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CELLULAR AND MOLECULAR PROPERTIES OF ALPHA-DYSTROBREVIN IN SKELETAL MUSCLE

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1. ABSTRACT

The dystrophin glycoprotein complex (DGC) is a large multisubunit complex located throughout the sarcolemma of striated muscle fibers. This complex is critical for maintaining the structural integrity of muscle fibers during muscle contraction and also provides a scaffold for signaling molecules. Defects in some components of the DGC, such as dystrophin and sarcoglycans, disrupt the complex and lead to muscular dystrophies. Alpha-dystrobrevin is a dystrophin-related component of the DGC that is localized to the cytoplasmic side of the sarcolemma. In skeletal muscle, alpha-dystrobrevin is also highly concentrated at the neuromuscular junction, a highly specialized region of the sarcolemma responsible for receiving motor nerve signals necessary for muscle contraction. Current evidence suggests that alpha-dystrobrevin plays an important role in signaling at the sarcolemma and in the maturation and maintenance of the postsynaptic apparatus at the neuromuscular junction. In this review, we summarize the currently known cellular and molecular properties of alpha-dystrobrevin in skeletal muscle and discuss its potential functions at both the sarcolemma and neuromuscular junction.

2. INTRODUCTION

Alpha-dystrobrevin was originally identified as an 87 kD postsynaptic protein that associated with the nicotinic acetylcholine receptor (AChR) in *Torpedo* electric organ (1), a tissue with morphological and biochemical similarities to mammalian skeletal muscle (2). Immunofluorescence microscopy showed that an antigenically related protein was present at the sarcolemma of rat and chick skeletal muscle with a higher concentration at the neuromuscular junction (1). Additional experiments showed that in *Torpedo* postsynaptic membranes, dystrobrevin was associated with dystrophin, the protein product of the Duchenne muscular dystrophy gene, and syntrophin, the 58 kD postsynaptic protein (3,4), thereby providing evidence that the 87 kD postsynaptic protein was a component of the dystrophin glycoprotein complex (DGC). cDNA cloning of *Torpedo* (4) and mammalian (5,6) 87 kD postsynaptic proteins revealed homology to the cysteine-rich and C-terminal domains of dystrophin, leading investigators to rename the protein “dystrobrevin” meaning a dystrophin-like protein of significantly smaller size. In addition, the *Torpedo* protein was shown to be phosphorylated *in vivo* (4), and the mammalian protein was

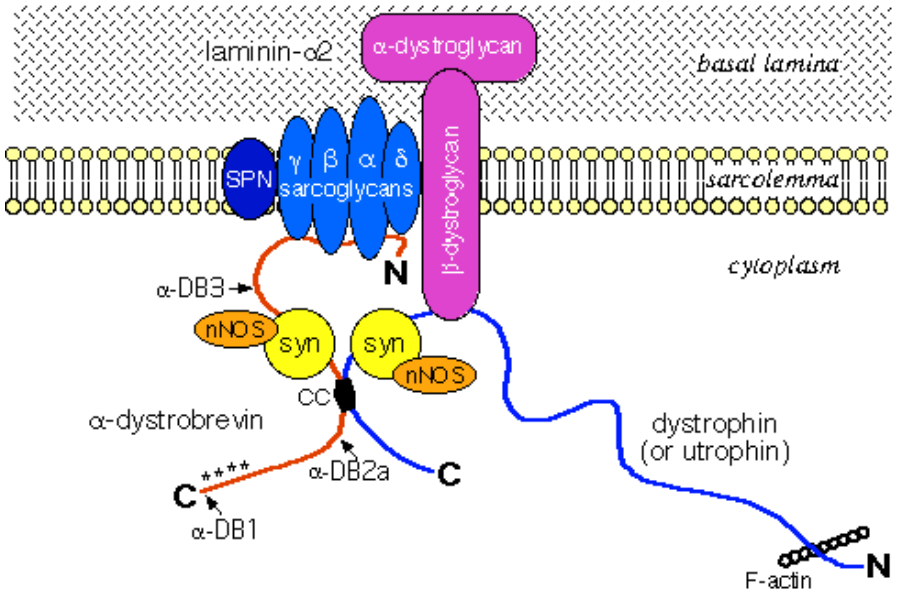

Figure 1. Model of the dystrophin glycoprotein complex (DGC) in skeletal muscle. The DGC spans the sarcolemmal membrane, linking the actin based cytoskeleton to the overlying basal lamina. Transmembrane components of the DGC include sarcoglycans, sarcospan (SPN), and beta-dystroglycan, the latter of which provides a direct link between dystrophin and laminin-alpha2 via the extracellular protein alpha-dystroglycan. The cytoplasmic components of the complex include dystrophin (or utrophin), alpha-dystrobrevin, and syntrophins (syn), the latter of which anchors the signaling molecule neuronal nitric oxide synthase (nNOS). At least three alpha-dystrobrevin isoforms (alpha-DB1, alpha-DB2a, and alpha-DB3) can associated with the DGC; arrows designate the positions of their C-termini. Alpha-DB1 and alpha-DB2a bind to dystrophin through the coiled-coil (CC) domains contained in each. Asterisks represent four potential tyrosine phosphorylation sites in alpha-DB1.

shown to be encoded by multiple transcripts generated by alternative splicing (5,6). Following the discovery of a second gene with significant homology to dystrobrevin (7-9), the nomenclature alpha-dystrobrevin and beta-dystrobrevin was adopted for the protein products of the first and second genes discovered, respectively. Beta-dystrobrevin is abundantly expressed in brain and other tissues, but does not appear to be expressed in muscle; therefore, the present review will focus solely on alpha-dystrobrevin. Although the precise physiological function of alpha-dystrobrevin remains unknown, recent analyses of mice carrying a targeted deletion of the alpha-dystrobrevin gene suggest that in skeletal muscle, alpha-dystrobrevin plays a role both in signaling at the sarcolemma (10) and in maturation of the postsynaptic apparatus at the neuromuscular junction (11).

