Human Molecular Genetics, 2018, Vol. 27, No. 17 2978–2985

doi: 10.1093/hmg/ddy197
Advance Access Publication Date: 22 May 2018
Original Article

ORIGINAL ARTICLE

Syntrophin binds directly to multiple spectrin-like repeats in dystrophin and mediates binding of nNOS to repeats 16–17

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Abstract

Mutation of the gene encoding dystrophin leads to Duchenne and Becker muscular dystrophy (DMD and BMD). Currently, dystrophin is thought to function primarily as a structural protein, connecting the muscle cell actin cytoskeleton to the extracellular matrix. In addition to this structural role, dystrophin also plays an important role as a scaffold that organizes an array of signaling proteins including sodium, potassium, and calcium channels, kinases, and nitric oxide synthase (nNOS). Many of these signaling proteins are linked to dystrophin via syntrophin, an adapter protein that is known to bind directly to two sites in the carboxyl terminal region of dystrophin. A search of the dystrophin sequence revealed three additional potential syntrophin binding sites (SBSs) within the spectrin-like repeat (SLR) region of dystrophin. Binding assays revealed that the site at SLR 17 bound specifically to the α isoform of syntrophin while the site at SLR 22 bound specifically to the β-syntrophins. The SLR 17 α-SBS contained the core sequence known to be required for nNOS–dystrophin interaction. *In vitro* and *in vivo* assays indicate that α-syntrophin facilitates the nNOS–dystrophin interaction at this site rather than nNOS binding directly to dystrophin as previously reported. The identification of multiple SBSs within the SLR region of dystrophin demonstrates that this region functions as a signaling scaffold. The signaling role of the SLR region of dystrophin will need to be considered for effective gene replacement or exon skipping based DMD/BMD therapies.

Introduction

Duchenne and Becker muscular dystrophies are caused by the absence of a full-length form of dystrophin and the subsequent loss of its associated proteins. A main function of dystrophin in normal muscle is to organize and localize a complex of proteins at specific sites of the sarcolemma. Many proteins bind directly to dystrophin including cytoskeletal proteins (actin, microtubules, intermediate filament proteins, ankyrin B, plectin), at least one transmembrane protein (β-dystroglycan) and several intracellular proteins (dystrobrevins, syntrophins, Par-1B) [reviewed in (1)]. Dystrophin organizes these proteins into a dynamic functional complex that prevents muscle degeneration and preserves muscle health.

Traditionally, dystrophin has been divided into four domains based on structure and binding properties (2). The N-terminal domain binds actin, the cysteine rich domain

Received: March 27, 2018. **Revised:** May 9, 2018. **Accepted:** May 14, 2018

contains the β-dystroglycan binding region and the C-terminal region binds dystrobrevin and syntrophin. The bulk of the protein is made of 24 spectrin-like repeats (SLRs) that originally were thought to function as a spring or shock absorber. However, recently this region has also been implicated in the binding of lipids, cholesterol and proteins. Specifically, the proteins binding the SLRs include actin filaments (3), microtubules (4), intermediate filaments (5,6), Par-1B (7) and neuronal nitric oxide synthase (nNOS) (8).

Proper localization of nNOS to the sarcolemma requires both α-syntrophin and the region of dystrophin forming SLRs 16/17. nNOS binds directly to the PDZ domain of α-syntrophin (9). Biochemical and crystallography studies show that the β-finger adjacent to the PDZ domain of nNOS binds with high affinity to the ligand-binding groove in the syntrophin PDZ domain. α-Syntrophin is required for sarcolemmal localization of nNOS as demonstrated in Snta$^{-/-}$ mice using both immunofluorescence and western blots of membrane enriched fractions (10,11). Furthermore, the Snta$^{-/-}$ mice fail to attenuate adrenergic stimulated vasoconstriction, indicating that nNOS function is also lost in the α-syntrophin$^{-/-}$ mice (12). The nNOS requirement for full-length dystrophin was demonstrated by similar experiments using mdx mice (a mouse model lacking dystrophin). In mdx mouse muscle, nNOS is not localized to the sarcolemma (13) and adrenergic stimulated vasoconstriction is not attenuated (14). The requirement that a portion of the rod domain of dystrophin was needed for nNOS localization was originally observed in DMD and Becker patients carrying genomic deletions that removed rod domain portions spanning SLRs 16 and 17. Muscle from these patients had sarcolemmal α-syntrophin but nNOS was absent from the sarcolemma (15). The region in dystrophin required for nNOS localization was further narrowed to SLRs 16/17 by experiments that restored this region of dystrophin in mdx mice (8).

