Identification of a novel actin binding site within the Dp71 dystrophin isoform

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Abstract The Dp71 dystrophin isoform has recently been shown to localize to actin filament bundles in early myogenesis. We have identified an actin binding motif within Dp71 that is not found in other dystrophin isoforms. Actin overlay assays and transfection of COS-7 cells with fusion proteins of wild type and mutated Flag epitope-tagged Dp71 demonstrate that this motif is necessary and sufficient to direct localization of Dp71 to actin stress fibers. Furthermore, this localization is independent of alternative splicing which alters the C-terminus of the protein. The identification of an actin binding site suggests Dp71 may function to anchor membrane receptors to the cytoskeleton.

Key words: Dp71; Dystrophin; Actin binding site; Duchenne muscular dystrophy; Stress fiber

1. Introduction

Duchenne muscular dystrophy (DMD) is caused by mutations in the dystrophin gene [1]. The dystrophin gene encodes at least seven tissue-specific and developmentally regulated transcripts which are subject to alternative splicing at the 3′ end, generating a family of isoforms which differ in size and structure [2]. Dystrophin is required to prevent muscle wasting and is also important for the normal function of the central nervous system as is evidenced by the mild cognitive impairment and abnormal retinal electrophysiology that is associated with patients with DMD [2]. The precise function of dystrophin is unknown. It has been postulated that full length dystrophin, Dp427, reinforces the muscle membrane by binding to cytoskeletal actin at the N-terminus and to a transmembrane glycoprotein complex at its C-terminus [3]. In brain and retina two smaller isoforms of dystrophin, Dp260 and Dp71, are required and may be involved in positioning receptors or channels at specialized sites in the membrane [4–6]. Distinct patterns of expression and localization of the dystrophin isoforms are seen in skeletal muscle, heart, brain, and retina suggesting that the isoforms have differences in their function which in turn are due to differences in protein interactions. Thus a complete understanding of the interactions of individual isoforms is necessary to determine the function of this gene.

We have focussed our efforts on the Dp71 isoform since this isoform is widely expressed and is the most structurally distinct of all of the isoforms. Dp71 lacks the N-terminal actin binding domain, rod domain, and WW domain found in full length dystrophin, but contains a unique seven amino acid N-terminus fused to the cysteine-rich and C-terminal domains common to all dystrophin isoforms [7]. Since Dp71 retains many of the binding sites which in Dp427 are involved in interactions with the dystrophin-associated glycoprotein complex, it may also interact with members of this complex, possibly to sequester receptor complexes to specific areas of the cell membrane. Recently, we have demonstrated that Dp71 is localized to actin filament bundles in muscle cell culture systems [8]. The mechanism by which Dp71 localizes to actin filament bundles is enigmatic, since Dp71 does not contain the actin binding sites found in Dp427. Nevertheless, our results suggest that Dp71 does interact directly with the actin cytoskeleton, possibly through one or more unique binding sites within the N- or C-terminal sequences that are absent in Dp427.

Here, we investigate the mechanism of Dp71 localization to actin filament bundles to characterize further the biological role of Dp71. Our results demonstrate that a hexapeptide motif within the Dp71 unique N-terminus is necessary and sufficient for the localization of Dp71 to stress fibers in COS-7 cells. These results provide the first evidence for a direct interaction between Dp71 and the actin cytoskeleton and raise important questions regarding the biological role of Dp71.

2. Materials and methods

2.1. Dp71 constructs

To generate the histidine-tagged Dp71 fusion protein in *Escherichia coli*, an *Eco*RI/*Stu*I fragment containing the N-terminal 1.4 kb of Dp71 was blunt end ligated into the *Nde*I site of the vector Pet 25b (Novagen). The final product was a chimeric construct containing the Dp71 N-terminus and a polyhistidine tract at the 3′ end. This vector was transformed into *E. coli* strain BL-21(DE-3). The fusion protein containing the Dp71 N-terminus was insoluble, therefore, cells were harvested by centrifugation, and lysed in cracking buffer (6 M urea, 150 mM NaCl, 50 mM Tris (pH 8.0), and 0.01% Triton X-100). The lysate was incubated on ice for 30 min, sonicated to shear bacterial genomic DNA, and cleared by centrifugation at $10\,000 \times g$. The supernatant was incubated with Ni^{2+} agarose beads pre-equilibrated in cracking buffer. The histidine-tagged fusion protein was eluted in cracking buffer containing 250 mM imidazole.

