Spectrin-like repeats from dystrophin and α-actinin-2 are not functionally interchangeable

Scott Q. Harper¹,², Robert W. Crawford¹, Christiana DelloRusso¹ and Jeffrey S. Chamberlain¹,²,*

¹Department of Neurology, University of Washington School of Medicine, HSB Room K243, Box 357720, Seattle, WA 98195-7720, USA and ²Program in Cellular and Molecular Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA

Received March 3, 2002; Revised May 8, 2002 and Accepted May 18, 2002

Mutations in the dystrophin gene result in Duchenne muscular dystrophy (DMD). Dystrophin is a multidomain protein that functions to stabilize the sarcolemmal membrane during muscle contraction. The central rod domain has been proposed to act as a shock absorber, as a force transducer or as a spacer separating important N- and C-terminal domains that interact with actin and the dystrophin–glycoprotein complex (DGC). Structure/function studies demonstrated that deletion of large portions of the rod domain can result in the production of smaller, yet highly functional, dystrophin proteins. In a dramatic example, a ‘micro-dystrophin’ transgene containing only four dystrophin spectrin-like repeats resulted in complete correction of most of the symptoms associated with dystrophy in the mdx mouse model for DMD. Dystrophin shares considerable homology with the multidomain, actin-crosslinking protein α-actinin. To explore the hypothesis that the dystrophin rod domain acts as a spacer region, a chimeric micro-dystrophin transgene containing the four-repeat rod domain of α-actinin-2 was expressed in mdx mice. This chimeric transgene was incapable of correcting the morphological pathology of the mdx mouse, but still functioned to assemble the DGC at the membrane and provided some protection from contraction-induced injury. These data demonstrated that different spectrin-like repeats are not equivalent, and reinforced the suggestion that the dystrophin rod domain is not merely a spacer but likely contributes an important mechanical role to overall dystrophin function.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder caused by mutations in the dystrophin gene (1). Dystrophin is expressed in a wide variety of tissues, but its absence in muscle is responsible for the devastating weakness and early lethality that characterize the disease (2–4). At present, there is no treatment that can reverse the pathology of DMD. The mdx mouse model for DMD has proven to be important for the study of dystrophin structure and function, as well as in the development of potential therapies for the disease (5). Muscles of mdx mice are highly susceptible to contraction-induced injury (6–9), undergo cycles of myofiber necrosis and regeneration, and are populated with large amounts of centrally nucleated myofibers, immune cells, fat deposits and fibrotic lesions (5,10–12). The demonstration that a full-length dystrophin transgene can correct these deficits in the mdx mouse suggests that dystrophin gene replacement may be an effective treatment for the disease (3). Despite this, gene therapy for DMD is complicated for several reasons, including the large size of the gene (2.4 Mb; 14 kb cDNA), which precludes its packaging into most known viral vectors (13).

Dystrophin is a member of the spectrin superfamily of proteins that includes α-actinin, utrophin and the spectrins (14–17). The full-length muscle isoform of dystrophin is composed of four domains: the N-terminal actin-binding domain (ABD), the central rod, and cysteine-rich (CR) and C-terminal (CT) domains (15). The association of dystrophin with actin and a collection of integral and peripheral membrane proteins, known as the dystrophin–glycoprotein complex (DGC), anchors the extracellular matrix to the internal cytoskeleton and prevents contraction-induced damage to the sarcolemma (18–21). The dystrophin rod domain contains 24 spectrin-like repeats (22), has been proposed to act as a ‘shock absorber’ at the cytoskeleton–membrane interface (23) and likely makes a major contribution to the sarcolemmal reinforcement accomplished by the DGC. The rod domain has also been proposed to function as a force transducer (24), or merely as a spacer between the N- and C-terminal domains (25).

*To whom correspondence should be addressed. Tel: +1 2062215363; Fax: +1 2066168272; Email: jsc5@u.washington.edu
We have recently described the engineering of extremely small dystrophin proteins lacking large portions of the rod domain that were shown to be highly functional in mouse muscles (26). These 'micro-dystrophins' were approximately 3.8 kb in size, contained only 4 of the 24 dystrophin spectrin-like repeats and were able to restore most of the dystrophic features in muscles of mdx mice. In addition to their potential clinical relevance, these micro-dystrophins provided knowledge about the function of the dystrophin rod domain and of the individual spectrin-like repeats. We also showed that dystrophin proteins lacking a rod domain were non-functional.