Following the initial identification of syntrophin as a dystrophin-binding protein, three independent labs identified a region in the C-terminal domain of dystrophin as the syntrophin binding site (SBS) (16–18). The SBS is also present in the dystrophin homologs, utrophin, DRP2 and dystrobrevin. Newey *et al.* (19), showed that the SBS was actually comprised of two adjacent sites, allowing two syntrophins to bind dystrophin (or utrophin, DRP2, dystrobrevin). However, none of these studies searching for the SBS used full-length dystrophin, only the C-terminal region. Also, Crawford *et al.* showed that otherwise full-length dystrophins lacking the C-terminal domain still co-localized with syntrophin in skeletal muscles (20). We therefore investigated if additional SBSs were present in other regions of dystrophin (the N-terminal and SLR regions). Here we identify and characterize additional SBSs in dystrophin.

The results presented in this paper allow three major conclusions. First, it is α-syntrophin that links nNOS to the SLR 16/17 region of dystrophin, not nNOS binding directly to dystrophin as reported by Lai *et al.* (8). Second, although α-syntrophin can bind to either or both of the dystrophin C-terminal SBSs, only the α-syntrophin bound to SLR 16/17 binds nNOS. Finally, we have discovered at least one more SBS in SLR 22 of dystrophin that specifically binds β-syntrophins. These results need to be considered when designing the most effective gene therapy construct and for correcting dystrophin mutations by exon skipping or gene replacement.

Results

We searched for potential SBSs within dystrophin by using the Newey study as a guide (19). A similar approach had been used previously to identify a third SBS in dystrobrevin present in an alternatively spliced exon (21). We compared the two known SBSs in the dystrophin C-terminal region with those in dystrobrevin, utrophin and DRP2. We used the sequence of these proteins from multiple species (including mammals, birds, fish and insects) to develop a consensus sequence for the dystrophin family SBS (Fig. 1). The consensus sequence shows an absolutely conserved histidine residue, designated Position 0. The consensus SBS occurs in a predicted amphipathic α-helix with hydrophobic residues at Positions 3, 6 and 10. The hydrophobic residue in Position 6 is almost always a tyrosine or phenylalanine. The −2 and −3 positions are primarily charged amino acids with the majority being negatively charged aspartic and glutamic acid residues. Using this consensus sequence, we scanned full-length human dystrophin and identified a total of 5 potential SBSs (Fig. 1). The first two sites listed in Figure 1 are the previously characterized sites located in the C-terminal region (Exons 74 and 72). The remaining three potential SBSs are located in SLRs 17, 22 and 23. Each occurs in a similar position in the A-helix of the SLR. Interestingly, the SBS in repeat 17 corresponds to the minimal 'nNOS binding' site reported by Lai *et al.* (22) (boxed sequence in Fig. 1).

To confirm that these putative SBSs are, in fact, capable of binding syntrophin we synthesized peptides corresponding to each of the new SBSs and two control peptides (randomized SLR 17 sequence, and the corresponding A-helix sequence of repeat 15). The synthesized peptides were biotinylated at the N-terminus and used to 'pull down' binding partners from mouse muscle homogenates. Western blot analysis of the proteins pulled down using each peptide is shown in Figure 2. The SBS in repeat 17 was the only SLR SBS to bind specifically to α-syntrophin. The SBS in repeat 22 bound to both β1 and β2 syntrophin but did not bind α-syntrophin. The isoform binding specificity provides additional evidence that the pulldown experiments are not artifactual. No syntrophin binding was observed with the potential SBS from repeat 23 under the conditions used.

After establishing that repeat 17 SBS can bind to α-syntrophin in wild-type mouse muscle homogenates, we tested if this binding was dependent on the presence of nNOS. This is necessary since it is possible that α-syntrophin only binds to the 17 SBS in the presence of nNOS. We repeated the pull down experiments using mouse muscle homogenates from wild-type (positive control), α-syntrophin KO (negative control) and nNOS KO mice (Fig. 3). Even in the absence of nNOS, α-syntrophin binds to repeat 17 SBS.

