Aberrantly spliced α-dystrobrevin alters α-syntrophin binding in myotonic dystrophy type 1

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ABSTRACT

Background: Myotonic dystrophy type 1 (DM1) is a multisystemic disorder caused by a CTG repeat expansion in the *DMPK* gene. Aberrant messenger RNA (mRNA) splicing of several genes has been reported to explain some of the symptoms in DM1, but the cause of muscle wasting is still unknown. By contrast, many forms of muscular dystrophy are caused by abnormalities of the dystrophin–glycoprotein complex (DGC). α-Dystrobrevin is a key component of the DGC in striated muscle and plays important roles in maturation and signal transduction by interacting with α-syntrophin. The goal of this study was to investigate alternative splicing of α-dystrobrevin in DM1 and examine α-syntrophin binding of different α-dystrobrevin splice isoforms.

Methods: Splicing patterns of α-dystrobrevin in DM1 muscle were studied by reverse-transcriptase PCR. Expression of the variant splice isoform was examined by immunoblotting and immunohistochemistry. Alternatively spliced isoforms were expressed in cultured cells to investigate interaction with α-syntrophin. α-Syntrophin expression was examined by immunoblotting.

Results: α-Dystrobrevin mRNA including exons 11A and 12 was increased in both skeletal and cardiac muscle of DM1 patients. The aberrantly spliced α-dystrobrevin isoform was localized to the sarcolemma, and showed increased binding with α-syntrophin. Furthermore, levels of α-syntrophin associated with the DGC were increased in DM1 muscle.

Conclusion: Alternative splicing of α-dystrobrevin is dysregulated in myotonic dystrophy type 1 (DM1) muscle, resulting in changes in α-syntrophin binding. These results raise the possibility that effects on α-dystrobrevin splicing may influence signaling in DM1 muscle cells.

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GLOSSARY

α-DB = α-dystrobrevin; **α-syn** = α-syntrophin; **ALS** = amyotrophic lateral sclerosis; **β-DG** = β-dystroglycan; **CC** = coiled-coil domain; **cDM** = congenital myotonic dystrophy type 1; **cDNA** = complementary DNA; **Cont** = control; **DAPC** = dystrophin-associated protein complex; **DBS** = dystrophin binding site; **DGC** = dystrophin–glycoprotein complex; **DM1** = myotonic dystrophy type 1; **EF** = EF hand region; **GAPDH** = glyceraldehyde 3-phosphate dehydrogenase; **IP** = immunoprecipitation; **LGMD** = limb-girdle muscular dystrophy; **MDRS** = muscular disability rating scale; **mRNA** = messenger RNA; **NA** = not available; **NMJ** = neuromuscular junction; **NT** = not tested; **P** = postnatal day; **PM** = polymyositis; **RT-PCR** = reverse-transcriptase PCR; **SBS** = syntrophin binding site; **SDS-PAGE** = sodium dodecyl sulfate polyacrylamide gel electrophoresis; **TBS** = Tris-buffered saline; **WCL** = whole-cell lysate; **vr** = variable region; **Y** = unique tyrosine kinase substrate domain; **ZZ** = zinc-binding domain.

Myotonic dystrophy type 1 (DM1) is the most common type of muscular dystrophy in adults and exhibits multisystemic involvement including myotonia, muscle wasting, insulin resistance, cardiac conduction defects, and cardiomyopathy.[1] Substantial evidence has accumulated to support the hypothesis that RNA containing expanded CUG repeats accounts for splicing abnormalities in DM1 by perturbing developmental control for splicing.[2] So far, disrupted messenger RNA (mRNA) alternative splicing has been reported in several genes, including cardiac troponin T (*cTnT*), insulin receptor (*IR*), muscle-specific chloride channel (*ClC-1*), *ZASP*, ryanodine receptor 1 (*RYR1*), and sar-

Supplemental data at www.neurology.org

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coplasmic/endoplasmic reticulum calcium ATPase (*SERCA*).[3-8] In particular, myotonia is attributed to the disruption of *ClC-1* splicing, and insulin resistance is attributed to abnormal alternative splicing of *IR*.[4-6] However, the pathomechanism of muscle wasting, the most disabling symptom in DM1, has not been well elucidated.

It is possible that misregulated splicing of other transcripts may relate to the mechanism of muscle wasting. We have been especially interested in the splicing abnormalities that affect cytoskeletal proteins of the dystrophin–glycoprotein complex (DGC), such as dystrophin,[9] because abnormalities of the DGC are well recognized to be responsible for many forms of muscular dystrophy. For this study, we have chosen α-dystrobrevin (α-DB), because 1) α-DB undergoes extensive alternative splicing that is under developmental control, 2) is linked to impairment of striated muscles, 3) is expressed several organs affected by DM1, and 4) is thought to be indispensable for signal transduction in muscle and neuromuscular synaptogenesis.[10] We examined the splicing patterns and expression levels of α-DB in skeletal and cardiac muscles of DM1 patients. Furthermore, the α-syntrophin binding to variant α-DB splice products was investigated.