3. ALPHA-DYSTROBREVIN, A DYSTROPHIN-RELATED PROTEIN, IS A COMPONENT OF THE DYSTROPHIN GLYCOPROTEIN COMPLEX

The DGC is located throughout the sarcolemma of striated (skeletal and cardiac) muscle fibers and links the cytoskeleton to the extracellular basal lamina (reviewed in 12,13). The complex (figure 1) consists of dystrophin, alpha-dystrobrevin, and syntrophins on the cytoplasmic side of the membrane, several transmembrane proteins (beta-dystroglycan, alpha-, beta-, gamma- and delta-sarcoglycan, and sarcospan), and alpha-dystroglycan on the extracellular side of the membrane. Many of the DGC components have more than one isoform, some generated by alternative splicing of a single gene and others originating from distinct genes. For example, there are three syntrophin genes and several splice variants of both dystrophin and alpha-dystrobrevin. This diversity can therefore generate a large number of DGCs that could have distinct physiological functions in particular tissues or subcellular regions.

Defects in genes encoding DGC proteins are associated with several forms of muscular dystrophy (reviewed in 14-19). In Duchenne and Becker muscular dystrophies, mutations in dystrophin disrupt the linkage between the extracellular matrix and cytoskeleton leading to contraction induced damage to the sarcolemma (20). Similarly, defects in any one of the four sarcoglycans lead to limb-girdle muscular dystrophy by disassembling the DGC. These studies have suggested that one role of the DGC is to provide structural stability to the sarcolemma; however, the DGC may also play a role in signaling. In support of this idea, signaling molecules such as Grb2, calmodulin, and neuronal nitric oxide synthase (nNOS) bind to DGC components (21-24), and mice lacking alpha-dystrobrevin display defects in nitric-oxide-mediated signaling (10). These knockout mice develop skeletal and cardiac myopathies despite the presence of a structurally intact DGC in the sarcolemma, suggesting that alpha-dystrobrevin and its signaling function are critical for maintaining muscle stability. The DGC also appears to play a role in the maturation and maintenance of the postsynaptic apparatus at the neuromuscular junction (11).

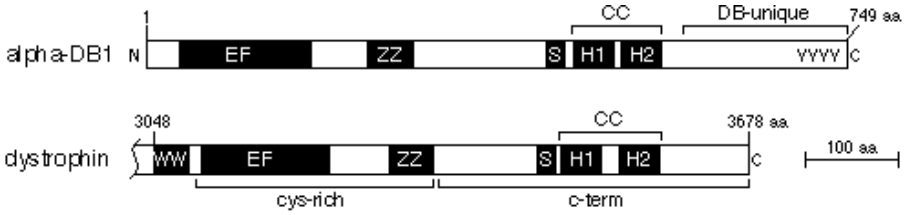

Figure 2. Schematic representation of alpha-dystrobrevin homology to dystrophin. Full length mouse alpha-DB1 protein is shown relative to the C-terminal 650 amino acids of mouse dystrophin which includes the cysteine-rich (cys-rich) and carboxyl-terminal (c-term) domains. Homologous protein domains include: an EF domain (EF) containing two EF hand motifs, a zinc finger domain (ZZ), a core syntrophin binding motif (S), and a coiled-coil domain (CC) containing two alpha helices (H1 and H2). The WW domain (WW) is unique to dystrophin, and the C-terminus of alpha-dystrobrevin (DB-unique), which includes 4 potential tyrosine phosphorylation sites (YYYY), is unique to alpha-DB1.

Alpha-dystrobrevin is a member of the dystrophin family of proteins which includes dystrophin, utrophin, dystrophin-related protein 2, and various short forms of these proteins (reviewed in 25-28). Full length alpha-dystrobrevin is homologous to the cysteine rich and C-terminal domains of dystrophin (figure 2) with 27% amino acid identity over this region. The two proteins have 4 modular domains in common: an EF domain containing two EF-hand motifs that may be involved in calcium binding (29), a ZZ zinc finger domain that binds calmodulin (21,30,31), a syntrophin binding domain (reviewed in 32), and a coiled-coil domain consisting of two alpha helices (33). The C-terminus of alpha-dystrobrevin is unique and contains tyrosine residues that are phosphorylated *in vivo* (4). Alpha-dystrobrevin appears to associate with the DGC through multiple sites (figure 1); the coiled-coil domain mediates its binding to dystrophin (34), while the N-terminal region mediates its interaction with the sarcoglycan subcomplex (35).

4. ALPHA-DYSTROBREVIN CLONING

Currently, alpha-dystrobrevin cDNAs have been isolated and characterized in *Torpedo* (4), human (6), and mouse (5,36,37). In contrast to a single *Torpedo* transcript, human and mouse tissues express multiple transcripts generated by alternative splicing of a single alpha-dystrobrevin gene (38,39). Comparison of the full length protein sequences shows 96% amino acid identity between human and mouse, and 83% identity between *Torpedo* and either human or mouse. Dystrobrevin cDNAs have also been described for rabbit (40) and *C. elegans* (41). Amino acid sequence comparison suggests that rabbit dystrobrevin is more similar to beta-dystrobrevin (9), while *C. elegans* dystrobrevin is 48% identical to both alpha- and beta-dystrobrevin.