To test these *in vitro* experiments in a more *in vivo* setting, we used several mdx mice lines expressing microdystrophins. Previous studies showed that nNOS was restored to the sarcolemma of mdx mice by AAV mediated expression of a microdystrophin construct that included repeats 16 and 17 (22,23). We investigated if nNOS restoration by this microdystrophin construct also required α-syntrophin. We expressed the same microdystrophin construct containing repeats 16/17 in both mdx and mdx/Snta$^{-/-}$ mice (mice lacking dystrophin or dystrophin and α-syntrophin, respectively). Immunofluorescence of muscle sections showed that restoration of nNOS at the mdx sarcolemma required α-syntrophin (Fig. 4).

Investigation of the binding of α-syntrophin to the SBS in SLR-17 *in vivo* presented several challenges. Endogenous α-SBSs on both dystrophin and on other dystrophin family member proteins (dystrobrevins, utrophin) must be considered. Previously, we generated a transgenic mouse line that expresses a modified dystrophin that is lacking the deleted C-terminal domain (dCT) on the mdx background (20). Both the previously

Exon	domain	aa	DE	DE	X	H	X	X	IL	X	X	YF	X	X	X	L
74	C-term1	3466	D	D	E	H	L	L	I	Q	H	Y	C	Q	S	L
72	C-term2	3431	D	D	T	H	S	R	I	E	H	Y	S	S	R	L
44	SLR 17	2107	R	R	F	H	Y	D	I	K	I	F	N	Q	W	L
55	SLR 22	2685	E	E	T	H	R	L	L	Q	Q	F	P	L	D	L
57	SLR 23	2808	K	R	L	H	L	S	L	Q	E	L	L	V	W	L

Figure 1. Dystrophin contains five SBS consensus sequences. In addition to the two known SBSs in the C-terminal region, dystrophin contains three SBSs located in the spectrin-like repeats (SLRs). The SBS located in SLR-17 encompasses the minimal sequence required for nNOS association with dystrophin (22) (boxed region).

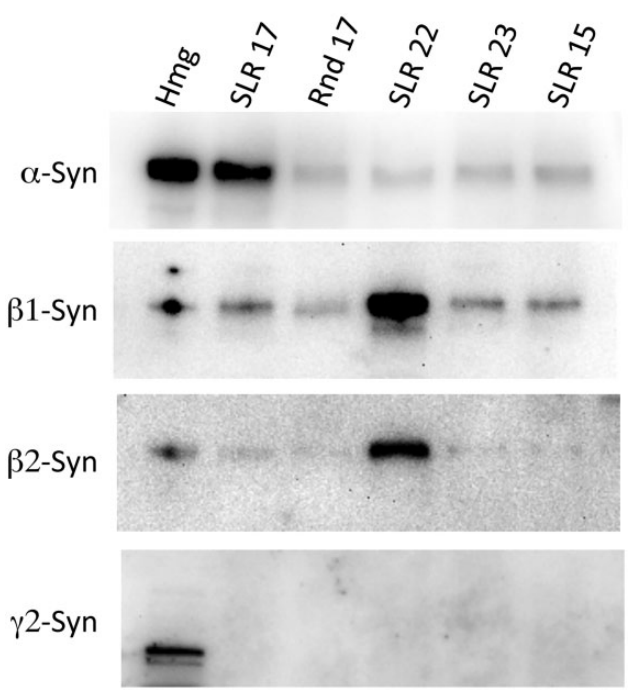

Figure 2. α- and β-Syntrophins bind specifically to different repeats. Peptides corresponding to the SBSs in SLRs were used to pull down syntrophins from mouse muscle homogenates. The associated syntrophins were identified by western blot. Hmg—muscle homogenate before pull down assay, Rnd 17—peptide from SLR-17 with amino acids in random order, SLR 15—negative control peptide with SLR 15 sequence that does not contain a SBS.

known SBSs and the dystrobrevin binding site are missing in these mice. Surprisingly, at the time, characterization of this mouse showed that it did retain some α-syntrophin on the sarcolemma. However, it could not be concluded that this α-syntrophin was binding to the SLR region since this mouse also retained some α-dystrobrevin at the sarcolemma that could potentially recruit the α-syntrophin. To address this issue, we bred this mouse onto the α-dystrobrevin KO background producing a dCT:mdx:dtn$^{-/-}$ mouse (see Fig. 5). Immunofluorescence microscopy of the TA muscle of these mice and control mdx:dtn$^{-/-}$ mice show that the dCT transgene restores both nNOS and α-syntrophin to the sarcolemma (Fig. 5). This association of α-syntrophin with dCT dystrophin *in vivo* is consistent with α-syntrophin binding to SLR-17. We did observe α-syntrophin sarcolemmal labeling in the mdx:dtn$^{-/-}$ control. In the absence of dystrophin (or in this case absence of dCT dystrophin),