A GFP-Dp71 fusion construct has been previously described [8]. To place the GFP-Dp71 cDNA under the regulation of the CMV promoter, fragments containing GFP-Dp71 (+exon 71/+exon 78) and GFP-Dp71 (−exon 71/−exon 78) were isolated from pRcCMV vector (Invitrogen, California) and ligated into the *Xba*I site in the adenovirus vector CMV-pΔE1Sp1b [9].

PCR amplification of pBluescript (Stratagene, La Jolla, CA) con-

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taining the full length Dp71 cDNA was used to generate Flag epitope-tagged wild type and mutant versions of the Dp71 N-terminus. The primers for the wild type construct were: Fwt: 5′-TCTGAATTCA-ATGAGGGAACAGCTCAAGGG-3′; and R1: 5′-TCTGGATCCG-CAGCTGACAGGCTCAAGAG-3′. For the Dp71(L→A/K→A)-Flag construct the following primers were used: Fmu: 5′-TCTGA-ATTCAATGAGGGAACAGGCGGCAGGCCAGG; and R1. The product of each reaction was separated on a 1.5% agarose gel, isolated and blunt end ligated into pBluescript. An *Eco*RI/*Bam*HI insert containing the Dp71 N-terminus was cloned into the pFlagCMV-2 vector (InterScience, Missisauga, Ont.).

2.2. Actin overlay

Actin was purified from chicken breast according to the method of Pardee and Spudich [10]. Fusion proteins (20 μg) were separated by SDS-PAGE, and transferred to PVDF membrane. Membranes were blocked overnight in 1×TBST (50 mM Tris (pH 8.0), 150 mM NaCl, 1% fetal calf serum, and 0.01% Tween-20) with 5% non-fat milk. The next day, membranes were incubated with 300 ng/ml actin in 1×TBST supplemented with 1 mM $MgCl_2$, and 10 mM ATP, washed briefly in 1×TBST, and incubated with a pan-actin monoclonal antibody # 4700 (dilution 1:100) (Sigma, St. Louis, MO). Membranes were washed 3×5 min with 1×TBST and incubated with an anti-mouse antibody conjugated to horseradish peroxidase (Amersham, UK). Actin binding was detected using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham, UK). For the Western blot using antibody Nme-1, the same blot used in the actin binding experiment was stripped and blocked overnight as indicated above. Antibody Nme-1 has been previously described and is specific for the first seven amino acids of the Dp71 N-terminus [5]. The blocked membrane was incubated with antibody Nme-1 (dilution 1:500) in 1×TBST and 1% non-fat milk for 1 h. Fusion proteins containing the Dp71 N-terminus were detected by ECL as described.

2.3. Cell culture and transfection

COS-7 cells were obtained from the American Tissue Culture Collection (ATCC CRL 1651, Rockville, MD). One day prior to transfection, COS-7 cells were plated at a density of 1000 cells per cm^2 into chamber slides (Nunc, Illinois). Cells were grown in growth medium (α-MEM 2×glucose) supplemented with 10% fetal calf serum and gentamicin (20 μg/ml) (Gibco BRL, New York). Supercoiled plasmids were diluted in serum-free media, and introduced into cells using lipofectamine as per the manufacturer's directions (Gibco BRL). After a 5 h incubation period, the transfection mixture was removed by washing with growth medium. Cells were maintained in growth medium for 48 h before being analyzed.