METHODS

Patients and muscle tissue samples. Human skeletal muscle tissues were obtained from 11 DM1 patients, 1 patient with congenital DM1 (cDM), 6 normal controls, 5 patients with amyotrophic lateral sclerosis (ALS), 5 patients with polymyositis (PM), and 2 patients with limb-girdle muscular dystrophy (LGMD). Cardiac muscle tissues from 3 DM1 patients were obtained at autopsy. This research was approved by the institutional ethics committee and informed consent for use of the specimens for research was obtained. Mouse skeletal muscle from wild-type and HSA^{LR} mice[11] was also examined. The experiments using mice were performed in accordance with the protocol complying with the Guidelines for the Care and Use of Laboratory Animals.

DM1 patients were classified according to the 5-point muscular disability rating scale (MDRS): 1, no clinical impairment; 2, minimal signs of impairment; 3, distal weakness; 4, mild or moderate proximal weakness; and 5, nonambulatory.[12] CTG trinucleotide repeat expansion sizes were determined by Southern blot analysis of genomic DNAs from muscles or blood of patients with DM1, as described previously.[13]

RNA preparation and cDNA synthesis. Total mRNA was extracted and first-strand complementary DNA (cDNA) was synthesized as described previously.[8] Total RNA purchased from vendors and used as normal control were as follows; one batch of fetal skeletal muscle (mixture of 5, Stratagene, La Jolla, CA), two lots of fetal cardiac muscle (mixture of 10, Stratagene; mixture of 34, Clontech, Mountain View, CA), and three lots of adult cardiac muscle (one from a single female donor, Stratagene; two lots of mixture of 10, Clontech).

PCR analysis of splicing pattern. The sequences of primers used for reverse-transcriptase PCR (RT-PCR) assays of α-DB splicing are listed in table e-1 on the *Neurology*® Web site at www.neurology.org. The program for amplification consisted of initial denaturation at 94 °C for 10 minutes, 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 minutes. The PCR products were electrophoresed and quantified as described previously.[8]

mRNA quantification. The expression levels of α-DB1 and α-DB2 mRNAs were measured by real-time PCR using TaqMan Gene Expression assays on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression.

Antibodies. A specific antibody against α-DB including variable region 3 (Ab-DBvr3) was prepared by injecting purified synthetic peptides into rabbits according to the standard protocols. Ab-DBvr3 was raised against the peptide CITRSSPPKDSEVEQNKLLAR (amino acids 362–381 in the α-DB1 vr3 region plus an amino-terminal cysteine). Other antibodies used were anti–α-DB1 goat polyclonal, anti–α-syntrophin goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), anti–α-DB2 mouse monoclonal (BD Biosciences, San Jose, CA), anti–β-dystroglycan (β-DG) mouse monoclonal (Novocastra Laboratories, Newcastle upon Tyne, UK), and horseradish peroxidase–conjugated anti-mouse, anti-rabbit (ICN Pharmaceuticals, Costa Mesa, CA), or anti-goat (Santa Cruz Biotechnology) secondary antibodies.

Protein analysis. Dystrophin-associated protein complex (DAPC) was prepared, as described previously,[9] from 12 human skeletal muscles: 4 from DM1 patients, 8 from non-DM individuals (4 normal controls, 2 with PM, 1 with ALS, and 1 with facioscapulohumeral muscular dystrophy), and mouse skeletal muscle. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described previously.[8] Blots were blocked with 5% (weight/volume) nonfat milk and then incubated with antibodies at the following dilutions: anti–α-DB1, 1:100; anti–α-DB2, 1:500; anti-DBvr3, 1:200; anti–α-syntrophin, 1:200; and anti–β-DG, 1:500. After repeated washings, the membranes were incubated with secondary antibodies. The membranes were then washed and developed to enhance the chemiluminescence (Amersham, Little Charfont, UK) and exposed to x-ray film. Densitometry was performed with FluorChem IS-8000 (Alpha Innotech, San Leandro, CA).

Immunohistochemistry. Serial 10-μm-thick transverse frozen sections were cut from the rectus abdominis muscle of DM1 patient and mouse hind limb muscle. The sections were treated with cool acetone and 0.3% hydrogen peroxide in methanol. The sections were incubated with Ab-DBvr3 diluted 1:500, and then reacted with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer (Envision+, DAKO, Carpinteria, CA), rinsed, and

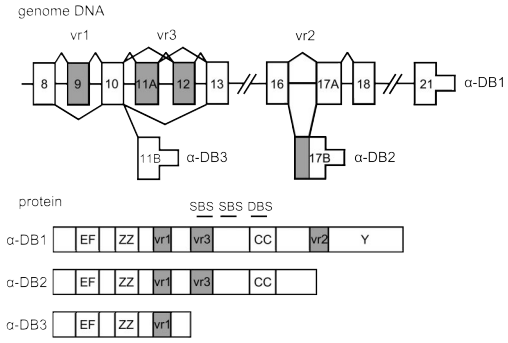
visualized by incubation with diaminobenzidine in the presence of hydrogen peroxide.