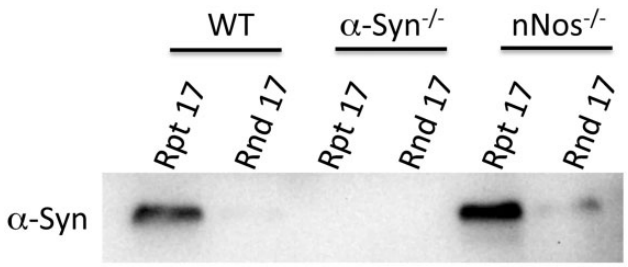

Figure 3. α-Syntrophin binding to SLR 17 does not require nNOS. The SLR-17 SBS peptide was used in mouse muscle homogenate pull down assays and α-syntrophin was detected by western blot. Homogenates were generated from C57bl6 (WT), α-syntrophin knockout (Snta$^{-/-}$) and nNOS knockout (Nos1$^{-/-}$) mice.

utrophin is upregulated. The α-syntrophin observed in the control most likely is binding to the C-terminal region of utrophin. While utrophin lacks the SBS consensus sequence analogous to that in dystrophin SLR 17 and does not recruit nNOS to the sarcolemma (15), the C-terminal SBS can still bind α-syntrophin.

The *in vitro* and *in vivo* experiments described above validate that the SBS consensus sequences in the SLR repeat region of dystrophin bind specifically to syntrophins. We searched for SBSs in other proteins. Table 1 lists 17 proteins that contain SBS that are conserved across species. The first three proteins listed (KIAA 0556, VPS13C and ATP11C) were previously identified as potentially linked to syntrophin. Lyssand *et al.* (24) used a TAP Tag strategy to isolate syntrophins expressed in HEK cells and identified potential interacting proteins by mass spectroscopy. These three proteins were identified in that study. The second three proteins (ATR, SULT1C3 and DST) have also been linked to syntrophin in a previous study. Steen (25) performed gene chip analysis comparing wild-type and α-syntrophin$^{-/-}$ muscle RNA expression. The levels of RNA encoding each of these three proteins showed significant differences in expression in the α-syntrophin$^{-/-}$ muscle. The remaining potential syntrophin binding proteins are listed in the table because their SBS consensus sequence is conserved across species. The authenticity and significance of syntrophin binding to these proteins remain to be determined.

Discussion

The findings described above lead us to three main conclusions. First, α-syntrophin specifically binds to SLR 17 in dystrophin and mediates the binding of nNOS in skeletal muscle. Second, the α-syntrophin bound to SLR 17 is the only dystrophin-associated syntrophin that binds nNOS: the syntrophins

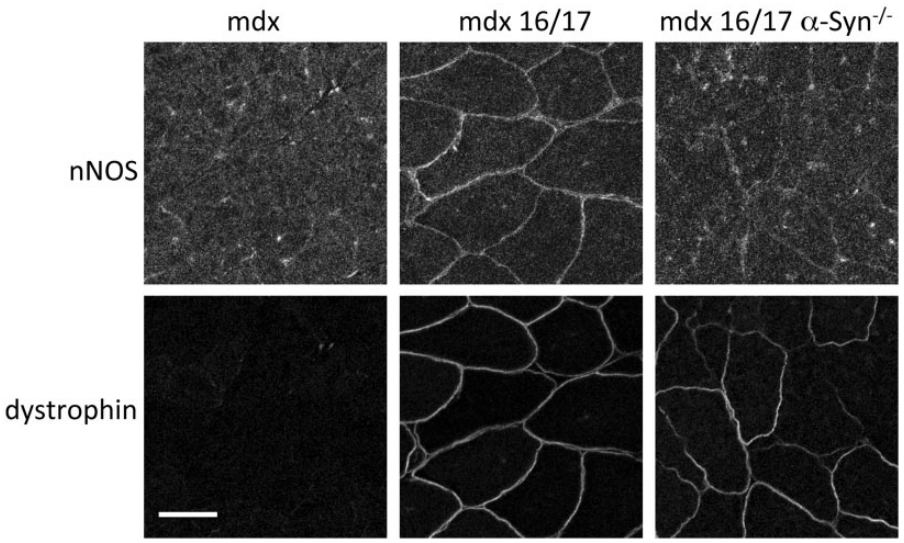

Figure 4. Restoration of nNOS to *mdx* sarcolemma by AAV-microdystrophin requires α-syntrophin. AAV-microdystrophin containing SLRs 16 and 17 was injected into the TA muscle of *mdx* and *mdx*/Snta$^{-/-}$ mice. Immunofluorescence of cryosectioned muscle showed that both treated strains expressed the microdystrophin but nNOS was only restored to the sarcolemma in the *mdx* strain that expressed α-syntrophin. Scale bar = 50 μm.

