Isoform Diversity of Dystrobrevin, the Murine 87-kDa Postsynaptic Protein*

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Dystrophin-related and associated proteins are important in the formation and maintenance of the mammalian neuromuscular junction. We have characterized mouse cDNA clones encoding isoforms of the dystrophin-homologous 87-kDa postsynaptic protein, dystrobrevin. In Torpedo, the 87-kDa protein is multiply phosphorylated and closely associated with proteins in the postsynaptic cytoskeleton, including the acetylcholine receptor. In contrast to Torpedo, where only a single transcript is seen, the mouse expresses several mRNAs encoding different isoforms. A 6.0 kilobase transcript in brain encodes a 78-kDa protein (dystrobrevin-1) that is very similar to the Torpedo sequence. A second transcript encodes a 59-kDa protein (dystrobrevin-2) that has a different C terminus, lacking the putative tyrosine kinase substrate domain. In skeletal and cardiac muscle, transcripts of 1.7 and 3.2/3.5 kilobases predominate and encode additional isoforms. Alternative splicing within the coding region and differential usage of untranslated regions produce additional variation. Multiple dystrobrevin-immunoreactive proteins copurify with syntrophin from mouse tissues. In skeletal muscle, dystrobrevin immunoreactivity is restricted to the neuromuscular junction and sarcolemma. The occurrence of many dystrobrevin isoforms is significant because alternative splicing and phosphorylation often have profound effects upon the biological activity of synaptic proteins.

Chemical synapses in the peripheral and central nervous systems mediate intercellular communication, controlling and coordinating an overwhelming range of cellular processes. Despite their importance, comparatively little is known about the structure and function of synapses in the brain. The majority of our knowledge of synapse structure originates from the study of the specific contact that occurs between a motor neuron and a muscle fiber: the neuromuscular junction (NMJ). The NMJ consists of a presynaptic nerve terminal separated from the postsynaptic muscle cell by the synaptic cleft containing the basal lamina. The morphology and molecular architecture of the NMJ are critical for efficient synaptic transmission.

The formation of dense clusters of acetylcholine receptors (AChRs) at the point of contact between the motor neuron and muscle fiber is an early event in postsynaptic differentiation. Of the various factors that influence AChR clustering, agrin, an extracellular matrix protein secreted by the motor neuron, appears to play a pivotal role (2). In addition to AChR clustering, agrin also induces the redistribution of many other postsynaptic (3, 4) and cytoskeletal (5) proteins. These localized cellular changes lead to the formation of microclusters of AChRs that are subsequently developing into the macroclusters characteristic of the mature synapse.

Central to the role of agrin in synaptogenesis is the cell-surface agrin receptor. α-Dystroglycan (156-kDa dystrophin-associated glycoprotein), a component of the dystrophin-associated glycoprotein complex (DGC), has recently been shown to be an agrin receptor in skeletal muscle (6–9). Extra-junctionally, α-dystroglycan binds to the laminin α3-chain (merosin) in the extracellular matrix and to components of the DGC that span the sarcolemma (10). One of these transmembrane proteins, β-dystroglycan (43-kDa dystrophin-associated glycoprotein), binds to the cysteine-rich domain of dystrophin (11). Since the N terminus of dystrophin binds to actin (12, 13), the dystroglycans effectively link the cytoskeleton of the muscle fiber to the extracellular matrix.

The immobilization of AChR clusters is thought to be accomplished by proteins in the junctional cytoskeleton (14). Although dystrophin is present in the cytoskeleton of the NMJ (15–17), utrophin, an autonomously encoded homologue of dystrophin (18), precisely colocalizes with the AChR clusters at the crests of the junctional folds (19–21). Urophin, like dystrophin, also binds to components of the DGC (22). An attractive hypothesis is that agrin binding to α-dystroglycan at the NMJ causes a local accumulation and reorganization of cytoskeletal proteins and, in particular, utrophin, eventually resulting in the immobilization of AChR clusters by a cytoskeletal scaffold. This hypothesis is supported by evidence showing that utrophin is present in spontaneous clusters of AChRs (23) and following agrin induction (7).