2.4. Immunofluorescence

COS-7 cells transfected with either pFlagCMV-2, pFlag-Dp71wt, or pFlag-Dp71mu vectors were washed with PBS ×3, and fixed for 10 min with cold acetone at −20°C. The slides were dried and incubated with primary antibody Flag-M2 (InterScience, Mississauga, Ont.) in PBS containing 1% fetal calf serum. Cells were washed with PBS and then incubated for 1 h with secondary goat anti-mouse IgG conjugated to biotin (Sigma). Cells were washed with PBS (3×10 min), and incubated for 1 h with streptavidin conjugated to CY3 (Sigma). Cells were washed as before, mounted with Immunoflo (ICN, Ohio), and examined with a Leica epifluorescent microscope.

2.5. Adenovirus preparation

Recombinant replicative-defective adenoviruses were prepared according to the method of Graham and Prevec [9]. Clonal populations of myogenic cells were isolated from 10 week human fetal muscle biopsies, as previously described [11]. GFP-Dp71 expression was examined in cells seeded onto Permanox chamber slides (Nunc, UK) at high density and allowed to recover for 24 h. To induce myogenic differentiation, cells were transferred to fusion medium (α-MEM, 16 mM glucose, 2% fetal calf serum and 100 U/ml penicillin and 0.1 mg/ml streptomycin), and grown for 3–5 days. Fused myotubes were infected with recombinant adenovirus bearing either GFP-Dp71(+71/+78) or GFP-Dp71(−71/−78) at a multiplicity of infection (MOI) of 100 infectious particles per cell. Cells were maintained in growth medium for 48 h, washed in PBS, and fixed with 3% paraformaldehyde in PBS for 30 min. Fixed cells were then mounted in 50% glycerol/PBS and analyzed for GFP expression using a Leica epifluorescent microscope with a HB103/W mercury lamp and standard FITC filter set.

2.6. Subcellular fractionation

Liver from C57BL/J6 was harvested and snap frozen in liquid nitrogen. Powdered extract was prepared by crushing the frozen sample using a chilled mortar and pestle. The powdered extract was then lysed in Triton X-100 lysis buffer (2% Triton X-100, 10 mM EGTA, 10 mM Tris-HCl, 2 μg/ml leupeptin, 10 mM benzamidine, 2 mM PMSF, pH 7.4) and fractionated as previously described [8]. Dp71 was detected using either antibody Act-1 (1:500 dilution) or antibody 1583 (1:1000 dilution). The preparation of antibody Act-1 and 1583 has been previously described [5,12]. Actin was detected with an anti-α-actin (A-2172, Sigma). GST-π was detected using anti-GST-π (Medical and Biological Laboratories, Nagava, Japan). Primary antibodies were detected using ECL according to the manufacturer's instructions (Amersham, UK).

3. Results

3.1. Actin overlay assay

To investigate whether Dp71 could interact directly with filamentous actin, actin overlay assays were performed on Dp71 fusion proteins. As shown in Fig. 1, both purified and crude extracts of a Dp71 N-terminus-polyhistidine fusion protein containing the first 510 amino acids of Dp71 bind actin filaments. No actin binding was detected in control lanes containing equal amounts of an unrelated polyhistidine PHR-1 (pleckstrin homology containing protein in the retina) fusion protein (generous gift from Rahim Ladak) or bovine serum albumin, suggesting that this interaction is specific. The histidine-tagged Dp71 fusion protein is also recognized by a polyclonal antibody specific to the N-terminal seven amino acids of Dp71, confirming that the fusion protein contains an intact Dp71 N-terminus (Fig. 1c). These results suggested that an actin binding site is located within the first 55 kDa of Dp71, possibly within the unique N-terminus of Dp71.