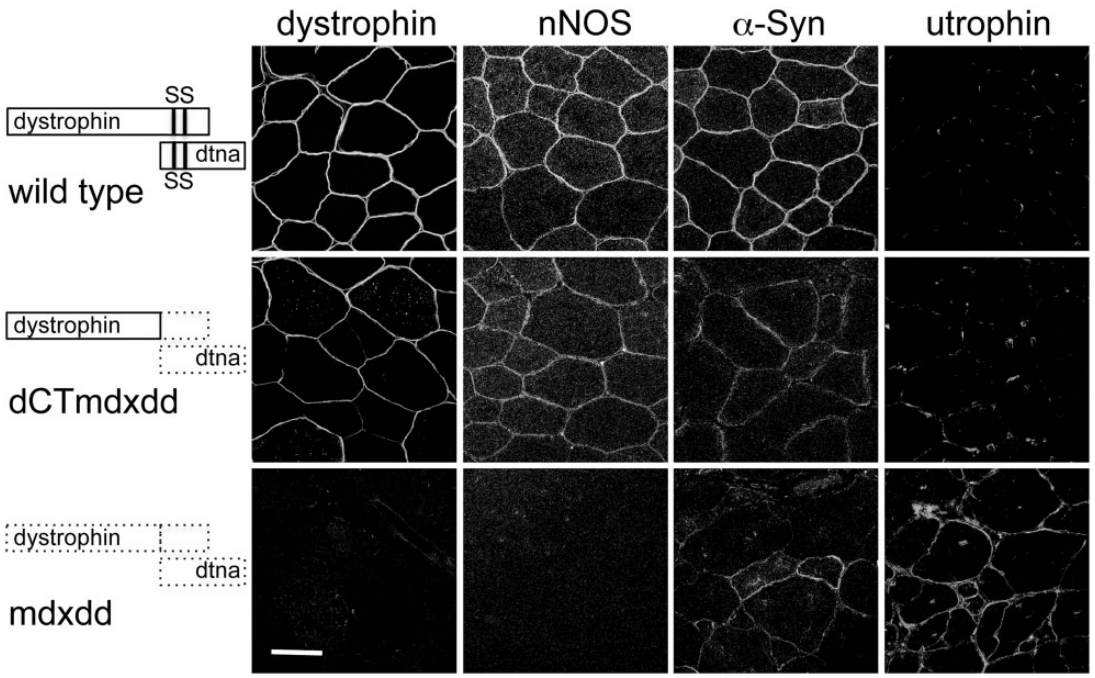

Figure 5. Both α-syntrophin and nNOS are restored to the *mdx* sarcolemma by a dystrophin transgene lacking the C-terminal SBSs and α-dystrobrevin. Immunofluorescence of gastrocnemious muscle cross sections shows that α-syntrophin and nNOS are restored to the sarcolemma of *mdx* mice expressing dystrophin lacking the C-terminal region (dCT) and lacking α-dystrobrevin (dd). Scale bar = 100 μm.

attached to the other SBSs do not bind nNOS. Third, the SLR region of dystrophin contains two additional SBS consensus sequences, one of which (SLR 22) specifically binds β-syntrophins. These findings strongly support the concept that the repeats are not simply 'springs' or 'shock absorbers', but are also capable of assembling adapters and signaling proteins that coordinate signaling pathways in time and space.

We have previously used pull down assays to determine which syntrophins bind to the two C-terminal SBSs in dystrophin family members (dystrophin, utrophin, dystrobrevin and DRP2) (26). Both sites in dystrophin bound α, β1 and β2 syntrophin with no indication of isoform specificity. In contrast, the current study shows specific syntrophin interactions among the SBSs located in the SLRs. SLR 22 bound only to the beta syntrophins (both β1 and β2) while SLR 17 bound only α-syntrophin. The potential SBS in SLR 23 did not show any binding to syntrophins in our pull down assays. This site (which contains a non-ideal leucine in Position 6) may not bind to syntrophins or do so only under specific (as yet unidentified) conditions.