Plasmid constructs. pMGT1-DB, constructed from subcloned α-DB1 containing the vr3 region, was kindly provided by Dr. Y. Mizuno (Gunma University, Gunma, Japan).[14] pMGT1-DB Δvr3 was constructed by cutting out the vr3 region from pMGT1-DB. The FLAG-tagged α-DB expression construct including the vr3 sequence was generated by digesting pMGT1-DB, and the construct excluding the vr3 was generated by pMGT1-DB Δvr3. Then both were inserted into the pCMV-3Tag-1 vector (Stratagene). Using the QuickChange Site Directed Mutagenesis Kit (Stratagene), the L416P mutation of α-DB1 was introduced.

Cell culture, transfection, and immunoprecipitation. HEK293 human embryonic kidney cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. FuGENE 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) was used for transfection with FLAG-tagged α-DB according to the manufacturer's protocol. After 48 hours, cells were collected in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM Na_3VO_4, 50 mM NaF, 1% Nonidate P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Whole-cell lysates (WCLs) were centrifuged for 10 minutes at 15,000*g*. The resulting supernatants were incubated with anti-FLAG mAb M2 agarose gel (Sigma-Aldrich). The anti-FLAG M2 agarose gel was washed with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl) and eluted with TBS containing 3× FLAG peptide (Sigma-Aldrich). The immunoprecipitates or WCLs were separated by SDS-PAGE and immunoblotted as described above, using anti-FLAG M2 antibody (Sigma-Aldrich) or anti–α-syntrophin antibody.

Statistics. Differences between two groups were evaluated by Mann–Whitney *U* test. Error bars indicate mean ± SEM.

Figure 1 Alternative splicing of human α-dystrobrevin



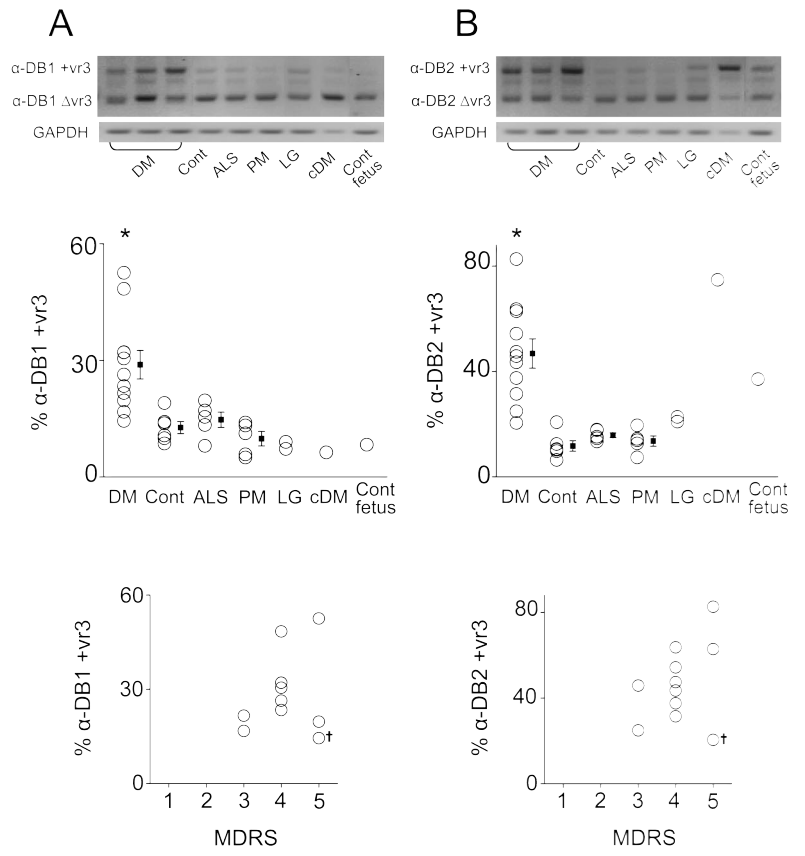
Exons represented as thick boxes are translated segments, and thin boxes indicate untranslated segments. Introns and downstream flanking regions are represented by horizontal lines. Gray boxes represent the alternatively spliced exons: variable regions 1 (vr1), 2 (vr2), and 3 (vr3). The different isoforms of protein are represented below. The identifiable domains are boxed: EF = EF hand region; ZZ = zinc-binding domain; CC = coiled-coil domain; Y = unique tyrosine kinase substrate domain. Suggested syntrophin binding sites (SBS) and dystrophin binding site (DBS) are indicated.[25] α-DB = α-dystrobrevin.