4.1. Alpha-dystrobrevin gene structure

The mouse and human alpha-dystrobrevin genes consist of at least 23 coding exons (figure 3B) ranging in size from 9 to ~1200 bp (38,39). Note that two different numbering systems were used for mouse and human alpha-dystrobrevin exons, both of which are shown in figure 3B; throughout this review, we will use the numbers designated for the mouse gene. All 23 exons have the conserved AG and GT dinucleotides at their 3' splice acceptor and 5' splice donor sites, respectively. In mouse, two additional exons (19 and Z, figure 3B) have been proposed based on their presence in cDNA clones (36,39). Exon 19 is probably rarely used since its 3' splice acceptor site does not fit the consensus, and most cDNA clones do not contain the exon (39). Exon Z was proposed to account for a unique 3' end that we observed in a mouse muscle cDNA (36). A polyclonal antibody raised against the sequence encoded by exon Z recognized a protein of 65 kD in mouse skeletal muscle, suggesting that this cDNA encodes an authentic alpha-dystrobrevin isoform (36). In the mouse gene, an additional 7 exons encoding the 5' untranslated region (UTR) were identified within the 270 kb upstream region (42) (figure 3A). Not all of these exons are present in each transcript; instead, they are preferentially used by different promoters (see below).

The human alpha-dystrobrevin gene has been mapped to chromosome 18q12 (43) and spans at least 180 kb (38), while the mouse alpha-dystrobrevin gene has been mapped to the proximal end of chromosome 18 and spans 130-170 kb (39). The physical map of the alpha-dystrobrevin locus (exons 1-24) is consistent with that for the homologous region in dystrophin (exons 63-79) which has been estimated at 160 kb (44). The intron-exon structure of alpha-dystrobrevin from exons 1 to 21 is remarkably similar to that of dystrophin from exons 63 to 77 with many of the intron-exon boundaries conserved (38,39). Interestingly, alpha-dystrobrevin exons 9-13, which includes 3 alternatively spliced exons, corresponds to dystrophin exons 71-73 which are also alternatively spliced (45,46). Thus, despite the relatively low 27% amino acid homology between alpha-dystrobrevin and dystrophin, the intron-exon arrangement is remarkably conserved, suggesting that both genes evolved from an ancestral duplication event (28,38,39).

Like dystrophin and utrophin (47,48), alpha-dystrobrevin expression is controlled by multiple promoters (42), presumably to provide transcriptional regulation at the tissue, cellular and/or developmental levels. In the mouse gene, three promoters have been identified within the 270 kb region upstream of the first coding exon (42). Adding this to the ~170 kb coding region gives the entire gene a minimum size of ~440 kb. Each promoter uses a different 5' UTR exon (exons A, B or C, figure 3A) as the first exon of its transcripts with multiple transcription start sites identified in each. In addition, the promoters lack canonical TATA boxes (42) which is consistent with the use of multiple transcription start sites (reviewed in 49).

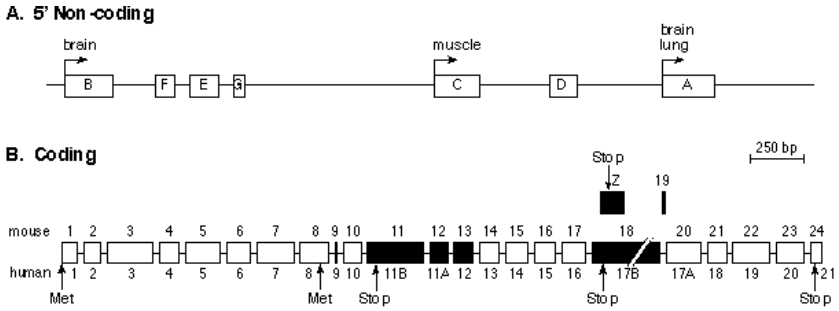

Figure 3. Genomic structure of the alpha-dystrobrevin gene. Non-coding (A) and coding (B) regions are shown with boxes respresenting exons drawn to scale and lines representing introns (not to scale). Coding exons are numbered according to both the mouse (39) and human (38) nomenclature. In the 5' non-coding region, three tissue selective promoters generate transcripts beginning with exons A, B and C as indicated by the arrows. In the coding region, exons 1 and 8 contain initiating methionines and exons 11, 18, Z, and 24 (mouse nomenclature) contain stop codons for the various alpha-dystrobrevin isoforms. Alternatively spliced exons are shown in black. Exons Z and 19, which have only been described in mouse, are offset because the position of exon Z is unknown and exon 19 is a putative exon.

The promoters do contain putative binding sites for transcription factors such as Sp1, Ap1 and Ap2 (42), but which factors actually bind to these promoters remains unknown. Interestingly, the muscle selective promoter C contains several cis-acting sequences known to mediate skeletal muscle expression (42).

4.2. Alpha-dystrobrevin cDNAs generated by alternative splicing

In contrast to the characterization of a single 4.6 kb alpha-dystrobrevin transcript in *Torpedo* (4), the cloning of mouse and human alpha-dystrobrevin cDNAs revealed multiple transcripts generated by alternative splicing. To date, 6 transcripts using different start and stop codons have been reported (5,6,36,37): alpha-DB1, alpha-DB2a, alpha-DB2b, alpha-DB3, alpha-DB4, and alpha-DB5 (figure 4). The coding regions of the first four transcripts begin with the same initiating methionine (in exon 1), but differ in their C-terminal tails because they use alternative 3' exons containing stop codons and 3' UTRs. The *Torpedo* alpha-dystrobrevin transcript is homologous to alpha-DB1. The alpha-DB4 and alpha-DB5 transcripts, which have only been reported in human (6), differ from the others in that their coding regions begin at a methionine in exon 8. At their 3' ends, alpha-DB4 and alpha-DB5 are identical to alpha-DB1 and alpha-DB2a, respectively. Importantly, only alpha-DB1 and alpha-DB4 transcripts encode the tyrosine kinase substrate domain, suggesting that these two isoforms may have a function that is regulated by phosphorylation. In skeletal muscle, the major transcripts appear to be alpha-DB1, alpha-DB2a, and alpha-DB3 (6,37,39), with protein products detected for all three (5,35,37).