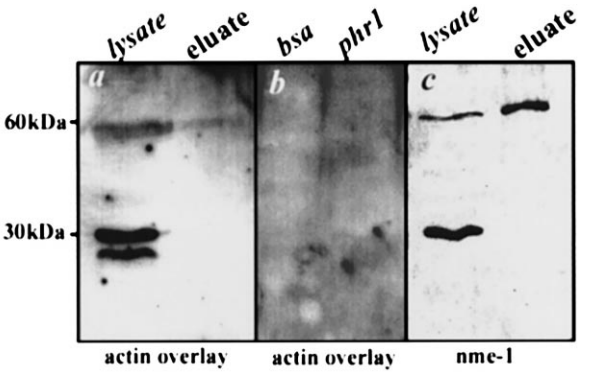

Fig. 1. Actin overlay. a: Overlay assay was conducted as described in Section 2.2. Dp71-histidine fusion protein bound to actin in both the crude extract (lysate) and purified eluate lanes. Two additional lower molecular weight bands were detected in the crude extract and correspond to truncated products of the full length fusion protein. b: Negative controls, bovine serum albumin (BSA) (66 kDa) and a purified polyhistidine-tagged protein (PHR-1) (28 kDa) were also probed for actin binding activity using identical conditions. No actin binding was detected for either protein. c: To confirm the identity of the bands detected in above, the blot used in a was stripped and probed with antibody Nme-1 specific for the first seven amino acids in the Dp71 N-terminus. Nme-1 detected identical bands corresponding to the full length fusion protein in both the lysate and eluate lanes. Also detected was a band corresponding to the middle band in the overlay experiment.

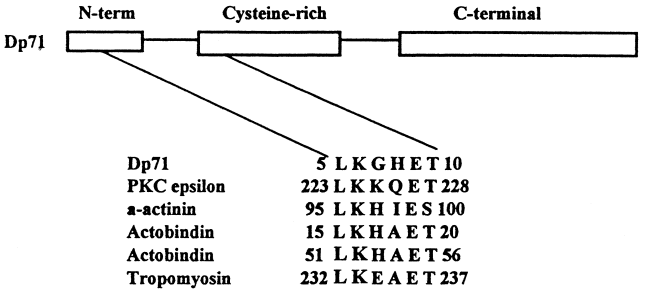

Fig. 2. Top: A diagram of the Dp71 protein indicating the position of the novel N-terminus, cysteine-rich domain, C-terminal domain, and the alternative C-terminus (ACT) encoded by exon 79. Bottom: Alignment of Dp71 N-terminus with the LKXXES/T motif present in many actin binding proteins. In bold are shown the conserved residues.

3.2. Actin binding motif within Dp71

A search of the Dp71 primary sequence for protein motifs which may account for interaction with the actin cytoskeleton identified a putative actin binding motif within the Dp71 unique N-terminus (Fig. 2). This motif (LKXXES/T) is present in several known actin binding proteins and has been shown to participate in actin binding [13–15]. The critical residues for actin binding are the conserved leucine and lysine residues, which have been shown to interact directly with the N-terminus of actin [14,15]. In most cases, the third and fourth positions contain a histidine and an alanine residue followed by a conserved glutamate, and either a serine, or threonine. In Dp71, the third and fourth positions are occupied by glycine and histidine respectively. As three of the six residues are encoded by exon one, this actin binding site is restricted to the Dp71 dystrophin isoform. A search of dystrophin primary sequences confirmed that this motif is not present in any other dystrophin isoform.

3.3. Expression of Dp71 N-terminus in COS-7 cells

To test whether the LKGHET motif within the N-terminus of Dp71 is a functional actin binding site, a eukaryotic expression vector containing the N-terminus of Dp71 fused to the Flag peptide (containing the epitope for monoclonal antibody Flag M2) was constructed and transfected into COS-7 monkey kidney cells. These cells were chosen for their high transfection efficiency and because they produce stress fibers, allowing actin co-localization to be readily identified. As shown in Fig. 3a,b, a Flag fusion protein containing the LKGHET motif was targeted to stress fibers in COS-7 cells, and gave a staining pattern that was identical to cells stained with phalloidin-FITC. In contrast, cells expressing the Flag epitope alone did not localize to stress fibers (Fig. 3c). To verify that the LKGHET motif is directly responsible for targeting the fusion protein to stress fibers, the first and second residues of this motif were mutated to alanine residues. These residues are highly conserved and are known to be involved in actin binding in the protein actobindin [14,15]. As shown in Fig. 3d, mutation of these residues abolished the localization of the fusion protein to stress fibers, indicating that the LKGHET motif within the N-terminus of Dp71 is directly involved in localizing the protein to stress fibers.