RESULTS

Splicing of α-DB in skeletal muscle. The human α-DB gene consists of 21 coding exons.[15] The alternative use of three exons—21, 17B, and 11B—generates transcripts of different lengths encoding three major α-DB isoforms in human skeletal muscle: α-DB1, α-DB2, and α-DB3 (figure 1).[15] α-DB1, which has a unique C-terminal tyrosine kinase substrate domain,[16,17] is localized in the sarcolemma and is abundant at the neuromuscular junction (NMJ).[18-20] α-DB2 is localized around the entire circumference of the sarcolemmal membrane, including the NMJ.[19] α-DB3 has been suggested to localize in cytoplasm.[21] Additional variability is observed as a result of alternative splicing within the coding regions referred to as variable regions 1 (vr1), 2 (vr2), and 3 (vr3).[17] First, vr1 consists of exon 9 encoding 3 amino acids. Second, vr2 consists of exons 17A and 17B. The first 21 nucleotides of exon 17B are also found in the α-DB1 transcript as a result of splicing at a cryptic site.[20] In mouse, the expression of the vr2 region in the α-DB1 transcript seems to be developmentally regulated.[22] Last, vr3 consists of exons 11A, 11B, and 12. Exon 11B encodes the unique C-terminal tail of α-DB3. In α-DB1 and α-DB2, four in-frame alternatively spliced transcripts may arise by joining exon 10 with either exon 11A, 12, or 13 or by joining exon 11A with either 12 or 13.[15] In mouse skeletal muscle, the splicing of vr3 has also been reported to be developmentally controlled.[18,22]

To investigate the splicing isoforms of α-DB, RT-PCR analysis of spliced transcripts was performed on skeletal muscles from patients with DM1 or cDM compared with non-DM controls (normal controls, ALS, PM, LGMD). The splicing patterns of vr1 and vr2 were not changed in DM1 skeletal muscle (data not shown). In a detailed investigation of the splicing of the vr3 region of α-DB1, two major PCR products representing α-DB1, including exons 11A and 12 (α-DB1+vr3) and excluding exons 11A and 12 (α-DB1 Δvr3), were found with other minor alternatively spliced variants in human skeletal muscle (figure 2A, top). These minor spliced variants consisted of isoforms including only exon 11A or a novel exon that was found in the EST database (sequence number BP348148). α-DB1+vr3 mRNA was observed in skeletal muscles from several DM1 patients, whereas α-DB1 Δvr3 mRNA was the predominant splice product in non-DM muscle. The proportion of α-DB1+vr3 mRNA was increased in DM1 patients in comparison with controls having normal muscle (p =

Figure 2 Analysis of α-dystrobrevin (α-DB) splice variants, showing an increase of α-DB1+vr3 and α-DB2+vr3 in DM1 skeletal muscle



(A) Reverse-transcriptase PCR (RT-PCR) analysis for variable region 3 (vr3) of α-DB1 messenger RNA (mRNA) in skeletal muscle tissue from myotonic dystrophy type 1 (DM, n = 11), normal control (Cont, n = 6), amyotrophic lateral sclerosis (ALS, n = 5), polymyositis (PM, n = 5), limb-girdle muscular dystrophy (LG, n = 2), and congenital myotonic dystrophy type 1 (cDM, n = 1) patients and normal fetus (Cont fetus). Top: Representative RT-PCR products. PCR products from α-DB1+vr3 (+exons 11A and 12) and α-DB1 Δvr3 (Δexons 11A and 12) are 690 and 519 bp. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplicons are shown as loading controls. Middle: The mean percentage of α-DB1+vr3 (% α-DB1+vr3/total α-DB1). *$p < 0.05$. Bottom: Relationship between the clinical involvement and the proportion of α-DB1+vr3 in skeletal muscle tissues from DM1 patients. The percentage of α-DB1+vr3 plotted against the muscular disability rating scale (MDRS). † Data from an autopsied patient who became nonambulant as a result of advanced ovarian cancer. (B) RT-PCR analysis for vr3 of α-DB2 mRNA. Top: Representative RT-PCR products. PCR products from α-DB2+vr3 and α-DB2 Δvr3 are 670 and 499 bp. Middle: The mean percentage of α-DB2+vr3 (% α-DB2+vr3/total α-DB2). *$p < 0.01$. Bottom: Relationship between the clinical involvement and the proportion of α-DB2+vr3. The percentage of α-DB2+vr3 plotted against the MDRS.

0.002), ALS ($p = 0.015$), or PM ($p = 0.002$). Similar to α-DB1, splicing of the vr3 region of α-DB2 was also altered in DM1 skeletal muscle (figure 2B). The proportion of α-DB2+vr3 mRNA was higher in DM1 muscles than in controls having normal muscle ($p = 0.001$), ALS ($p = 0.002$), or PM ($p = 0.002$). Moreover the relative amount of α-DB2+vr3 was strikingly increased in cDM muscle compared with normal fetal muscle. Next, we assessed mRNA expression levels of total α-DB1 and α-DB2 by real-time PCR analysis. The total amount of α-DB1 and α-DB2 mRNA did not differ between DM1 and controls (α-DB1: 1.70 ± 0.19 in 8 subjects with DM1, 1.62 ± 0.26 in 6 controls; α-DB2: 1.94 ± 0.56 in DM1, 0.93 ± 0.11 in controls, expressed in arbitrary units normalized to GAPDH expression). In addition, we studied the relationships between the splicing abnormalities of α-DB and the severity of muscular involvement of DM1 patients, as measured by the MDRS[12] or the number of CTG repeats in muscle tissue (table). A strong relationship was found between the proportion of α-DB2+vr3 and muscular disability, with the exception of an autopsied patient who became nonambulant as a result of advanced ovarian cancer (Pearson correlation coefficient $R = 0.729$, $p = 0.017$) (figure 2, bottom). Moreover, muscles with higher splicing abnormalities tended to have longer CTG repeats (figure e-1).