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In Torpedo electric organ, two proteins with molecular masses of 87 and 58 kDa (syntrophin) are associated with dystrophin and postsynaptic complexes. The 87-kDa protein is a minor component of the postsynaptic membrane that copurifies with the AChR in the electric organ membranes (24). Monoclonal antibodies (mAbs) raised against this protein label Torpedo electric organ, mammalian end plates, and, to a lesser extent, the extrasynaptic sarcolemma (24). The 87-kDa protein colocalizes with dystrophin and syntrophin (25, 26). Three mammalian syntrophin genes have been identified by molecular cloning (27–29). The β-form of syntrophin is highly localized to the neuromuscular postsynaptic membrane, whereas α-syntrophin is ostensibly located at the sarcolemma (30).

Cloning of the Torpedo 87-kDa cDNA revealed moderate but significant sequence similarity to the cysteine-rich and C-terminal domains of the dystrophin protein family (1, 31). Similar regions in dystrophin, utrophin, and the 87-kDa protein contain the binding sites for the mammalian homologues of syntrophin (32–35). In Torpedo, the 87-kDa postsynaptic protein is the product of a single 4.6-kb transcript expressed in the electric organ, brain, and in skeletal muscle (1). While the function of the 87-kDa protein is unresolved, a role in the formation and stability of synapses has been proposed (1). The 87-kDa protein is also a major phosphotyrosine-containing protein in Torpedo electric organ (1). This is particularly significant because agrin-induced AChR clustering is blocked by inhibitors of tyrosine kinase (14, 36, 37). In mammalian tissues, multiple 87-kDa cross-reactive proteins copurify with syntrophin from many different rat tissues (26). The 87-kDa protein may therefore have other functions in addition to its role in synaptogenesis.

To investigate the molecular organization of utrophin- and dystrophin-containing complexes in mouse brain and muscle, we have cloned the murine homologue of the Torpedo 87-kDa postsynaptic protein. In this paper, we show that there are many different murine 87-kDa protein isoforms produced from alternatively spliced transcripts. This is at variance with the situation in Torpedo where only a single transcript and protein have been described (1). Many of these 87-kDa isoforms are associated with syntrophin in different mouse tissues. In rat skeletal muscle, 87-kDa immunoreactivity is restricted to the NMJ and sarcolemma. Our studies parallel the heterogeneity and localization of the mammalian dystrophin/utrophin family and the syntrophin family (38), all of which are potential ligands for the 87-kDa isoforms described herein. In deference to the 87-kDa protein being a low molecular mass protein with homology to dystrophin and in agreement with other researchers studying this protein, we have adopted the name dystrobrevin for proteins encoded by the murine 87-kDa gene.

**EXPERIMENTAL PROCEDURES**

**RNA Extraction and Northern Blotting**—RNA was extracted from mouse tissue or cultured cell lines following the method of Chomczynski and Sacchi (49). mRNA was prepared from ~250 μg of total RNA using Oligotex (QIAGEN, Inc.) following the manufacturer’s instructions. Northern blots were prepared as described previously (50). All Northern blots were washed at the same stringency (2 × SSC, 0.1% SDS at 65°C for 30 min).

**cDNA Library Construction and Screening**—A cDNA library was made from 5 μg of adult mouse brain mRNA following an adaptation of the method of Gubler and Hoffman (51). Briefly, denatured mRNA was reverse-transcribed in a 100-μl solution containing 150 units of avian myeloblastosis virus reverse transcriptase, 14 mM dithiothreitol, 100 μM dNTPs, and 1 × RTI buffer (5 × RTI buffer = 250 mM Tris-HCl (pH 8.2 at 42°C), 250 mM KCl, and 30 mM MgCl₂) for 1 h at 42°C. The second strand was synthesized in a final volume of 400 μl by diluting the first strand reaction with 80 μl of 5 × RT2 buffer (100 mM Tris-HCl (pH 7.5), 500 mM KCl, 25 mM MgCl₂, and 0.25 mg/ml bovine serum albumin), 10 μl of 10 mM NAD⁺, 20 units of Escherichia coli DNA ligase, 4 units of RNase H, and 100 units of E. coli DNA polymerase I. The volume was adjusted to 400 μl with diethyl pyrocarbonate-treated water, and the reaction was incubated for 90 min at 16°C followed by 30 min at 22°C. The DNA was treated with 4 units of T4 DNA polymerase (Boehringer Mannheim) for 15 min at 37°C. BstXI adapters were ligated to the blunt-ended cDNA at room temperature for 16 h. The adapters and short cDNAs (<175 base pairs) were removed using GeneClean (BIO 101, Inc.). Purified adapter cDNA was ligated into the BstXI sites of pcDNAII (Invitrogen). The cDNA library was used to transform electrocompetent or chemically competent E. coli XL1-Blue. The cDNA library was plated at high density on Hybond-N membranes (Amersham Corp.) and screened with [α-32P]dCTP-labeled probes.