3.4. Expression of GFP-Dp71 isoforms

In contrast to full length dystrophin, the majority of endogenous Dp71 transcripts in myoblasts undergo splicing which removes exon 78, and changes the reading frame of the transcript, such that a 13 amino acid hydrophilic C-terminus is replaced with a 31 amino acid primarily hydrophobic C-terminus [8]. Alternative splicing of exon 71 has also been shown to be a common event in Dp71 transcripts [16]. To investigate whether alternative splicing of exons 71 or 78 plays a role in the interaction of Dp71 with actin filaments, primary myotubes were transduced with recombinant adenoviruses, expressing GFP fusion constructs corresponding to either Dp71 (+exon 71/+exon 78) or Dp71 (−exon 71/−exon 78). Stress fiber-like structure (SFLS) localization was clearly evident in myotubes expressing either Dp71(+exon 71/+exon 78) or Dp71 (−exon 71/−exon 78), suggesting that splicing of exons 71 and 78 does not influence actin binding (Fig. 4). GFP-Dp71 fluorescence was detected along the lengths of the actin filaments and at the ends of the myotubes, where they adhere to the substrate. This is consistent with our previous analysis of Dp71 localization in C_2C_{12} cells, and confirms that Dp71 interacts with SFLS in myogenic cells [8].

3.5. Biochemical fractionation

Actin filaments are inherently insoluble in Triton X-100 and are often sufficiently crosslinked that they will pellet under low centrifugal force [17]. Fig. 5A,B shows Western blots of Triton X-100 extracts of mouse liver probed with either antibody Act-1, which recognizes the hydrophobic carboxy-terminus encoded by exon 79 [5], or antibody 1583, which recognizes the hydrophilic carboxy-terminus encoded by exon 78

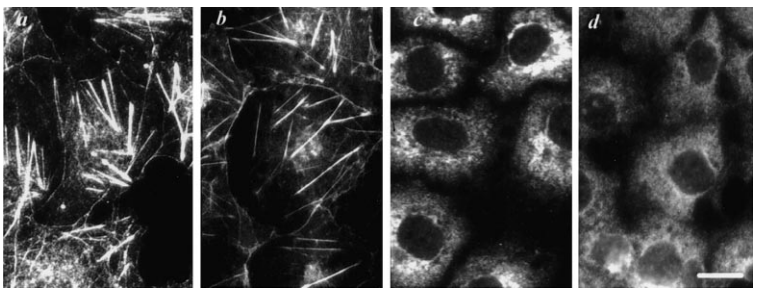

Fig. 3. Transfection of COS-7 cells. COS-7 cells were transfected with either Flag-Dp71wt (a), Flag vector alone (c), or Flag-Dp71mu (L→A/K→A) (d), and immunostained with the anti-Flag antibody M2. Wild type Flag epitope-tagged Dp71 localized to stress fibers and appeared identical to cells labeled with phalloidin-FITC (b). No actin filament was identifiable in cells transfected with either Flag vector alone or the mutated Flag Dp71 construct. Bar = 10 μm.