Additional variability is observed within the 6 transcripts described above due to alternative splicing within the coding region at sites referred to as variable regions 1, 2 and 3 (figure 4) (5,6,38,39), as well as alternative splicing within the 5' and 3' UTRs (39,42). Variable region 1 (vr1) consists of exon 9 encoding 3 amino acids (DTW). In human and mouse, exon 9 is primarily restricted to brain transcripts (5,36,38), suggesting that this sequence may have a specific function in brain. The *Torpedo* cDNA, which was isolated from an electric organ library (4), lacks the exon 9 sequence.

Variable region 3 (vr3) consists of exons 11, 12 and 13 which encode 9, 26 and 31 amino acids, respectively. Exon 11 encodes the unique C-terminal tail of alpha-DB3 and its complete 3' UTR which is ~0.24 kb in length (6,37). In alpha-DB1 and alpha-DB2, exon 11 is absent, and exons 12 and 13 may be present together, present individually, or absent (36,38,39). Although the functional significance of these two exons remains unknown, they are predominantly expressed in muscle transcripts (5,36,38,39), and their splicing is developmentally controlled (36,37) as discussed below. Interestingly, *Torpedo* alpha-dystrobrevin lacks the region encoded by exon 12, but retains the region encoded by exon 13.

Variable region 2 (vr2) consists of exons 18, 19 and Z which encode 16, 2 and 12 amino acids, respectively. Exon 18 encodes the unique C-terminal tail of alpha-DB2a and its complete 3' UTR which is ~1.2 kb in length (36,38). The first 21 nucleotides of exon 18, which we refer to as 18*, are also found in mouse alpha-DB1 and alpha-DB2b transcripts as a result of splicing at a cryptic site within exon 18 (5,36,39). The sequence encoded by exon 18* (EEELKQG) is conserved in *Torpedo* alpha-dystrobrevin (EEEMRQE). Similar to exons 12 and 13 at vr3, the expression of exon 18* in alpha-DB1 transcripts appears to be muscle specific and developmentally regulated (36) as discussed below. Exon 19 of vr2 was identified in a single cDNA clone and appears to be rarely used (5,39). Finally, exon Z was identified in a mouse muscle cDNA clone following exon 18* (36). It encodes the unique C-terminal tail of alpha-DB2b, as well as a full length 3' UTR which is only 66 bp in length. Sequence analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) products generated from mouse skeletal muscle RNA confirmed that exon Z was indeed expressed in skeletal muscle and not the result of a cloning artifact (36).

Alternative splicing within the coding region of alpha-dystrobrevin can generate a large number of potential

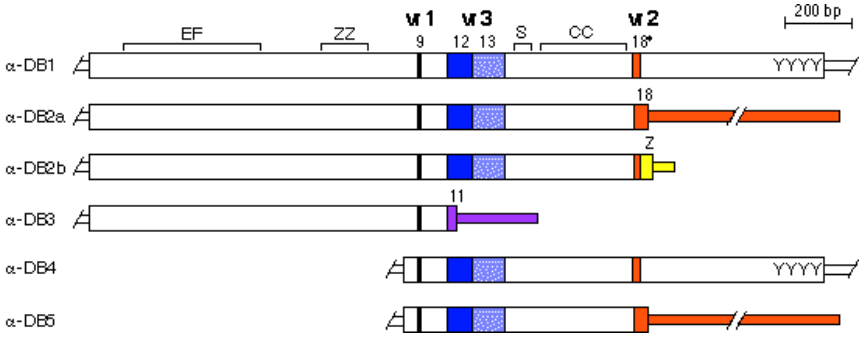

Figure 4. Schematic diagram of alpha-dystrobrevin transcripts generated by alternative splicing. The use of two different initiating methionines and four different stop codons (see **figure 3**) generates 6 classes of alpha-dystrobrevins as shown. Large and small boxes designate coding and non-coding regions, respectively. Shaded boxes denote alternatively spliced exons at three variable regions designated vr1 (exon 9), vr2 (exons 18, 18* and Z), and vr3 (exons 11, 12 and 13).

alpha-dystrobrevin transcripts and proteins. If one considers all possible variations observed at each variable region within each of the 6 alpha-dystrobrevins, 48 different isoforms of the alpha-dystrobrevin protein can potentially be generated. However, since much of the alternative splicing appears to be either tissue specific or tissue selective, a much smaller number of alpha-dystrobrevin isoforms probably exists in any single tissue. In skeletal muscle, protein products of the alpha-DB4 and alpha-DB5 transcripts have not been detected. In addition, most skeletal muscle transcripts do not contain exon 9, but do contain exons 12, 13 and 18*. Taken together, these data suggest that there are 4 major alpha-dystrobrevin isoforms in mature skeletal muscle, alpha-DB1$_{0,57,7}$, alpha-DB2a$_{0,57,16}$, alpha-DB2b$_{0,57,19}$ and alpha-DB3$_{0,9}$, where the subscripts refer to the amino acid lengths of the inserts at vr1, vr3 and vr2, respectively. An additional 3 isoforms, alpha-DB1$_{0,0,0}$, alpha-DB2a$_{0,0,16}$, and alpha-DB2b$_{0,0,19}$, are present in significant amounts during early muscle cell differentiation bringing the total number of alpha-dystrobrevin isoforms in muscle to 7.

