2 and MMP-9 were as follows: MMP-2, forward, 5′-GACCTTGACCAGAACACCATCG-3′, reverse, 5′-GCTGTATTCCCGACCGTTGAAC-3′; MMP-9, forward, 5′-CCCCACTTACTTTGGAAACGC-3′, reverse, 5′-AGCCACGACCATACAGATGCTG-3′. PCR products were analyzed on a 2% agarose gel, sub-cloned into the T vector and sequenced using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia) on the DSQ-1000L DNA sequencer (Shimazu).

Results

Degradation of the extracellular domain of βDG by the concentrated RT4 cell culture medium

We have reported previously that the putative MMP activity that processes βDG is expressed in RT4 cells [6].

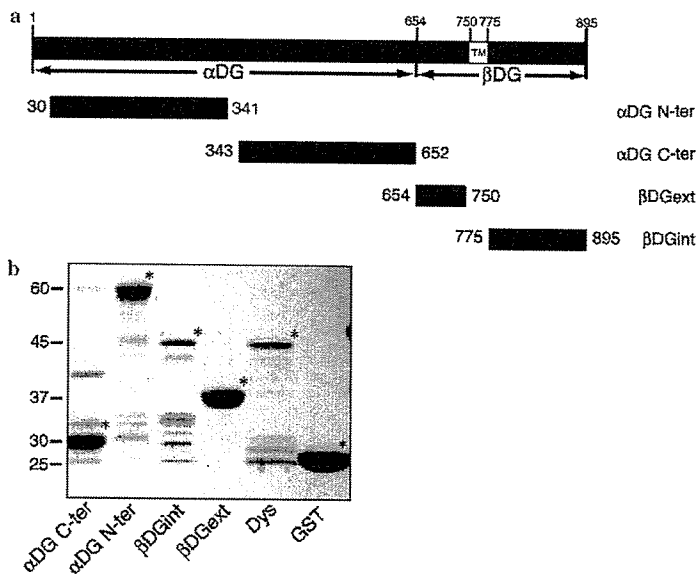

Fig. 1. DG fusion protein constructs. (a) Shown are the fusion protein constructs of DG. TM indicates the single transmembrane domain of βDG. (b) GST fusion proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue. GST fusion proteins corresponding to αDG C-ter, αDG N-ter, βDGint, βDGext, Dys, and GST are indicated by asterisks. Molecular mass standards (Da × 10^3) are shown on the left.