**RT-PCR**—10 μg of denatured total RNA was converted into first strand cDNA with 50 units of avian myeloblastosis virus reverse transcriptase in a final volume of 100 μl in a solution containing 14 mM dithiothreitol, 40 units of RNase inhibitor, 1 μg of oligo(dT)₃₉₋₁₄, 1 mM dNTPs, and 20 μl of 5 × RTI buffer for 1 h at 42°C. 2.5 μl of a 1:10 dilution of the first strand cDNA was used in 50-μl PCR reactions, performed under standard conditions using Taq DNA polymerase (Perkin-Elmer).

**Sequence Analysis**—cDNA clones were prepared for sequencing by making nested deletions with exonuclease III and S1 nuclease following the manufacturer’s instructions (Erase-a-Base, Promega). Deleted plasmids were assayed by PCR with Sp6 and T7 primers were assayed using the Genetics Computer Group Version 7.3 software package.

**Antibody Production**—Polyclonal antibodies were prepared in rabbits using synthetic peptides according to standard methods. Ab 308 and Ab 433 were generated against peptides CASREPLHPMFPDQJKPLN and CRVEHEQASQPTPEKQAQNQ (corresponding to amino acids 308–328 and 433–451 plus an amino-terminal cysteine for coupling), respectively. Each antibody was affinity-purified from serum using peptide-coupled Affi-Gel 10 (Bio-Rad) as described previously (26).

**Immunoblots**—Preparations enriched for syntrophins and associated proteins were immunostaining from Triton X-100 extracts of mouse tissues using mAb SYN1351 as described previously (26). Samples were resolved on 8% SDS-polyacrylamide gels, transferred to Immobilon-P (Millipore Corp.), and blotted (26). Blots were incubated with mAb SYN1351 (60 μg), Ab 308 (30 μg), or Ab 433 (30 μg) followed by appropriate horseradish peroxidase-coupled secondary antibody (1:3000; Jackson ImmunoResearch Laboratories, Inc.). Blots were developed using enhanced chemiluminescence (Pierce) and exposed to Bio-Max film (Eastman Kodak Co.).

**Immunofluorescence**—Cryosections (8 μm) of fixed rat sternomastoid were labeled with Ab 308 (20 μg) followed by rhodamine-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) as described previously (16). Labeling was examined in regions of NMJs identified with BODIPY–a-bungarotoxin (1:3000; Molecular Probes, Inc.). As a control, Ab 433 was preincubated with its corresponding peptide (100 μg) for 1 h prior to use. Labeling with each fluorophore was photographed and processed equivalently.

**RESULTS**

**Isolation of cDNAs Encoding the Murine Orthologue of the Torpedo 87-kDa Postsynaptic Protein**—Nucleic acid sequence data bases were searched with the Torpedo sequence encoding the 87-kDa postsynaptic protein. This search identified several human expressed sequence tags that had significant similarity to the query sequence. Oligonucleotide primers were designed to one of the sequences, EST000891 (39), and used to amplify a single DNA fragment from first strand cDNA made from human fetal brain and dorsal root ganglia RNAs. This amplified cDNA was used to screen a human fetal brain cDNA library. Two positively hybridizing clones, 87e1 (1.6 kb) and 87e3 (0.5 kb), were picked and partially sequenced. Both clones were similar to EST000891 and the Torpedo sequence. Because 87e1 was the larger of the two clones, it was used for Northern blotting and cDNA library screening.

Clone 87e1 was hybridized to a Northern blot of mRNAs from...
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FIG. 1. Northern blot hybridized with a cDNA encoding part of the human dystrobrevin. 5 µg of mRNA prepared from the following sources was hybridized with 87e1: a mouse BC3H-1 smooth muscle-like cell line, monkey kidney COS-7 cells, embryonic mouse brain (EMB), and adult mouse brain (AMB). The 6.0-kb full-length transcript was present in all samples except embryonic mouse brain mRNA. Additional transcripts of 4.3, 3.5, 3.3, and 1.7 kb (represented by asterisks) were also detected in adult mouse brain mRNA.