In this study, we further explored the role of the dystrophin rod domain by asking whether different spectrin-like repeats are functionally interchangeable. We assessed the level of dystrophic correction achieved in mdx mice through transgenic expression of a chimeric micro-dystrophin in which the rod domain was replaced with the homologous region from α-actinin-2 (27). The α-actins are major cytoskeletal components in many cell types, and share considerable homology with dystrophin (28,29). The α-actin-2 isoform is found only in skeletal and cardiac muscles (27), and, like the micro-dystrophins, binds actin and contains a four-repeat rod domain. Because of its size, muscle-specific expression and homology with dystrophin, we tested whether the four-repeat rod-domain of α-actinin-2 could functionally substitute for that of dystrophin.

We found that the dystrophin/α-actinin-2 chimera was expressed at high levels, assembled the DGC complex at the sarcolemma and partially protected myofibers from contraction-induced injury, but was incapable of preventing the morphological abnormalities associated with dystrophy in mdx mice. These data, taken together with our previous micro-dystrophin expression and localization of dystrophin/actinin chimera in transgenic mdx mice

To explore the function of the dystrophin rod domain, we generated transgenic mice that expressed a fusion protein in which the dystrophin rod domain was replaced by the homologous region from α-actinin-2. The encoded protein retained the N- and C-terminal domains of dystrophin, as well as hinges 1 and 4, but the rod domain was derived from the four-repeat domain of α-actinin-2. The chimera protein (Fig. 1A) was expressed at its predicted molecular weight of 169 kDa (Fig. 1B). Densitometric analysis revealed chimera expression to be about 6-fold greater than that of wild-type dystrophin—a level similar to that obtained with our most highly functional four-repeat micro-dystrophin [ΔR4-R23; Fig. 1B and (26)]. Like wild-type dystrophin, the dystrophin/actinin chimera was correctly localized to the muscle plasma membrane in both quadriceps and diaphragm (Fig. 1C).

Histopathological analysis of mdx mouse muscles expressing dystrophin/actinin chimera

The dystrophin/actinin chimera was unable to prevent the fibrosis, fat deposition, mononuclear cell infiltration or hypertrophy associated with muscular dystrophy in mdx mice, and myofibers of chimeric/mdx mice were permeable to Evans blue dye at similar levels as in mdx muscles (Figs 1C, 2B and 2C). Consistent with this finding was the high percentage of centrally nucleated myofibers detected in the muscles of the transgenic mice (Fig. 2A). This feature of dystrophic muscle is indicative of myofiber necrosis and regeneration, and muscles from dystrophin/actinin and mdx mice were not different by this measure. A further indication of myofiber necrosis, regeneration and hypertrophy is the wide variation in myofiber size observed in both mdx and dystrophin/actinin muscles (Fig. 2B). Interestingly, dystrophin/actinin myofibers were significantly larger on average than mdx controls in both quadriceps and diaphragm, despite similar degrees of myofiber size variation. In contrast, normal control muscles displayed uniform myofiber diameters, nuclei located almost entirely at the periphery of the myofiber, and no evidence of fibrosis, fat deposition, mononuclear cell infiltration or hypertrophy, and myofiber membranes were impermeable to Evans blue (Figs 1C and 2).

Mechanical properties of mdx mouse muscles expressing dystrophin/actinin chimera

mdx TA muscles subjected to an in situ contraction-induced injury protocol demonstrated a significantly reduced ability to generate force following two lengthening muscle contractions (LC1 and LC2) compared with wild-type control muscles [(6) and Fig. 2D; the transgene was expressed uniformly in the TA at a similar level as in quadriceps; data not shown]. Chimera/mdx muscles were no different from control mdx muscles following LC1, but demonstrated a significant protection from contraction-induced injury following LC2.

The dystrophin/actinin chimera has a mild dominant-negative effect on endogenous dystrophin in wild-type C57BL/10 mice