Table 1. Potential SBSs in proteins other than members of the dystrophin family

Gene name	Description	Method	Sequence
KIAA 0556	Katanin-interacting protein	MS	EDEHMWLIPFSPGL
VPS13C	Vacuolar protein sorting 13C	MS	EEFHVPLDSYRCQL
ATP11C	ATPase phospholipid transporting	MS	DRLHELLIEYRKKL
ATR	ATR serine/threonine kinase	GC	EDGHFYLAKYYDKL
SULT1C3	Sulfotransferase family 1C	GC	KDMHRILYLFYEDI
DST	Dystonin	GC	MEFHNSLQDFINWL
ABHD14B	Abhydrolase domain containing 14B		EEWHTGLLDFLQGL
APPL1	Adaptor protein, phosphotyrosine interaction		KKQHQTMMHYFCAL
KIF1A	Kinesin family member 1A		KDMHDWLYAFNPLL
LTN1	Listerin E3 ubiquitin protein ligase		KKEHEDIFLFSCNL
PARD3	Par-3 family cell polarity regulator		KENHADLGIFVKSI
S1PR1	Sphingosine-1-phosphate receptor 1		KKFHRPMYYFIGNL
SHANK1	SH3 and multiple ankyrin repeat domains 1		DDAHFSMMVFRIGI
SLC8A2	Solute carrier family 8 member A2		DDDHAGIFSFQDRL
TIMELESS	Timeless circadian clock		RRMHLALKAYQELL
UBR1	Ubiquitin protein ligase E3-alpha-1		ERKHPVLCLFCGAI
ZZEF1	Zinc finger ZZ-type and EF hand domain 1		RKMHMFIARYCDLL

MS, mass spectroscopy; GC, gene chip.

The specific syntrophin binding to sites in SLR 17 and 22 may lead to the localization of unique PDZ ligands to these locations. The specific ligand binding to the beta syntrophins at SLR 22 is not known but several PDZ ligands are known to preferentially bind to the beta syntrophins, including ABCA1 (27) and ICA512 (28). The most obvious PDZ ligand binding to α-syntrophin at SLR 17 is nNOS. The sarcolemmal localization of nNOS depends on the presence of α-syntrophin as demonstrated in α-syntrophin null mice (10,11). Sarcolemmal nNOS is also dependent on the presence of the SLR 17 as demonstrated in both Becker patients (15) and mouse studies (8,22). In fact, the core amino acid sequence responsible for nNOS association with dystrophin (22) is embedded within the SLR-17 SBS (Fig. 1). The N-terminal region of nNOS and the α-syntrophin PDZ domain have been co-crystalized and the crystal structure revealed that the α-syntrophin PDZ domain binds directly to a β-finger region of nNOS (9). Together, these data demonstrate that α-syntrophin bound to SLR 17 binds nNOS and localizes it to the sarcolemma.

Additional data indicate that only the α-syntrophin bound to the SLR 17 SBS localizes nNOS. α-Syntrophin bound to either of the C-terminal two binding sites does not appear to bind nNOS. Transgenic mdx mice expressing the dystrophin C-terminal SBSs restore α-syntrophin to the sarcolemma but fail to restore nNOS (29). Similarly, AAV constructs that express C-terminal SBSs but not the SLR 17 SBS fail to restore nNOS but do express α-syntrophin on the sarcolemma (30). Lai *et al.* (22) showed that removal of the C-terminal dystrophin region (including both SBSs) does not appreciably affect the levels of nNOS associated with the membrane. This explains why some Becker patients have syntrophin on the sarcolemma but are missing nNOS (15).

The α-syntrophin bound to SLR 17 binds nNOS while α-syntrophin bound to the C-terminal SBSs does not bind nNOS (Fig. 6). How does the location of the SBS affect the PDZ ligand affinity? While we cannot fully answer this question here, we do have several clues as to how this may happen. First, one must remember that the interaction of the internal nNOS β-finger with the α-syntrophin PDZ domain is unique and different than the normal C-terminal PDZ ligand utilized by other syntrophin PDZ domain interactors (sodium channels, potassium channels, aquaporin-4, etc.). The nNOS β-finger is a loop and requires more space than a C-terminal ligand therefore the α-syntrophin PDZ domain must be in a slightly different conformation to accommodate the nNOS β-finger. Second, the SBS consensus sequence shows that the −2 and −3 positions are normally negatively charged amino acids (D, E) but in the SLR 17 SBS they are both positively charged arginine. This may alter the α-syntrophin confirmation to facilitate binding of the nNOS β-finger. Finally, the N-terminal region of α-syntrophin (PH1a) appears to be involved in the nNOS binding. In a previous study we showed that transgenic mice that express PH1a-deleted α-syntrophin fail to restore sarcolemmal nNOS in Snta$^{-/-}$ mice (31). This is despite the transgene localizing to the sarcolemma and expressing a functional PDZ domain. Functionality of the PDZ domain was demonstrated by its ability to restore sarcolemmal aquaporin-4 which is normally absent in Snta$^{-/-}$ mice.