5. EXPRESSION OF ALPHA-DYSTROBREVIN

5.1. Tissue expression

Similar to other dystrophin-related proteins, alpha-dystrobrevin is expressed in multiple tissues as revealed by Northern blot analysis. In *Torpedo*, one major alpha-dystrobrevin transcript was detected in electric organ, muscle and brain with additional minor species detected in electric organ (4); it is not clear whether these minor bands represent degradation products or alternative transcripts. In mouse, alpha-dystrobrevin transcripts were detected in skeletal muscle, heart, brain and lung, but not in other tissues (5,39). Similarly, analysis of human tissues revealed abundant alpha-dystrobrevin transcripts in skeletal muscle, heart and brain, and much less abundant transcripts in lung, liver and pancreas (6). In each mammalian tissue expressing alpha-dystrobrevin, several different sized bands were detected reflecting the presence of alternatively spliced transcripts. Refined northern blot analysis using probes specific for the 3' UTRs showed alpha-DB1, alpha-DB2a and alpha-DB3 transcripts in skeletal muscle and brain, although alpha-DB3 was just barely detectable in brain (6,39). In addition, alpha-DB2a and alpha-DB3 transcripts, but not alpha-DB1 transcripts, were detected in heart. An additional small transcript was detected in human skeletal muscle, heart and brain that may represent alpha-DB4 and alpha-DB5 transcripts (6). Consistent with these results, our RT-PCR analysis of mouse tissue RNA using primers homologous to the unique coding region of each isoform showed that alpha-DB2a, alpha-DB2b, and alpha-DB3 transcripts were all expressed in skeletal muscle, heart and brain; however, alpha-DB2b and alpha-DB3 were only weakly expressed in brain (36). In contrast to northern blot results, we detected significant levels of alpha-DB1 in heart, as well as in skeletal muscle and brain. This difference may reflect our use of primers homologous to the coding regions rather than the 3' UTRs. Taken together, these data suggest that skeletal muscle, heart and brain are the major tissues expressing alpha-dystrobrevin and that alpha-DB2b and alpha-DB3 are muscle selective, while alpha-DB1 and alpha-DB2a are more broadly expressed.

Within each alpha-dystrobrevin isoform, alternative splicing at the three variable regions also appears to be either tissue specific or tissue selective. Exons 12 and 13 of vr3 are predominantly expressed in skeletal and cardiac muscle (5,36,38,39), but they have also been detected in brain transcripts by RT-PCR (36). In brain, the splicing of exons 12 and 13 is differentially regulated depending on the isoform; these exons are extremely rare in alpha-DB2a transcripts, but common in alpha-DB1 and alpha-DB2b transcripts (36). Exon 9 of vr1 appears to be brain specific (5,36), although weak signals have also been detected in muscle (38). Exon 18* of vr2 is muscle specific based on RT-PCR analysis (36).

In the mouse gene, alpha-dystrobrevin expression is controlled by at least three promoters, and the transcripts generated by each promoter begin with either exon A, B or C (figure 3A) (42). Analysis of the expression of these exons in various tissues showed that exon A is expressed in brain and lung, exon B is brain specific, and exon C is predominantly expressed in skeletal muscle and heart with much lower expression in brain and lung (42). In addition, promoter C, but not promoter B, can direct high levels of

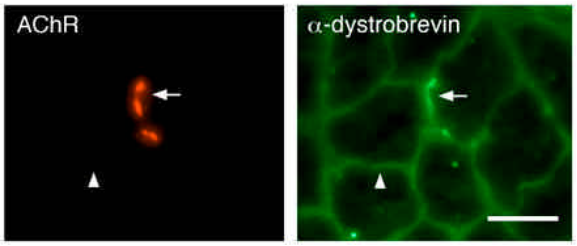

Figure 5. Immunostaining of alpha-dystrobrevin in adult mouse skeletal muscle. A frozen cross section (10 microns) of mouse tibialis muscle was double labeled with rhodamine conjugated alpha-bungarotoxin (left panel) and alpha-dystrobrevin-specific polyclonal antibody 692 (10) followed by a fluorescein conjugated secondary antibody (right panel). Alpha-dystrobrevin is present at the sarcolemma of the muscle fibers (arrowhead, right panel) and is concentrated at the neuromuscular junction as identified by AChR staining with alpha-bungarotoxin (arrows in both panels). Scale bar, 40 microns.

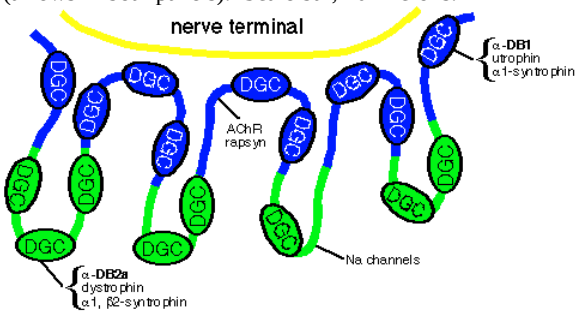

Figure 6. Model of the poststynaptic membrane of the neuromuscular junction in adult skeletal muscle. The postsynaptic membrane is invaginated repeatedly to form membrane folds with the crests and troughs shown in blue and green, respectively. Alpha-dystrobrevin isoforms differentially associate with molecularly distinct DGCs within the neuromuscular junction: alpha-DB1 colocalizes with utrophin and alpha1-syntrophin at the crests of the postsynaptic folds, whereas alpha-DB2a colocalizes with dystrophin and alpha1- and beta2-syntrophin in the troughs. Similarly, AChR and rapsyn are restricted to the crests of the postsynaptic folds, while voltage gated sodium channels are restricted to the troughs; these proteins may also associate with the DGCs.

reporter gene expression in myotubes (42). These data suggest that the three promoters are tissue selective rather than tissue specific. Of the 4 remaining 5' UTR encoding exons, exon D is expressed in all 4 tissues (in combination with either exon B or C), and exons E, F and G are only expressed in brain. However, it is not clear whether exons E, F and G are expressed from promoter B or another as yet unidentified promoter (42). Although the promoters are tissue selective, their activity does not appear to regulate the formation of individual splice variants since similar splice variants were associated with each promoter (42).