We observed that the chimeric protein had a deleterious effect on the function of endogenous dystrophin when expressed in wild-type mice. Quadriceps muscles expressing both the dystrophin/actinin chimera and wild-type dystrophin (chimera/C57BL/10) contained elevated levels of centrally nucleated myofibers compared with control C57BL/10 (Figs 1C and 2A). In addition, there was a slight, yet significant increase in average myofiber diameter and size variance in chimera/C57BL/10 mouse quadriceps compared with those of wild-type control mice (Fig. 2B). In the diaphragm of chimera/C57BL/10 10 mice, there was no increase in the percentage of centrally nucleated myofibers; however, we measured a significant increase in myofiber diameter and size variance relative to wild-type and mdx mice (Fig. 2B). Interestingly, all of our previously tested micro-dystrophins demonstrated similar myofiber hypertrophy in the diaphragm when expressed
Figure 1. Expression and localization of the dystrophin/actinin chimeric protein in transgenic mice. (A) Schematic illustration of the structure of the dystrophin/actinin chimeric protein. Domains in the full-length dystrophin, ΔR4-R23 micro-dystrophin and skeletal muscle α-actinin-2 are shown for reference. ABD, N-terminal actin-binding domain; ABD2, internal actin-binding domain; CR, cysteine-rich domain; CT, C-terminal domain. Dystrophin spectrin-like repeats are gray and indicated. The arrow points to repeat 24. Numbers in black boxes indicate hinge domains. EF, α-actinin-2 EF hand domains; the homologous region in dystrophin is located in the CR domain. Four α-actinin-2 spectrin-like repeats are white with confetti markings. (B) Western analysis of dystrophin expression in quadriceps. Full-length dystrophin is 427 kDa; chimera, 169 kDa; ΔR4-R23, 168 kDa. Protein expression levels compared with full-length dystrophin are indicated. Fifty micrograms of protein was loaded per sample set. (C) Anti-dystrophin immunofluorescence and H&E staining of 6-month-old C57BL/10, mdx, transgenic/ mdx and transgenic/C57BL/10 muscle sections. Examples of centrally nucleated myofibers in quadriceps are indicated by arrows. The bottom row displays Evans blue dye uptake (red fluorescence) by quadriceps muscles in the indicated mouse strains. Scale bar: 100 μm.
on the mdx background, but were highly functional in this muscle (26).

**DISCUSSION**

Although the pathological consequences of mutations in dystrophin are well characterised, the exact functions of the
protein remain unclear. Mutations in any one of the four dystrophin domains can have variable and sometimes profound myopathic effects (30–34). Expression of dystrophin transgenes carrying deletions in the CT domain resulted in normal muscle morphology and function in \( \textit{mdx} \) mice (35,36). Conversely, transgenic \( \textit{mdx} \) mice expressing dystrophins deleted for the DGC anchoring site in the CR domain were severely dystrophic (36). Furthermore, mutation of DGC proteins such as the sarcoglycans and dystroglycan can lead to other forms of muscular dystrophy or embryonic lethality, respectively (37,38). One critical function of dystrophin, therefore, is its role in mediating assembly of the DGC at the sarcolemmal membrane. Similarly, the N-terminal domain of dystrophin plays an important role in linking the DGC to the subsarcolemmal cytoskeleton, although a second actin-binding domain (ABD2) in the rod domain facilitates this linkage (39,40). Deletion of the internal ABD is without obvious consequence in mice and humans, while deletion of the N-terminal ABD typically destabilises dystrophin and leads to BMD (26,31,41,42).

The role and importance of the central rod domain of dystrophin are not well understood. While this region of the protein could act as a spacer between the flanking protein interaction domains, studies in mice and humans illustrate that much of the rod domain is not critical to the function of dystrophin (26,41,43–45). There appears to be some redundancy in the dystrophin rod domain, and certain regions are dispensable. However, there is a requirement for at least some

---

**Figure 3.** Immunofluorescence staining of dystrophin–glycoprotein complex (DGC) members. The \( \textit{mdx} \) panel demonstrates a reduction in muscle membrane localization of \( \beta \)-dystroglycan (\( \beta \)-Dg), \( \alpha \)-, \( \beta \)-, and \( \gamma \)-sarcoglycans (\( \alpha \)-, \( \beta \)-, and \( \gamma \)-Sarc), \( \alpha \)-1-syntrophin (\( \alpha \)-1-Syn) and \( \alpha \)-dystrobrevin-2 (\( \alpha \)-Db2) resulting from the absence of dystrophin. DGC members are present at the membrane in muscles expressing full-length dystrophin, dystrophin/actinin chimeric protein or \( \Delta R4-R23 \) micro-dystrophin. Scale bar: 100 \( \mu \)m.
portions of the rod domain, since complete removal of this region resulted in a severe dystrophic phenotype in transgenic mdx mice (26). Deletion of 20 of the 24 spectrin-like repeats caused a variable impact on the dystrophic phenotype of mdx mice, ranging from nearly complete correction of dystrophic symptoms to severe myopathy (26). These data demonstrated that the organization of the rod domain plays a critical role in supporting the function of dystrophin and that individual spectrin-like repeats may be structurally distinct within the context of the rod. The inability of the dystrophin/actinin chimeric protein to prevent the dystrophic characteristics of mdx mice further suggests that the mere presence of any rod domain is not sufficient to support proper dystrophin function, and demonstrates that different spectrin-like repeats are not functionally equivalent. In addition, our data with the chimeric transgene support previous studies showing that restoration of the DGC is not sufficient to prevent muscular dystrophy in mdx mice (46). Both the dystrophin/actinin chimera and the four-repeat AR4-R23 micro-dystrophin were able to prevent contraction-induced injury and restore the DGC in transgenic mdx mice; however, only the latter construct prevented the development of morphological abnormalities in muscle. Therefore, the rod domain must do more than physically separate the protein binding domains at the N- and C-termini of dystrophin. Indeed, the rod domain may participate in force transduction out of the cell (24), and in providing mechanical stabilization of the sarcolemmal membrane during muscle contraction (6–9).