Splicing of α-DB in cardiac muscle. We also analyzed the splicing of α-DB in human cardiac muscle. α-DB1 Δvr3 and α-DB2 Δvr3 mRNAs were the predominant splice products in nondisease controls (figure 3, A and B). The proportion of α-DB+vr3 mRNA was increased in DM1 cardiac muscle in comparison with nondisease controls for α-DB2 ($p < 0.05$), and showed a trend for increase in α-DB1+vr3 ($p = 0.13$). Thus, alternative splicing of the vr3 region was abnormally regulated not only in skeletal but also in cardiac muscle from DM1 patients.

Protein expression of aberrantly spliced α-DB. We next investigated whether the protein of the alternatively spliced α-DB is expressed in human muscle. We first prepared DAPC from human skeletal muscles: 3 from DM1 patients and 6 from non-DM individuals (3 normal controls, 2 with PM, and 1 with facioscapulohumeral muscular dystrophy). We analyzed the expression of α-DB protein in the purified DAPC by immunoblotting. Two bands were observed in each analysis for α-DB1 and α-DB2, which show the expected size difference for vr3 inclusion (figure 4A). The larger bands were increased in DM1 muscles compared with non-DM controls (α-DB1, $p = 0.02$; α-DB2, $p = 0.02$) (figure 4B).

To confirm that larger bands correspond to α-DB+vr3 splice isoform, we raised a specific antibody against this isoform (Ab-DBvr3) by immunizing rabbits with purified synthetic polypeptides encoded by exons 11A and 12 (figure e-2). In the immunoblot analysis of DAPC from human skeletal muscles, Ab-DBvr3 reacted with only the larger band of α-DB2 (figure 4A), and also weakly with the larger band of α-DB1 after longer expo-

Table Clinical features and molecular data of DM1 patients

Patient no./sex	Age at biopsy/ onset, y	MDRS	Cardiac involvement: Conduction defects	Cardiac involvement: Cardiomyopathy	CTG repeats: Skeletal muscle	CTG repeats: Heart
1/M	40/38	3	+	NA	1,100	NT
2/M	58/45	3	+	–	3,900	NT
3/M	49/22	4	–	–	3,500	NT
4/F	36/21	4	+	–	5,500	NT
5/F	51/41	4	NA	NA	4,900	NT
6/F	42/33	4	–	–	3,900	NT
7/F	31/17	4	NA	NA	6,500	NT
8/F	48/36	4	+	–	NT†	NT
9*/M	63/28	5	+	+	3,500	4,800
10*/F	56/36	5	+	+	4,300	5,800
11*/F	58/50	5	+	–	3,500	4,300

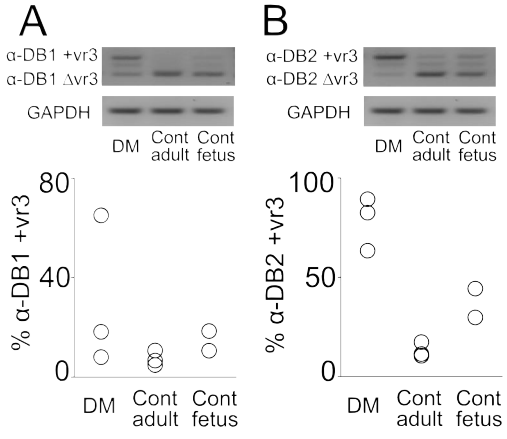
* Autopsied case.
† Diagnosis was confirmed by CTG repeats expansion in lymphocytes.
DM1 = myotonic dystrophy type 1; MDRS = muscular disability rating scale; NA = not available; NT = not tested.

sure, representing a lower expression level of α-DB1 in skeletal muscle relative to α-DB2 (data not shown). Thus, we confirmed that α-DB+vr3 corresponds to the larger bands that were relatively increased in DM1 muscle. Using this antibody, we also quantified α-DB2+vr3 in DM1 skeletal muscle, normalized to β-DG loading control. The amount of α-DB2+vr3 was higher in DM1 muscle than in non-DM muscle ($p = 0.02$) (figure 4C).

To investigate the subcellular localization of the α-DB+vr3 protein isoform in DM1 skeletal muscle, Ab-DBvr3 was used in immunohistochemical studies. As shown in figure 4D, α-DB+vr3 was detected in the sarcolemma of all fibers. This result shows that the α-DB+vr3 isoform localizes to the sarcolemma of DM1 muscle, as is the case for the normally spliced isoform (α-DB Δvr3) in human muscles,[20,21] and probably is associated with the DAPC.