5.2. Developmental expression in muscle cell cultures

In order to study the developmental regulation of mRNAs and proteins in skeletal muscle, cell culture systems in which myoblasts fuse into terminally differentiated myotubes are frequently used. Analysis of alpha-dystrobrevin expression during myoblast to myotube differentiation in both C2C12 and H-2K^b-tsA58 muscle cells showed that alpha-DB1 and alpha-DB3 transcripts are expressed early in differentiation, while alpha-DB2a and alpha-DB2b transcripts are expressed later (36,42). This is consistent with the early and late expression of alpha-DB1 and alpha-DB2a proteins, respectively (37,42). In addition, the expression of exons 12 and 13 at vr3 and exon 18* at vr2 is upregulated after 1-2 days of differentiation (36,42), again consistent with the appearance of slightly larger alpha-DB1 and alpha-DB2a protein isoforms late in differentiation (37,42). Exon C, the first exon transcribed by the muscle selective promoter, is upregulated during myoblast to myotube differentiation with the same time course as the early alpha-DB1 and alpha-DB3 transcripts as expected (42). Thus, the mouse alpha-dystrobrevin gene is transcriptionally activated by promoter C upon differentiation of myoblasts into myotubes, with a subset of the alpha-dystrobrevin isoforms expressed late in differentiation. This suggests that some isoforms have specific functions in myotubes, while others have specific functions in myoblasts.

6. PROPERTIES OF ALPHA-DYSTROBREVIN IN SKELETAL MUSCLE

6.1. Subcellular localization

Examination of the subcellular distribution of alpha-dystrobrevin in skeletal muscle provided the first clues as to its potential function in muscle. Immunostaining of chick, rat and mouse skeletal muscle sections with alpha-dystrobrevin-specific antibodies established that it is localized to the sarcolemma with higher concentrations at the neuromuscular junctions (figure 5) (1,5,11), suggesting a functional role at both sites. More recently, isoform-specific antibodies showed that alpha-DB1 was restricted to the neuromuscular junction (50,51), although another study showed alpha-DB1 in the sarcolemma as well (37). Alpha-DB2a was present throughout the sarcolemmal membrane including the neuromuscular junction (51). Within the neuromuscular junction, alpha-DB1 was restricted to the crests of the junctional folds, while alpha-DB2a was concentrated in the troughs of the folds (figure 6) (51). Thus, the subcellular distributions of alpha-DB1 and alpha-DB2a appear to parallel those of utrophin and dystrophin, respectively (52-54), suggesting that alpha-DB1 and alpha-DB2a may interact specifically with utrophin and dystrophin (however, see discussion in next section). The subcellular distributions of alpha-DB2b and alpha-DB3 have not yet been determined, although alpha-DB3 appears to interact with the DGC (35) suggesting that it localizes to the sarcolemma.

6.2. Protein-protein interactions

Early biochemical studies demonstrated that alpha-dystrobrevin was associated with dystrophin and syntrophin (3,4), while later studies showed additional interactions with utrophin (37,51) and the sarcoglycan-sarcospan complex (35). These alpha-dystrobrevin binding proteins are all components of the DGC (figure 1),

suggesting that alpha-dystrobrevin has an important function in this complex. The binding sites for these proteins have been mapped to specific regions within alpha-dystrobrevin. The dystrophin binding site is contained within the highly conserved coiled-coil domain (figure 2) (34) consisting of two alpha helical stretches (H1 and H2) separated by a short proline-rich linker (33). Using the yeast two hybrid system, the dystrophin binding site was mapped to the first helix, H1 (34). Similarly, the alpha-dystrobrevin binding site in dystrophin was mapped to the H1 helix, suggesting the H1 alpha helices form a heteromeric coiled-coil between the two proteins (34) as depicted in figure 1. In contrast, *in vitro* studies of the association between dystrobrevin and dystrophin from *C. elegans* demonstrated a requirement for the H2 alpha helices rather than H1, suggesting that the two species may use different mechanisms of association (55). Additional studies of specific alpha-dystrobrevin isoforms showed that both alpha-DB1 and alpha-DB2a can bind directly to dystrophin as expected since both contain the coiled-coil domain (51). Interestingly, no evidence for homodimerization of either dystrophin or alpha-dystrobrevin could be detected in the yeast two hybrid system (34,51), suggesting a specificity in the heteromeric interaction of the H1 helices.

Utrophin (56-58), a ubiquitous dystrophin-related protein with very similar overall structure to dystrophin, also binds to alpha-dystrobrevin. Coimmunoprecipitation of utrophin with alpha-DB1 from muscle cell cultures demonstrated an *in vivo* association (37), while *in vitro* association and yeast two hybrid experiments showed that alpha-DB1 could bind to a C-terminal fragment of utrophin (51). Further mapping refined the site of interaction in both proteins to the region encompassing the syntrophin binding and coiled-coil domains (figure 2) (31). Thus, utrophin and alpha-DB1 may associate through their respective coiled-coil domains as observed for dystrophin. Surprisingly, alpha-DB2a did not bind to the C-terminal fragment of utrophin despite the presence of the coiled-coil domain in both proteins (51), demonstrating a functional difference between alpha-DB1 and alpha-DB2a. The molecular basis for this difference is unknown; however, the two isoforms have different C-termini adjacent to their coiled-coil domains, which could potentially regulate their interactions with utrophin (51). Combined with the results obtained for dystrophin, these experiments indicate that dystrophin binds to both alpha-DB1 and alpha-DB2a, while utrophin binds to only alpha-DB1.