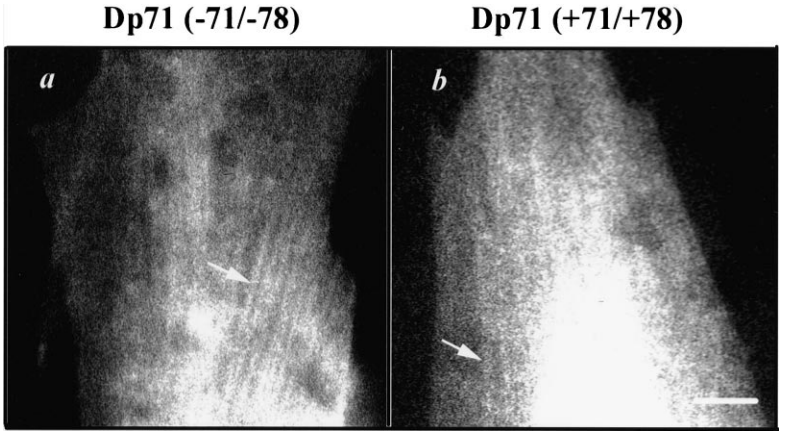

Fig. 4. Comparison of GFP-Dp71 isoform expression in primary myotubes. a: A primary myotube expressing GFP-Dp71 (−exon 71/−exon 78). b: A primary myotube expressing GFP-Dp71 (+exon 71/+exon 78). Both GFP-Dp71 chimeric proteins targeted to SFLS (arrows) in primary myotubes. Bar = 5 μm.

[12]. Both antibodies identified Dp71 in the low speed pellets, indicating that Dp71 co-sediments with actin filaments. A GST-π control included in these experiments demonstrated that cytosolic proteins fractionated as expected. These experiments confirm the results of our GFP-Dp71 expression studies, and together indicate that the Dp71 dystrophin isoform associates with actin filament bundles in vivo, and that alternative splicing of exons 71 and 78 does not affect this localization.

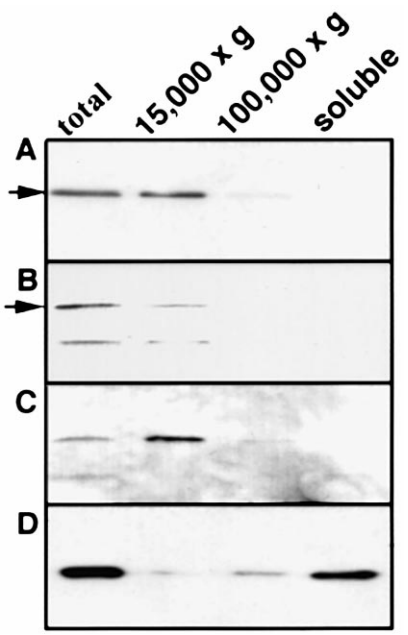

Fig. 5. Western analysis of fractionation. Fractionation of liver extracts was conducted as described in Section 2.5. A: Antibody 1583 (Dp71 hydrophilic C-terminus). B: Antibody Act-1 (Dp71 hydrophobic C-terminus). C: Anti-α-actin. D: Anti-GST-π. Lane 1, liver control sample (whole cell lysate) showing migration of Dp71 (arrows). Lane 2, pellet fraction from 15 000×*g* spin showing low speed actin pellet. Lane 3, pellet fraction from 100 000×*g* spin showing high speed pellet. Lane 4, supernatant from final spin showing the cytosolic fraction. The majority of Dp71 with either the hydrophilic C-termini or the hydrophobic C-termini was found in the low speed pellets and appeared very similar to the distribution of actin. In contrast, the majority of GST-π was found in the cytosolic fraction, indicating non-actin bound cytosolic proteins fractionated as expected.

4. Discussion

We have identified a binding site for filamentous actin in the N-terminus of Dp71 that is unique to this isoform of dystrophin. The site is encoded partially by the novel first exon of Dp71, and partially by exon 63 of the dystrophin gene. The LKGHET motif is very similar to an actin binding motif originally identified in the actin binding protein, actobindin, where an LKHAET motif has been shown to form a direct interaction with actin through the leucine and lysine residues [13–15]. Several independent lines of evidence indicate that the localization of Dp71 to SFLS occurs through a direct interaction between the LKGHET motif in the Dp71 N-terminus and actin filament bundles. First, actin overlay experiments demonstrated that actin binds to the Dp71 N-terminal fusion proteins containing the motif, but not to controls. Second, addition of the N-terminal 59 residues of Dp71 to the flag peptide was sufficient to target the flag epitope to stress fibers in COS-7 cells. Third, mutation of the leucine and lysine residues within the LKGHET motif abolished the co-localization activity. Finally, Dp71 was shown to co-fractionate with filamentous actin from mouse liver extracts. Taken together, these results demonstrate that Dp71, like Dp427, associates with the actin cytoskeleton.