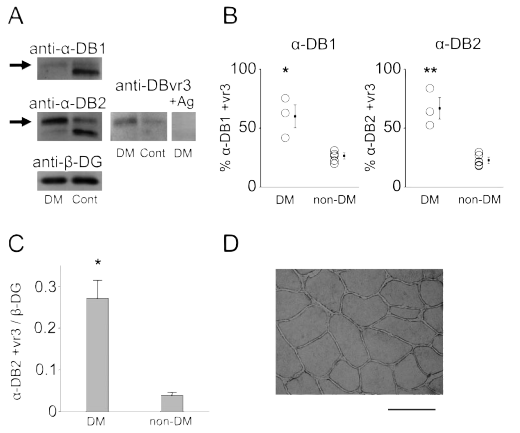
In vitro binding of α-DB and α-syntrophin. Next, we examined the functional difference between α-DB+vr3 and α-DB Δvr3. α-DB has been suggested to play an important role in intracellular signal transduction by interacting with syntrophin.[23] The binding motif for syntrophin was reported to be located downstream from the domain encoded by vr3.[24] Yeast two-hybrid anal-

Figure 3 Analysis of α-dystrobrevin (α-DB) splice variants, showing an increase of α-DB1+vr3 and α-DB2+vr3 in myotonic dystrophy type 1 cardiac muscle



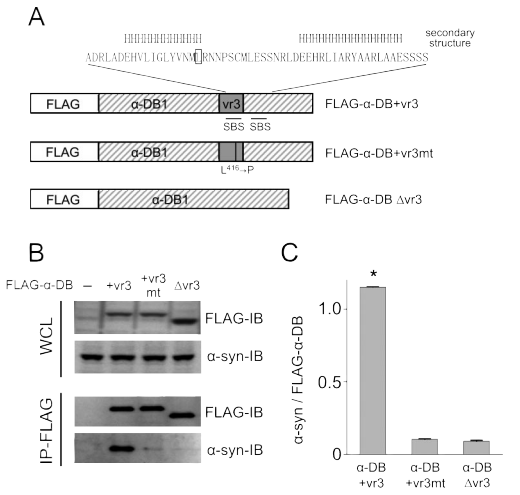
(A) Reverse-transcriptase PCR (RT-PCR) analysis for variable region 3 (vr3) of α-DB1 messenger RNA (mRNA) in cardiac muscle tissue from myotonic dystrophy type 1 patients (DM, n = 3), normal adult (Cont adult), and normal fetus (Cont fetus). Top: Representative RT-PCR products. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) amplicons are shown as loading controls. Bottom: The mean percentage of α-DB1+vr3 (% α-DB1+vr3/total α-DB1). (B) RT-PCR analysis for vr3 of α-DB2 mRNA. Top: Representative RT-PCR products. Bottom: The mean percentage of α-DB2+vr3 (% α-DB2+vr3/total α-DB2).

Figure 4 Immunoblot analysis of α-dystrobrevin (α-DB) from human skeletal muscles, showing an increase of α-DB1+vr3 and α-DB2+vr3 isoforms in myotonic dystrophy type 1



(A) Immunoblot analysis for dystrophin-associated protein complex from human skeletal muscles from myotonic dystrophy type 1 patients (DM, n = 3) and non-DM controls (non-DM, n = 6), with anti-α-DB1, α-DB2, and β-dystroglycan (β-DG) antibodies, and Ab-DBvr3 antibody with or without the specific antigen. Arrows indicate α-DB isoforms including variable region 3 (vr3). (B) The mean percentages of α-DB1+vr3 (left) and α-DB2+vr3 (right) isoforms measured as the densities of the larger bands in left panel of A (% α-DB+vr3/total α-DB). *$p < 0.05$. **$p < 0.05$. (C) The densities obtained by immunoblot assay of α-DB2+vr3 with the specific antibody (Ab-DBvr3) normalized to levels of β-DG. *$p < 0.05$. (D) Immunohistochemical analysis of α-DB+vr3 in DM1 skeletal muscle with Ab-DBvr3. Bar, 100 μm.

Figure 5 Immunoprecipitation analysis of α-dystrobrevin, showing interaction with α-syntrophin via the variable region 3 domain



(A) Schematic representation of the FLAG-tagged fusion α-dystrobrevin (α-DB) proteins. H represents α helix indicated by PROF analysis. Bars indicate suggested syntrophin binding sites (SBS). Leu^{416} is boxed. Hatched boxes represent the region encoding α-DB1. (B) Anti-FLAG immunoprecipitation (IP) and whole-cell lysates (WCLs) from HEK293 cells transfected with plasmids for FLAG-α-DB+vr3 (+vr3), or its mutant L416P (+vr3mt), or FLAG-α-DB Δvr3 (Δvr3) were subjected to immunoblot analysis. (C) The relative amounts of α-syntrophin immunoprecipitated with FLAG-tagged α-DB+vr3, α-DB+vr3mt, and α-DB Δvr3. *$p <$ 0.001.