Similar to the *in vitro* experiments described above, analysis of alpha-dystrobrevin complexes in muscle extracts showed that, alpha-DB2a preferentially copurifies with dystrophin, while alpha-DB1 preferentially copurifies with utrophin (51). Although the vast majority of utrophin complexes in muscle extracts are derived from non-muscle cells, and not from neuromuscular junctions (59), these data still demonstrate the preferential association of alpha-DB1 with utrophin. This preferential copurification is consistent with the subcellular localization of these proteins: dystrophin and alpha-DB2a are localized to the sarcolemma and the troughs of the junctional folds at the neuromuscular junction, while utrophin and alpha-DB1 are restricted to the crests of the junctional folds (see figure 6). These results are also consistent with analyses of mutant mice lacking dystrophin (*mdx* mice), utrophin or both proteins. Alpha-DB1 is retained at the neuromuscular junction in mice lacking either utrophin or dystrophin, but is absent from the neuromuscular junction in mice lacking both proteins (51). This suggests that alpha-DB1 can interact with either utrophin or dystrophin at the neuromuscular junction. Alpha-DB2a is absent from the sarcolemma of mice lacking dystrophin or both dystrophin and utrophin, but is present in the sarcolemma of utrophin knockout mice consistent with dystrophin interaction at the sarcolemma (51). Similarly, alpha-dystrobrevin staining of the sarcolemma is severely reduced in biopsies from Duchenne muscular dystrophy patients who express little if any dystrophin (60). However, alpha-DB2a is retained at the neuromuscular junction in mice lacking either dystrophin or both dystrophin and utrophin suggesting that alpha-DB2a can interact with some other protein at the neuromuscular junction. As described below, an interaction with the sarcoglycan-sarcospan complex could explain this result.

Alpha-dystrobrevin also binds to syntrophins which are modular adapters that recruit signaling molecules, such as nNOS, to the sarcolemma via the DGC (figure 1) (reviewed in 32). There are three syntrophin isoforms, alpha1, beta1 and beta2, encoded by three separate genes (61-64). Similar to dystrophin and utrophin, alpha-dystrobrevin can bind all three isoforms *in vitro* (61,65). The syntrophin binding site has been mapped to the region upstream of the coiled-coil domain in both alpha-dystrobrevin and dystrophin, with a core 16 amino acid consensus binding motif in each (reviewed in 32). Strikingly, this core sequence is 100% conserved in all alpha- and beta-dystrobrevins identified to date except for the *C. elegans* homologue whose core is 69% identical and 88% similar (41). In skeletal muscle, alpha1-syntrophin is present on the sarcolemma and throughout the neuromuscular junction, while beta2-syntrophin is mainly restricted to the neuromuscular junction within the troughs of the junctional folds (figure 6) (66,67). Beta1-syntrophin is found in a subset of muscle fibers with a staining pattern similar to alpha1-syntrophin, and is also abundant in presynaptic structures and blood vessels (67,68). Although the dystrophin-related proteins can bind all three syntrophins, their differential localization within skeletal muscle suggests that they may form distinct ternary complexes as part of distinct DGCs at specific subcellular sites. For example, the sarcolemma contains mainly alpha-DB2a, alpha1-syntrophin and dystrophin, while the crests of the postjunctional folds contain mostly alpha-DB1, alpha1-syntrophin and utrophin. Thus, distinct alpha-dystrobrevin complexes may mediate specialized functions at the sarcolemma and neuromuscular junction.

The most recent addition to the list of alpha-dystrobrevin binding proteins is the sarcoglycan-sarcospan subcomplex of the DGC (figure 1). An interaction between alpha-dystrobrevin and the sarcoglycan complex was originally proposed based on the loss of alpha-dystrobrevin

from the sarcolemma of patients with sarcoglycan-negative limb girdle muscular dystrophy in which dystrophin is present at the sarcolemma (60). More recently, Yoshida et al. isolated a DGC subcomplex from skeletal muscle that consisted of the four sarcoglycans, sarcospan, syntrophin, and three alpha-dystrobrevins corresponding in molecular weight to alpha-DB1, alpha-DB2 and alpha-DB3 (35). The alpha-dystrobrevins associated with this complex through their common N-terminal regions. Furthermore, skeletal muscle DGCs prepared from beta-sarcoglycan knockout mice, which lack the sarcoglycan-sarcospan subcomplex (69), retained alpha-DB1 and alpha-DB2, but not alpha-DB3 (35). Presumably, alpha-DB3 is lost from the DGC because its sarcoglycan-sarcospan binding partner is absent and it lacks the coiled-coil domain that mediates alpha-DB1 and alpha-DB2 interactions with dystrophin (34). The functional significance of alpha-DB3 remains unknown; however, a potential function of alpha-DB1 and alpha-DB2 may be to provide a link between the sarcoglycan-sarcospan complex and the signaling molecule nNOS via alpha-syntrophin (35).