The sequence of m24 was identical to the m871 sequence throughout the region of overlap. The sequences of m24 and m871 3′ of the TGA stop codon overlapped with the 5′-end of m22 (Fig. 2A). The other end of m22 showed significant similarity to the 3′-UTR of the Torpedo cDNA including the proposed polyadenylation site (data not shown). The m22 cDNA is therefore part of the 3′-UTR of the same transcript from which m24 and m871 are derived. Because the m24 and m22 cDNAs are 2.8 and 3.2 kb, respectively, and they overlap by ~50 base pairs, it was concluded that they are derived from the 6.0-kb brain transcript (Fig. 1). This was confirmed by Northern blotting (see below) and by RT-PCR (data not shown). We have named the 78-kDa protein product of the 6.0-kb transcript “dystrobrevin-1” to distinguish it from the other proteins described in this study.

The extreme 5′-end of m32 had no homology to the Torpedo sequence or to the m24 sequence. In common with m24, the homology to the Torpedo sequence started at nucleotide 135. Upstream of this sequence are stop codons in every reading frame, indicating that this sequence is not translatable and therefore is the 5′-UTR. Conceptual translation of the m32 sequence yielded a long open reading frame encoding a 59-kDa protein. There are some significant differences between the sequences of m32 and m24 and the Torpedo sequence. The m32 sequence diverges from m24 at amino acid 504 of the m32 sequence (Fig. 3C). A TAA stop codon occurs after 30 nucleotides, resulting in a protein with a different C terminus (Fig. 3B). m32 therefore encodes a protein devoid of the tyrosine kinase substrate domain described by Wagner et al. (1).

In common with the 5′-UTRs, m32 and m24 also have different 3′-UTRs. Comparison of the m24 and m32 sequences revealed two other minor differences. m32 has an insertion of 9 nucleotides that code for the tripeptide DTW. This sequence is not present in m24 or in the Torpedo sequence (Fig. 3C). Additionally, there is a single base difference between the two sequences, resulting in a neutral amino acid difference of Leu-251 in m32 and Val-251 in m24. Valine is found at this position in the Torpedo sequence (Fig. 3C). Like m24, the m32 sequence contains a deletion of amino acids 370–400 of the Torpedo sequence (Fig. 3C). This observation is investigated below.

The sequences of m32 and m872 3′ of the stop codon are identical. The 3′-ends of m32, m21, and m11 also overlap, suggesting a common 3′-UTR in all the clones. In contrast, the extreme 5′-end of m21, within the predicted 5′-UTR, differs from the sequence of m32, although the remainder of the 5′-UTR is identical in these clones. This result indicates that the 5′-UTRs of some of the dystrobrevin transcripts may be encoded by more than one exon. Because clones m32 and m21 are both ~3.0 kb and their 5′-UTRs are different, the corresponding transcript sizes (4.3, 3.5, or 3.3 kb) cannot be directly deduced. We have named the 59-kDa protein encoded by the m32 cDNA clone “dystrobrevin-2.”

Alternative Splicing—Sequence analysis of other clones isolated from the adult mouse brain cDNA library and of RT-PCR products cloned from different mouse tissues revealed the presence of at least four different variants. These differences are most likely to be the result of alternative splicing; however, it is formally possible that this variation is the consequence of sequence divergence between very closely related genes.

In addition to the sequence differences in the 5′-UTRs of clones m24, m32, and m21 described above, there are three variable regions within the protein coding sequence. An extra 3 amino acids (DTW) occur at position 335 of the m32 peptide sequence that are not present in the m24 sequence. We have designated this site “variable region (vr) 1.”
A stretch of charged residues (EEELKQGTR) corresponding to amino acids 495–503 of the m24 sequence (Fig. 3A) is replaced by the sequence TQG encoded by m871 (Fig. 4A). We have designated this region “vr2.” vr2 covers the point where the predicted C termini of the brain dystrobrevin-1 and dystrobrevin-2 sequences diverge and is immediately adjacent to the coiled-coil region conserved in the dystrophin-related protein family (31). The Torpedo sequence is also similar to the 9 amino acids at vr2 (Fig. 3C).