Based largely on the observation that α-syntrophin at the C-terminal SBSs does not bind nNOS and on experiments with microdystrophin containing SLR16/17, the idea that nNOS binds directly to dystrophin at this region was proposed. The direct binding of nNOS to dystrophin has been generally accepted despite the inability of this model to address the fundamental question of why α-syntrophin would be required for sarcolemmal localization if nNOS binds directly to dystrophin. The only evidence for a direct interaction comes from yeast two hybrid experiments that showed that repeats 16/17 gave a positive Y2H interaction while repeat 16 or 17 alone did not (8). The more appropriate control would have been another repeat dimer (e.g. 14/15) with a similar coiled-coil structure. The repeats are coiled coils which are notorious for providing false positive Y2H results (32). In light of our discovery of an SBS in dystrophin SLR 17, it is now apparent that the nNOS/dystrophin interaction is mediated via α-syntrophin at the SLR 17 site.

Discovery of at least two SBSs embedded within the SLR domain unleashes new possibilities for potential protein interactions with dystrophin. While the SLRs are known to interact with Par-1b (SLR 8/9) (7) and with cytoskeletal proteins (actin, intermediate filaments and microtubules) this dystrophin region has been largely viewed as a structural 'spring'. Our data suggest that the SLR region may also serve as a scaffold for protein interactions that may be crucial for the function of dystrophin in muscle. Identifying and characterizing proteins/pathways interaction with the SLRs of dystrophin will be important for optimizing DMD gene therapy constructs and other treatments of this disease. AAV constructs with both the SLR 16/17 and SLR 22 SBSs may show greater efficacy than current constructs.

Finally, the refinement and validation of the consensus syntrophin binding sequence has allowed us to search for SBS in proteins outside of the dystrophin protein family. Based on our identification of six proteins previously linked to syntrophin, it is likely that many proteins other than the dystrophins utilize syntrophin as an adapter protein to increase functionality.

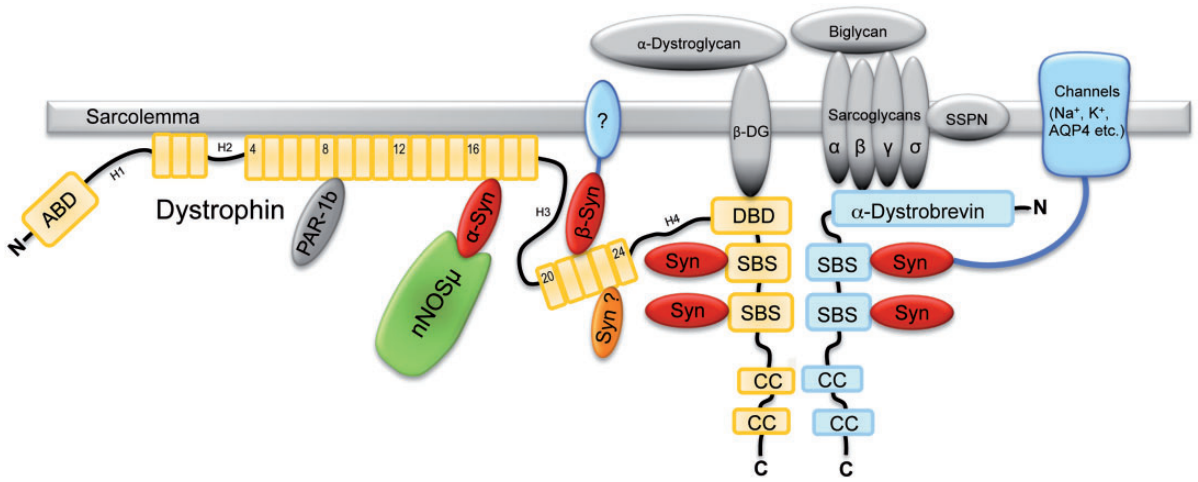

Figure 6. Model of syntrophin/dystrophin interactions. α-Syntrophin can bind to SLR-17 and to either C-terminal SBSs but only the α-syntrophin at SLR-17 binds nNOS. β1/β2-Syntrophins can bind to SLR-22 and to either C-terminal SBSs.