6.3. Phosphorylation

Tyrosine phosphorylation plays an important role in synaptogenesis at the neuromuscular junction (reviewed in 70,71). Neural agrin, a signaling molecule released by the motor nerve terminal, initiates the reorganization of proteins in the postsynaptic membrane by activating the muscle specific tyrosine kinase MuSK. MuSK activation leads to a series of tyrosine phosphorylation events that ultimately results in aggregation and cytoskeletal anchoring of postsynaptic proteins at the synaptic site. Because alpha-dystrobrevin is one of the major phosphotyrosine containing proteins in *Torpedo* postsynaptic membranes, its phosphorylation may be important in synaptogenesis (4). *Torpedo* alpha-dystrobrevin has four consensus sites for tyrosine phosphorylation within the unique C-terminus (4), all of which are conserved in mammalian alpha-DB1 and alpha-DB4 (figure 4). Tyr-693 and Tyr-710 are the major sites of phosphorylation by endogenous *Torpedo* kinases, while an additional site, Tyr-685, can be phosphorylated by src kinase *in vitro* (50). In mouse myotubes, tyrosine phosphorylation of alpha-DB1 can be detected if the cells are first treated with pervanadate, a phosphatase inhibitor; however, alpha-DB1 was not phosphorylated in response to agrin treatment, suggesting that it is not a target of phosphorylation in the MuSK signaling cascade (37). Interestingly, agrin treatment did result in the co-aggregation of alpha-DB1 and AChRs into macroclusters, indicating that alpha-DB1 is one of the postsynaptic proteins that is reorganized in response to agrin (37). Currently, the physiological function of alpha-DB1 phosphorylation in skeletal muscle remains unknown.

7. POTENTIAL FUNCTIONS OF ALPHA-DYSTROBREVIN IN SKELETAL MUSCLE

7.1. Muscular dystrophy in alpha-dystrobrevin deficient mice

Recent analyses of alpha-dystrobrevin knockout mice has provided clues to its potential functions in skeletal muscle at both the sarcolemma and neuromuscular junction (10,11). Mice homozygous for a deletion of exon 3 of the alpha-dystrobrevin gene were viable, fertile and outwardly healthy. However, histopathological analysis of cardiac and skeletal muscles revealed mild muscular dystrophy similar to that displayed by *mdx* mice which lack dystrophin, providing direct evidence that alpha-dystrobrevin is required for muscle fiber stability (10). Immunostaining of the sarcolemma showed no alpha-dystrobrevin as expected, but did reveal the presence of other DGC components (dystrophin, alpha- and beta-sarcoglycan, beta-dystroglycan and alpha1-syntrophin) indicating that the structure of the DGC is essentially intact with the cytoskeleton still linked to the basal lamina (10). In contrast, these proteins were all severely reduced in *mdx* mice. Additional immunostaining of skeletal muscles from alpha-dystrobrevin knockout mice revealed a dramatic displacement of nNOS from the sarcolemma and impaired nitric-oxide-mediated signaling (10), suggesting that alpha-dystrobrevin plays a role in the localization and function of nNOS at the membrane. However, the loss of nNOS function alone cannot explain the dystrophic phenotype since nNOS knockout mice do not exhibit muscular dystrophy (72,73). Taken together, these results suggest that alpha-dystrobrevin is an important component of the DGC that mediates signaling rather than structural functions of the complex.

7.2. Neuromuscular defects in alpha-dystrobrevin deficient mice

Examination of neuromuscular junctions in alpha-dystrobrevin knockout mice revealed subtle structural defects although movement was not impaired in these mice (11). At one week of age the high density patches of AChRs in the postsynaptic membrane appeared normal, but by one month, the normally even distribution of AChRs in these patches had become granular in appearance with ragged edges. The cytoskeletal proteins utrophin and rapsyn had a similar granular appearance indicating that alpha-dystrobrevin is required for proper organization of cytoskeletal as well as transmembrane postsynaptic proteins (11). Ultrastructural analysis of the neuromuscular junctions showed a decrease in density of postsynaptic folds and an expansion in AChR staining such that it was no longer restricted to the crests of the junctional folds (11). Myotube cultures prepared from alpha-dystrobrevin negative muscles were able to form essentially normal AChR clusters in response to agrin; however, these clusters were quickly dispersed into microaggregates when agrin was withdrawn indicating that they were unstable (11). Taken together, these results suggest that alpha-dystrobrevin is not required for the initial formation of the neuromuscular junction, but is required for its maturation and stabilization. The molecular mechanism underlying this process is unclear, although it may involve signaling through the DGC. Most DGC components were present in the postsynaptic membrane at normal levels; however, alpha-dystrobrevin, syntrophins and nNOS showed significant decreases (11). In addition, very weak alpha-DB2a staining was observed which could represent either upregulated alpha-DB4 or beta-dystrobrevin (11). Taken together, these results suggest that alpha-dystrobrevin may anchor syntrophins and nNOS to the cytoplasmic face of

the postsynaptic membrane and thereby provide signaling function to the DGC that is important for synapse maturation and stabilization.

8. PERSPECTIVES

Recent studies have clearly established an important role for alpha-dystrobrevin in skeletal muscle at both the sarcolemma and neuromuscular junction. Its association with the DGC provides strong evidence that it functions through this complex, most likely in a signaling role rather than a structural role. Furthermore, characterization of the expression patterns of the various alpha-dystrobrevin isoforms and their splice variants suggests that specific isoforms have distinct functions within skeletal muscle and at different stages of differentiation; however, what these functions are remains unknown. Future work will need to focus on functional differences between the isoforms and their splice variants to learn what their specific roles are in skeletal muscle. Questions to address include: what is the role of the alternatively spliced inserts that are upregulated during differentiation? Are they involved in subcellular localization and/or specific protein-protein interactions? What is the role of phosphorylation of alpha-DB1 and what signaling pathway controls this phosphorylation? Which alpha-dystrobrevin isoforms mediate maturation and stabilization of the postsynaptic apparatus, and what is the mechanism involved? And finally, how do the various alpha-dystrobrevin isoforms function within the context of the DGC? As we learn more about the physiological functions of the DGC in skeletal muscle, we will gain new insights into the potential functions of alpha-dystrobrevin isoforms, and likewise, the more we learn about alpha-dystrobrevin isoforms the better we will understand the function of the DGC.

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