Computer-assisted alignment of the predicted protein sequences of m24 and m32 with the Torpedo peptide sequences shows that the region encompassing amino acids 363–400 of the Torpedo sequence is not present in the two mouse sequences (Fig. 3C). The mouse brain cDNA clones only have a small additional stretch of nonhomologous sequence (FITRSM) before the similarity with the Torpedo sequence is restored after amino acid 400. Oligonucleotides were made that flanked this region and were used to amplify first strand cDNA prepared from various mouse tissues. Products of the predicted size were obtained in brain cDNA; however, the products in skeletal and cardiac muscle were ~200 base pairs larger (data not shown). The products from both tissues were cloned and sequenced. Sequence analysis showed that the additional sequence, accounting for the increased mobility of the PCR products in heart and muscle, was very similar to the region encoding amino acids 364–399 of the Torpedo sequence, but absent in m24 and m32. The region of similarity between the Torpedo and mouse sequences is not continuous, but is interrupted by two insertions of 5 and 21 amino acids in the mouse sequence (Fig. 4B). RT-PCR showed that this region, which we have called “vr3,” appears to be specific to skeletal and cardiac muscle (data not shown).

Since the dystrobrevin-1 and dystrobrevin-2 transcripts differ in their 3′-ends (Fig. 3, A and B) and hybridize to transcripts in heart and skeletal muscle (Fig. 5, A and C), it was important to determine whether vr3 was specific to either transcript. RT-PCR using a forward primer in the common coding sequence and a reverse primer in the 3′-UTR of either the dystrobrevin-1 or dystrobrevin-2 sequence only gave products that included the vr3 sequence. This was confirmed by cloning and sequencing (data not shown). These data indicate that the major dystrobrevin-encoding transcripts in heart and skeletal muscle contain vr3 and are thus different from the transcripts present in brain. We have named the protein that contains the vr3 sequence and is encoded by a transcript with the same 3′-UTR as the dystrobrevin-1 transcript “dystrobrevin-3.” Similarly, we have named the protein that contains the vr3 sequence but is encoded by a transcript with the same 3′-UTR as the dystrobrevin-2 transcript “dystrobrevin-4.” The organization of dystrobrevin-1, -2, -3, and -4 is summarized in Fig. 4C.

**Figure 2.** Restriction map of the murine cDNA clones predicted to encode brain dystrobrevin-1 (A) and brain dystrobrevin-2 (B). The structure of the Torpedo transcript is shown for reference and is not meant to indicate regions of homology. hI + hII denotes the location of the α-helices predicted to form the coiled-coils conserved in proteins with homology to the C terminus of dystrophin (31), and TYR in the Torpedo 87-kDa sequence denotes the tyrosine kinase substrate domain (1). The polyadenylation consensus sequence, ATTTAA, is shown in parentheses because it is only homologous to the Torpedo sequence and may not be the true polyadenylation signal. The dashed line at the end of the dystrobrevin-2 restriction map indicates that the entire dystrobrevin-2-encoding transcript(s) has not been cloned. B, BamHI; E, EcoRI; H, HindIII; Sc, SacI; S, SphI; X, XhoI.

**Figure 3.** Tissue distribution of the murine dystrobrevin transcripts. The Torpedo 87-kDa postsynaptic protein is encoded by a single 4.6-kb transcript expressed in the electric organ, in brain, and in skeletal muscle (1). Preliminary Northern blot analysis indicated that several transcripts in mouse brain RNA hybridized to a cDNA clone encoding part of human dystrobrevin (Fig. 1). Furthermore, at least two families of cDNA clones were obtained from the adult mouse brain cDNA library. To examine the tissue distribution of these transcripts, Northern blots prepared from mouse tissue RNA were hybridized with different cDNA clones and subclones.

Northern blots hybridized with clone m872 (Fig. 2), encoding the majority of dystrobrevin-2, gave a complex band pattern (Fig. 5A). The m872 cDNA clone was chosen because it contains coding sequence that is common to both dystrobrevin-1 and dystrobrevin-2. In brain RNA, m872 hybridized strongly to transcripts of 6.0, 4.3, 3.5, 3.3, 1.7, 1.2, and 0.7 kb. The hybridization pattern in the other tissues is less complex. In heart, m872 hybridized to two transcripts of 3.3 and 1.7 kb. In skeletal muscle and lung, m872 hybridized predominantly to three transcripts of 6.0, 3.5 (3.3 kb in muscle), and 1.7 kb. No strongly hybridizing transcripts were detected in RNA from other tissues. The 6.0-kb full-length transcript as well as the 4.3-, 3.5-, 3.3-, and 1.7-kb transcripts are also present in the adult mouse brain mRNA (Fig. 1) used in the cDNA library construction.
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A