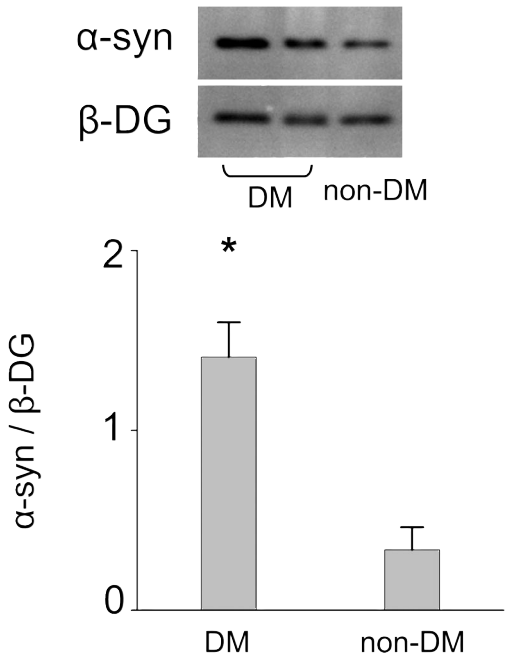
ysis and binding studies of peptide fragments suggested an additional binding motif that forms a tandem pair of predicted α helices within the vr3 region of mouse α-DB.[25] To test for an interaction between human syntrophin and α-DB+vr3 or α-DB Δvr3, FLAG-tagged α-DB expression constructs including vr3 (pCMV-FLAG-α-DB+vr3) or excluding vr3 (pCMV-FLAG-α-DB Δvr3) were generated from pMGT1-DB and pMGT1-DB Δvr3. These constructs were used to transfect HEK293 cells (figure 5A). Only the α-DB construct was transfected because HEK293 cells express abundant α-syntrophin intrinsically (figure 5B, first lane). The resulting cell extracts were prepared and subjected to immunoprecipitation analysis. It should be noted that overexpression of FLAG-tagged α-DB in HEK293 cells did not result in alteration of endogenous α-syntrophin expression (figure 5B). Using anti-FLAG M2 agarose gel, endogenous α-syntrophin was immunoprecipitated from cell lysates transfected with pCMV-FLAG-α-DB+vr3, whereas little was immunoprecipitated with pCMV-FLAG-α-DB Δvr3 (figure 5B, second and fourth lanes, and figure 5C). These results suggest that α-DB including the vr3 region has a strong ability to bind α-syntrophin.

It has been suggested that the vr3 region of α-DB forms an α helix that is indispensable for syntrophin binding.[25] Leu^{416} within vr3 is highly conserved in many species, and computer analysis using PROF (Profile Network Heidelberg, www-.predictprotein.org/) indicates that it is crucial for maintaining the α helix structure of the vr3 region. To clarify the specific interaction between the vr3 region of α-DB and α-syntrophin, we next constructed a FLAG-tagged mutant α-DB+vr3 expression vector (pCMV-FLAG-α-DB+vr3mt) carrying a Leu/Pro substitution at Leu (416) (figure 5A). The amount of α-syntrophin immunoprecipitated with the mutant α-DB+vr3 was dramatically reduced to the same level of that with pCMV-FLAG-α-DB Δvr3 (figure 5B, third lane, and figure 5C). Taken together, these results demonstrate strong interaction between α-syntrophin and the vr3 region of α-DB.

Expression of α-syntrophin in DM1 muscle. From in vitro studies of binding between α-DB and α-syntrophin, it is hypothesized that aberrantly spliced α-DB alters the amount of α-syntrophin expression in the sarcolemma in vivo. To investigate the expression of α-syntrophin in DM1, immunoblot analysis was performed using purified DAPC from human skeletal muscles (4 from DM1 patients and 5 from non-DM controls). The amount of α-syntrophin in DAPC was increased in muscles from DM1 patients compared with non-DM controls ($p = 0.014$) (figure 6). Because α-DB+vr3 is increased and localized at the sarcolemma in DM1 muscle and binds strongly to α-syntrophin, the increased amount of α-syntrophin in DM1 supports the possibility that the aberrantly spliced α-DB+vr3 recruits more α-syntrophin to the DGC.

Splicing of α-DB in mouse muscle. Previously, several groups reported that mouse skeletal muscle expresses α-DB+vr3 exclusively,[18,22] but in the present study, α-DB+vr3 showed low expression in normal human muscles. To confirm the species difference in the splicing of the vr3 region, we examined the developmental regulation of α-DB splicing in mouse skeletal muscle at postnatal days (P) 0, 7, 14, 28, and in adult mice. There was an increase in vr3 inclusion of α-DB1 between P0 and P28 (figure e-3A). As described previously, the skeletal muscle from adult mouse expressed only α-DB+vr3 mRNA. The splicing pattern was not changed in the HSA^{LR} transgenic mouse, where

Figure 6 Immunoblot analysis of α-syntrophin from human skeletal muscles, showing an increase of α-syntrophin in myotonic dystrophy type 1



Top: Immunoblot analysis of α-syntrophin (α-syn) in myotonic dystrophy type 1 and non-DM controls. Bottom: The densities obtained by immunoblot assay of α-syntrophin normalized to levels of β-dystroglycan (β-DG) (DM, n = 4; non-DM, n = 5). *$p < 0.05$.

the expression of an expanded CUG repeat in skeletal muscle leads to a DM-like phenotype without severe muscle degeneration.[11] We also investigated the protein expression in mouse by immunoblot and immunohistochemistry. As suggested by the mRNA expression patterns, α-DB1+vr3 and α-DB2+vr3 protein isoforms were expressed in adult mouse skeletal muscle and localized to the sarcolemma (figure e-3, B and C).