There are differences between the rod domains of dystrophin and α-actinin-2 that complicate interpretation of our results. The α-actinin-2 rod domain is capable of self-dimerization (47) and contains binding sites for Z-disk proteins, including titin, ALP, myotilin and FATZ (48–51), as well as several transmembrane receptors (e.g. NMDA, β1-integrin, and L-selectin (52–55). Dystrophin does not dimerize (56–58), and to date the full-length rod domain has only been shown to interact with actin (39). It is possible, therefore, that the rod domain of the dystrophin/actinin chimera could dimerize and/or bind other proteins and that these interactions may interfere with dystrophin function. This scenario may be unlikely, however, since two dystrophin/actinin chimeric proteins present as antiparallel dimers might be expected to sterically hinder the formation and localization of the DGC, which was not observed (Figs 3 and 4). It is also unlikely that the insertion of the α-actinin-2 rod domain changed the conformation of the dystrophin ABD such that it abolished actin binding. Dystrophin and α-actinin share their highest homology in the 246 N-terminal amino acids comprising the ABD, and are functionally interchangeable in this region (59–61). One study demonstrated actin binding in vitro from a dystrophin/α-actinin chimera in which the ABD of chicken α-actinin was replaced with the homologous region of dystrophin (60). Furthermore, deletion of the N-terminal ABD in mdx mice caused only a mild dystrophy that did not approach the pathology observed in dystrophin/actinin chimeric mice (62). The ability to protect mdx muscles from contraction-induced injury also suggests that the dystrophin/actinin chimera was at least partially able to anchor the actin cytoskeleton to the ECM (Fig 2D).

An interesting finding was the observation of a mild, dominant-negative effect on wild-type dystrophin arising from overexpression of the non-functional dystrophin/actinin chimeric transgene. We have previously observed dominant-negative effects of dystrophin expression in wild-type muscles only when the transgenic dystrophin was non-functional (42). In the present study, there was no detectable morphological pathology in the diaphragm of the transgenic/wild-type mice except for an increase in myofiber diameter. We speculate that this hypertrophy was due to the ability of the chimera to displace wild-type dystrophin from the sarcolemma such that a mild hypertrophy without necrosis was produced. A similar increase in myofiber diameter was observed in the quadriceps, along with an elevation of the percentage of centrally nucleated fibers. These differential degrees of morphological pathology in the quadriceps compared with the diaphragm were consistent with our previous studies of mini- and micro-dystrophins, in which diaphragm function was less dependent than limb muscles on dystrophin expression (26).

Finally, the combination of basic protein structure and function studies and the recent advances made in genomics

---

**Figure 4.** Western analysis of DGC members from muscle microsome extracts. The mdx panel demonstrates reduced expression of DGC proteins in muscles lacking dystrophin. DGC proteins were detected in membrane preparations from C57BL/10, chimera/mdx or AR4-R23 micro-dystrophin muscles. DGC protein abbreviations are as indicated in Figure 3. Fifty micrograms of total protein were loaded per lane.
will likely contribute to the rational design of proteins with novel functions that may be used for a variety of therapeutic purposes. Although de novo synthesis of such proteins may someday be successful (63), the most obvious approach to introduce novel function into proteins is through generation of chimeras that combine known functional elements from pre-existing proteins. The most successful examples of this to date have been attempts to alter specific amino acids in the active sites of enzymes in order to change substrate specificity (64). The dystrophin/actinin chimeric protein represents an attempt to rationally engineer a structural chimeric protein in a modular fashion, such that large domains were replaced instead of a few amino acids or peptides. We attempted to create a protein that could maintain dystrophin function and present fewer foreign epitopes to the immune system when delivered to muscles of a DMD patient. While this chimera was unable to meet these expectations, it nonetheless provided insight into the structure and function of the dystrophin rod domain.