B

C

FIG. 3.
To establish the molecular basis of this complex pattern of transcripts, Northern blots were hybridized with cDNA subclones. Using the 1.1-kb EcoRI restriction fragment from m871, composed of sequence unique to the dystrobrevin-1 transcript, the major transcript detected is 6.0 kb (Fig. 5B). This transcript is abundant in brain, but can also be detected at lower levels in muscle and lung and very weakly in kidney. Additionally, a weakly hybridizing 3.3-kb transcript is also detected in brain, lung, and muscle RNAs. This confirms that the brain transcript encoding dystrobrevin-1 is 6.0 kb. m22 gave the same hybridization pattern as the 1.1-kb EcoRI subclone of m871 (data not shown).

The 0.9-kb XhoI-EcoRI fragment of m21 covers part of the 3'-UTR of the transcript(s) that encodes dystrobrevin-2. Hybridization of this probe to Northern blots gave a complex band pattern (Fig. 5C). In brain, this probe hybridizes to transcripts of 6.0, 4.3, 3.5, 3.3, 1.7, 1.2, 0.8, and 0.7 kb. In skeletal muscle, heart, and lung, the 0.9-kb XhoI-EcoRI fragment of m21 predominantly hybridizes to 3.5-kb (3.3-kb in muscle) and 1.7-kb transcripts. In muscle and lung, this probe also hybridizes weakly to a 6.0-kb transcript. Additionally, in muscle, a 4.3-kb transcript can also be detected. This complex pattern is very similar to the pattern of hybridization of m872 to the same Northern blots. m872 also contains part of the dystrobrevin-2 3'-UTR as well as the majority of the coding region. This indicates that the 3'-UTR of the brain dystrobrevin-2 transcript, present in m872, m32, m21, and m11, cross-hybridizes to several different dystrobrevin-encoding transcripts. This hybridization pattern may be due to a repetitive element within the 3'-UTR or may reflect the number of dystrobrevin-encoding transcripts that utilize this UTR or a UTR with a very similar sequence.

Expression of Dystrobrevin Isoforms—Expression of the dystrobrevin isoforms was determined by immunoblot analysis. Two anti-peptide antibodies were made using synthetic pep-
Dystrobrevin isoforms were identified with Ab 308 (middle panel) and Ab 433 (lower panel). The sizes of the molecular mass markers (in kilodaltons) are indicated.

**Fig. 6. Immunoblot analysis of dystrobrevin gene products in mouse tissues.** Dystrobrevin isoforms associated with syntrophin were purified from Triton X-100 extracts of tissues using mAb SYN1351. Sample loadings were adjusted so that the amount of syntrophin in each lane was approximately equal (upper panel, anti-syn). Dystrobrevin isoforms were identified with Ab 308 (middle panel) and Ab 433 (lower panel). The sizes of the molecular mass markers (in kilodaltons) are indicated.

This paper describes the isolation and characterization of cDNA clones encoding isoforms of the murine homologue of the 87-kDa postsynaptic protein originally identified in the electric organ of *Torpedo californica*. In contrast to the situation in *Torpedo*, where there appears to be a single transcript, in the mouse, there are multiple transcripts encoding different protein isoforms. In mouse brain, the “full-length” transcript is 6.0 kb and encodes a protein with a predicted molecular mass of 78 kDa. We have called this protein dystrobrevin-1. In addition to the dystrobrevin-1 transcript, several other mRNAs can be detected in brain. One of these transcripts encodes a protein with a predicted molecular mass of 59 kDa; we have called this product dystrobrevin-2. The dystrobrevin-1 transcript is most similar to the *Torpedo* mRNA because there is significant sequence similarity between the 3′-UTRs as well as in the coding sequence.