DISCUSSION α-DB is abundantly expressed in skeletal and cardiac muscle and brain,[26] the major organs affected in DM1. Mice lacking α-DB exhibit skeletal and cardiac myopathies, defects of NMJ maturation, and abnormal myotendinous junctions.[27-29] Our study shows that alternative splicing of α-DB is dysregulated in skeletal muscle of DM1 patients, and that the splicing abnormality is correlated with the muscular disability. We also found aberrant splicing of α-DB in cardiac muscle of DM1. Although several genes show misregulated alternative splicing in DM1 heart,[3,30,31] the cause of the cardiac symptoms in DM1 is not well understood. It is noteworthy that a mutation in the α-DB gene was reported in a family with left ventricular noncompaction,[32] a cardiac abnormality associated with arrhythmia that occurs in some patients with DM1.[33] In addition, we investigated splicing of α-DB in human brain tissues (5 from DM1 patients and 2 from non-DM individuals). However, we found that α-DB Δvr3 isoform was the predominant splice product in brain, with no difference between DM1 and non-DM (data not shown).

A key biochemical defect in DM1 is "spliceopathy," i.e., a trans-effect on the alternative splicing of many RNAs.[2,34] In general, the effect on splicing involves abnormal expression in adults of splice isoforms that normally occur in fetal development, and these effects are conserved in mouse models of DM1. We confirmed previous reports that vr3 splicing is developmentally regulated in mice,[18,22] and we also showed that the frequency of vr3 inclusion increases during normal postnatal development. However, the predominant isoform expressed in normal adult human muscle is the α-DB Δvr3 splice product. Therefore, in the case of α-DB, DM1 has caused a transition to the splicing pattern that normally predominates in adult mice. These results suggest a different, or possibly an opposite, mode of regulation of vr3 alternative splicing between humans and mouse. Among many abnormally spliced mRNAs in DM patients, α-DB is not the only case that shows different splicing regulation between human and mouse. It was reported that the pattern of misregulated *IR* splicing in DM1 patients is not conserved in mouse models of DM1.[35] Such discrepancies may contribute to the phenotypic differences between DM1 and mouse models. However, the possibility remains that CUG expansion is not responsible for the misregulated α-DB splicing.

α-DB is associated with the sarcoglycan complex, dystrophin, and syntrophin. α-DB1, α-DB2, and α-DB3 bind to the sarcoglycan complex via the N-terminal region.[36] α-DB1 and α-DB2 bind dystrophin through the highly conserved coiled-coil domain (figure 1).[37] The syntrophin binding site was mapped to the region upstream of the coiled-coil domain on α-DB1 and α-DB2.[38] Skeletal muscle contains several isoforms—α-, β1-, β2-, and γ2-syntrophin—encoded by different genes, and α-syntrophin is the major isoform in human skeletal muscle, being expressed in the sarcolemma.[20,39,40] With regard to the syntrophin binding site on Torpedo 87K protein, a homolog of human α-DB1, the binding site for α-syntrophin was mapped to a region similar to exons 12 and 13 of human α-DB.[41] A yeast two-

hybrid study identified another syntrophin binding site within the mouse vr3 region, suggesting that α-DB contains two independent syntrophin binding sites in tandem.[25]

Several lines of evidence suggest that alternative splicing of vr3 may influence the function of α-DB. Syntrophin proteins are recruited to the sarcolemma through interactions with α-DB. These proteins coordinate the assembly of several important proteins, such as neuronal nitric oxide synthase, stress-activated protein kinase 3, and calmodulin to the DGC.[23] Our results show, in the context of mammalian cells, that vr3 inclusion enhances the interaction of α-syntrophin with α-DB. Therefore, the increased recruitment of α-syntrophin to the sarcolemma might lead to a disturbance of intracellular signal transduction. In addition, syncoilin, a member of the intermediate filament family of proteins, interacts with α-DB via vr3 and its flanking region.[42] Finally, α-DB1 Δvr3 has been shown to contain more phosphotyrosine than α-DB1+vr3.[18] α-DB1 is one of the major phosphotyrosine-containing proteins at the NMJ,[43] and phosphorylation of α-DB1 is suggested to play an important role in synaptogenesis.[16,29] Taken together, these observations raise the possibility that effects of DM1 on alternative splicing may influence protein interactions of α-DB and muscle function.

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New Guideline Identifies Those Most at Risk of Falls

A new AAN guideline finds certain neurology patients are at a high risk of unintentional falls and should be screened regularly to help prevent the high number of fall-related injuries and deaths in the United States each year. The guideline, "Assessing Patients in a Neurology Practice for Risk of Falls," has been published in the February 5, 2008, issue of *Neurology*®.

The guideline found people with stroke, dementia, and walking and balance disorders are at the highest risk of falling. A history of falling in the past year also strongly predicts that a person will fall again. People with Parkinson disease, peripheral neuropathy, weakness in the legs or feet, and substantial vision loss are also likely to fall. Routinely asking patients about falls will ultimately help reduce fall-related injuries and deaths and lead to better quality of life for patients at risk.

To view the full guideline, visit *www.aan.com*.