Materials and Methods

Mice

The control strain C57Bl/6, nNOS$^{-/-}$ mice and mdx mice were obtained from Jackson Labs (Bar Harbor, ME). Dtna$^{-/-}$ mice (33) were a gift from Drs Josh Sanes and Mark Grady. We have previously generated the Snta$^{-/-}$ mice (34) and the transgenic mice expressing dystrophin with the C-terminal region deleted (designated dCT) (20). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Washington.

Antibodies

We have previously characterized our rabbit antibodies to α-syntrophin, β1-syntrophin, β2-syntrophin (35), γ2-syntrophin (26) and utrophin (36). The dystrophin antibody used was MANEX1011b (1C7) (U. of Iowa, Developmental Studies Hybridoma Bank, deposited by Morris, G.E.) conjugated with Alexa Flour-488 (DyLight, Thermo). The nNOS rabbit antibody was purchased from ThermoFisher Scientific (Waltham, MA, USA).

Peptides

Biotinylated peptides were synthesized (Anaspec, Fremont, CA, USA) with sequence corresponding to the SBS regions of human dystrophin SLRs 17, 22 and 23. Additionally two control peptides were synthesized, one corresponding to the N-terminal region of SLR 15 [The A-helix (37) part of the SLR three-helix bundle that contains the SBS sequence in SLR17], and a peptide with the same amino acid composition as the SLR 17 peptide but in random order. The peptide sequences are:

SLR 17—Biotin-GSVEKWRRFHYDIKIFNQWLTEAE

SLR 22—Biotin-GREAALEETHRLLQQFPLDLEKFL

SLR 23—Biotin-GSSDQWKRLHLSLQELLVWLQLKD

SLR 15—Biotin-GISHQWYQYKRQADDLLKCLDDIE

SLR 17 randomized—Biotin-GHFIRSKNWFWEQERIETDYVLAK

Pull down assays

Streptavidin magnetic beads (ThermoFisher Scientific) were incubated 12–16 h at 4°C with mouse muscle membrane enriched homogenates. Homogenates were generated by homogenizing mouse skeletal muscle and bone in 10 volumes of buffer 1 [phosphate buffered saline (PBS), pH 7.4, 1 mM EDTA, with protease inhibitors (ThermoFisher Scientific)], centrifuging for 15 min at 8000×*g*, removing the supernatant and resuspending the membrane enriched pellet in buffer 1 plus 0.5% Tween 20 and re-centrifuging for 25 min at 37 000×*g*. Beads were washed six times with 1 ml of buffer 1 and the sample eluted in 70 μl of sample prep buffer for western blot analysis performed as described previously (38).

Immunofluorescence

Mouse tibialis anterior (TA) and gastrocnemius muscle was snap frozen in liquid nitrogen cooled isopentane. Cryosections (10 μm) were treated as described (34) and incubated with the designated primary antibody and detected with a secondary antibody (goat anti-rabbit) labeled with either Alexa 555 or Alexa 488 fluorophores (ThermoFisher Scientific). Imaging was performed using a Leica SL confocal microscope (W.M. Keck Microscopy Center, University of Washington).

AAV

The expression construct, adapted from a second generation microdystrophin (39,40) (ΔR2–R15/ΔR18–R23/ΔCT) was driven by the immediate early promoter and enhancer of cytomegalovirus (CMV), and contained the N-terminal domain, SLRs 1, 16, 17 and 24 followed by the CR domain and the rabbit β-globin polyadenylation signal. Thirty units of AAV (1×10^{11} vector genomes) were injected intramuscularly in the TA and gastrocnemius muscles of the right leg of 8-week-old mdx or mdx/Snta$^{-/-}$ mice. The left (uninjected) muscle served as a negative control. Four weeks following injection, the hindlimb muscles were analyzed by immunofluorescence.

Funding

This work was supported by grants from Parent Project Muscular Dystrophy; The Raymond and Beverly Sackler Foundation; and National Institutes of Health [NS14871 to S.F., HL122332 to J.C.]. Viral vectors were developed and produced in the University of Washington Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center [U54 AR065139 to J.C.].

Conflict of Interest statement. None declared.

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