To date, five actin binding sites (ABS) have been identified within the dystrophin gene: ABS1–3 are located within the first 230 amino acids of Dp427 and are encoded by exons 2–7 [18–21]; ABS4 is located in a section of the rod domain encoded by exon 56 and is present in both Dp427 and Dp260 [22]. The fifth actin binding site, ABS5, is described here. The only other known protein binding sites in dystrophin lie within the C-terminus of the protein, in a region that is common to all dystrophin isoforms [23–25]. That three of five dystrophin isoforms (Dp427, Dp260, and Dp71) bind to the actin cytoskeleton suggests that these isoforms share similar functions. This is contrary to experimental evidence from both in vitro and in vivo studies indicating that these isoforms exhibit differences in cellular localization and function [5,8,26,27]. We

have shown that Dp71 occupies a subcellular compartment that is distinct from Dp427, and in the retina, Dp71 is localized to a cellular layer that is distinct from Dp427 or Dp260 [5,8]. Furthermore, Dp71 does not rescue the muscle phenotype in transgenic *mdx* mice, indicating that Dp71 cannot functionally replace Dp427 [26,27]. Therefore, although each of these isoforms associates with actin, differences in protein interactions mediated by other binding sites within each isoform are likely responsible for differences in localization and function.

Previously, we have shown that an alternatively spliced isoform of Dp71 is associated with SFLS in myogenic cells, suggesting that alternative splicing may influence Dp71 isoform localization [8]. We have tested by two independent means whether splicing could affect actin binding and localization to actin filament bundles. GFP-Dp71 fusion proteins bearing either spliced or unspliced forms of Dp71 localized to SFLS. Secondly, antisera to either the hydrophilic C-terminus (1583) or the hydrophobic C-terminus (Act-1) both detected Dp71 isoforms co-sedimenting with actin. These results suggest that Dp71 actin binding is independent of the splicing of exons 71 or 78.

The functional significance of the actin binding site in Dp71 is almost certainly related to the protein interactions occurring at the C-terminus of the molecule. Recently several dystrophin interacting proteins have been identified. The syntrophins (α1, β1, β2, γ) are a family of proteins that bind to the C-terminal domain of dystrophin [28–30]. These proteins also contain additional binding sites, including two pleckstrin homology (PH) domains, a PDZ domain (post-synaptic density-95 kDa, discs large tumor suppressor protein, zona occludens), and a syntrophin unique domain, and have been shown to bind to the cytoplasmic C-termini of the voltage-gated sodium channels, skm1 and skm2 [31,32]. Although syntrophins, and by extension dystrophin, are suggested to be involved in the clustering of these channels, studies of *mdx* mice transgenic for mutant Dp427 constructs deleted for syntrophin binding sites have shown that the interaction is not required for Dp427 function in muscle [31–33]. The identification of an actin binding site within Dp71 suggests that Dp71 may participate in the clustering of sodium channels by anchoring the syntrophin/channel complex to the actin cytoskeleton. This hypothesis is consistent with recent data showing that Dp71 is required for normal electrophysiology in the neural retina [5,6], and suggests that loss of Dp71 expression in the retina may disrupt clustering of retinal-specific ion channels at the inner limiting membrane. Obviously, identification of Dp71 interacting proteins will be necessary for understanding the role of Dp71 in this process.

In summary, we have shown that Dp71 forms a direct interaction with the actin cytoskeleton through a hexapeptide actin binding motif located in the N-terminus of Dp71. The identification of an actin binding site within the N-terminus of Dp71 provides important insights into the potential role of this isoform in membrane/protein complexes.

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