**DISCUSSION**

The major difference between dystrobrevin-1 and dystrobrevin-2 is that they have different C termini. Dystrobrevin-2 lacks the proposed tyrosine kinase substrate domain (1) and may not be a suitable substrate for tyrosine phosphorylation. However, dystrobrevin-2 may still be phosphorylated on serine and threonine residues in a similar manner to the *Torpedo* 87-kDa protein (40). The other major differences between the dystrobrevin-1 and dystrobrevin-2 transcripts occur in the untranslated regions. The cDNA clones m32, m24, and m21 all have different 5′-UTRs. The occurrence of several 5′-UTRs is suggestive of independent regulation. Each transcript may be regulated by a different promoter and may have separate 5′-
noncoding regions, but still utilize the same coding regions and initiator methionine. The rat gene for brain-derived neurotrophic factor has a similarly complex organization. Four short 5' non-coding exons can be spliced to a common coding exon and are each regulated by separate promoters (41). These promoters confer tissue-specific, axotomy- and neuronal activity-induced expression in transgenic mice (42). It is therefore possible that several of the dystrobrevin transcripts in the brain are transcribed in a region-specific manner from different promoters. The 6.0-kb transcript is most abundant in adult brain, but is also expressed at lower levels in skeletal muscle and lung. In muscle, heart, and lung, two transcripts of 3.3/3.5 and 1.7 kb predominate. The major dystrobrevin-encoding isoforms in muscle and heart differ from their brain counterparts and include an additional 57 amino acids (vr3) preceding the coiled-coil domain (Fig. 4C). The vr3 sequence is only present in skeletal and cardiac muscle and thus represents a significant difference between the products in brain and muscle. In addition, the vr3 sequence is also present in muscle transcripts that in addition to encode proteins with different C termini, i.e. with C termini identical to those of dystrobrevin-1 and dystrobrevin-2. We have called these muscle isoforms dystrobrevin-3 and dystrobrevin-4 (Fig. 4C). An important difference between dystrobrevin expression in mouse and Torpedo is that dystrobrevin is not expressed in Torpedo heart (1). In mouse heart, the 3.5- and 1.7-kb dystrobrevin transcripts are expressed at levels comparable to those in brain and skeletal muscle. This finding is consistent with our hypothesis that the dystrobrevins are ligands for dystrophin/utrophin and syntrophin since these proteins and their transcripts are also abundant in mouse heart (43). 2

In addition to the differences within the noncoding regions and the major tissue-specific splice variant, vr3, alternative splicing at two other sites, vr1 and vr2 (Fig. 4C), occurs. vr1 is an insertion of 3 amino acids, whereas the vr2 splice is a replacement of 3 amino acids with a stretch of 9 amino acids. While the function of this splice variation is unknown, it is noteworthy that an insertion of 8 amino acids into the coding sequence of neurona!y secreted agrin is associated with a 1000-fold stimulation in AchR clustering activity (44, 45).

Several features have shown that the dystrobrevins are components of one or more protein complexes. Our data demonstrate that multiple isoforms of the mammalian dystrobrevin are associated with syntrophin (Fig. 6). Because mAb 1351 detects all three forms of syntrophin, it is not known whether one form of syntrophin is preferentially associated with the dystrobrevins. It is a distinct possibility that there are other dystrobrevin isoforms that are not associated with syntrophin, presumably because they lack the syntrophin-binding domain and would therefore not be present in the affinity-purified syntrophin complex. In addition to syntrophin, dystrophin and utrophin are also present in dystrobrevin-containing protein complexes (25, 26). In this study, we have not established whether the dystrobrevins bind directly to dystrophin and utrophin or bind via syntrophin. The assembly of complexes containing dystrophin/utrophin, syntrophin, and the dystrobrevins is currently being investigated.

Finally, a recent paper by Yoshida et al. (46) describes the expression cloning of a partial cDNA for A0, a 94-kDa protein that copurifies with dystrophin and other components of the DGC including the syntrophin triplet (47, 48). The derived sequence has high similarity to the Torpedo 87-kDa protein sequence and is therefore likely to be the rabbit orthologue of the Torpedo gene. Interestingly, the mAb used to screen the library detects a 62-kDa protein in addition to the 94-kDa A0 protein on immunoblots of purified DGC from skeletal muscle. This observation supports our description of two predominant dystrobrevin isoforms, both of which we have cloned. Both forms of A0 are components of the DGC (46), and both are associated with syntrophin (this study).

In conclusion, we have described several isoforms of murine dystrobrevin. Multiple dystrobrevin isoforms are present in syntrophin preparations from different tissues. In muscle, anti-dystrobrevin antibodies label the NMJ and sarcolemma, in common with other components of the DGC. We predict that the dystrobrevins will be general ligands for both dystrophin/utrophin and syntrophin at the NMJ and sarcolemma, but may have additional roles in nonmuscle tissues.

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