**MATERIALS AND METHODS**

**Construction of chimeric transgene**

Dystrophin cDNA sequences (Genbank accession no. M18533) were obtained from pD1717#2, a full-length human cDNA cloned into the plasmid vector pBSX. The human α-actinin-2 rod domain (Genbank accession no. NM 001103.1) was amplified by PCR from IMAGE cDNA clone 627840 (Research Genetics, Inc.) The α-actinin-2 rod domain was fused to dystrophin hinges 1 and 4 by recombinant PCR using Pfu polymerase and conventional cloning techniques. The sequence-verified, 4722 bp chimeric cDNA (pDysAct) was ligated into an expression vector containing the human skeletal actin promoter, a splice acceptor from the SV40 VP1 intron, and tandem SV40 polyadenylation signals (35).

**Generation of mini- and micro-dystrophin transgenic mdx mice**

The pDysAct expression cassette was excised from the plasmid backbone and injected into wild-type C57BL/6 × SJL F2 hybrid embryos. Positive F0 mice were identified by PCR screening of extracted tail DNA (65) using deletion-specific primers, and then backcrossed onto the C57BL/10ScSn-Dmd<sup>mdx/J</sup> (Jackson Labs) background in a specific pathogen-free (SPF) facility for three generations prior to analysis. Controls for all studies used age- and sex-matched C57BL/10 mice (wild-type) or mdx and transgene-negative/mdx mice, unless explicitly stated otherwise (no differences were observed with any of the tests performed between mdx and transgene-negative/mdx littersmates).

**Histopathological analysis**

Muscles were excised from 6-month-old mice and immediately frozen in OCT embedding compound (Tissue-Tek) using liquid nitrogen-cooled isopentane. Five micrometer cryosections were stained with Gills #3 hematoxylin and eosin–phloxine (Fisher Scientific) and mounted with Permount (Fisher Scientific).

Stained sections were imaged with a Nikon E1000 microscope and a Spot-2 CCD camera. ImagePro software (Media Cybernetics) was used to determine muscle fiber diameters as well as the percentage of centrally nucleated myofibers compared with the total number of muscle fibers per section. To assess membrane permeability, 5-month-old mice were injected intraperitoneally with 600 µl Evans blue dye (10 mg/ml). After 18 hours, the mice were sacrificed, and 5 µm cryosections from the quadriceps were photographed by fluorescence microscopy for dye uptake.

**Analysis of protein expression**

**Rabbit polyclonal antisera.** Immunofluorescence was performed as previously described (36), using antibodies against the dystrophin N terminus (1:1200 dilution) (35), α-dystrobrevin-2 (1:100) (66) and α1-syntrophin (1:400) (67). Following primary antibody incubations and washes, slides were incubated with an Alexa-488-coupled goat anti-rabbit secondary antibody (1:1200; Jackson Labs), washed again, and overlaid with Vectashield (Vector Labs).

**Mouse monoclonal antisera.** The MOM Immunodetection kit (Vector Laboratories) was used for immunofluorescence using mouse monoclonal antibodies against β-sarcoglycan (1:100), β-sarcoglycan (1:50), γ-sarcoglycan (1:100) and δ-sarcoglycan (1:50) (all from Novocastra Laboratories). Manufacturer’s instructions were followed for all steps except acetone fixation, which was not performed.

**Western analysis.** Total muscle and microsome extract preparations and western transfer were performed as previously described (21,36). Total protein concentration was determined by the Bradford method (68). Western blots were incubated with rabbit polyclonal antisera to α-dystrobrevin-2 (1:50) (65) and α1-syntrophin (1:200) (66) or mouse monoclonal antibodies to the dystrophin C terminus (Dys-2, 1:500), α-sarcoglycan (1:50), β-sarcoglycan (1:50), γ-sarcoglycan (1:100) and δ-sarcoglycan (1:50) (all from Novocastra Laboratories). Following washes, blots were incubated with a 1:50,000 dilution of HRP-coupled donkey anti-mouse or anti-rabbit IgG secondary antibody (Jackson Labs) and developed using chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Pharmacia Biotech). Densitometry was performed using GeneSnap software (Syngene BioImaging).

**Contraction-induced injury**

The susceptibility of TA muscles to contraction-induced injury was determined by measuring the deficit in maximum isometric force (P<sub>0</sub>) resulting from stretches of maximally stimulated muscles (lengthening contractions) of 1.4 × optimal fiber length. Force deficits after one lengthening contraction were calculated as the difference between P<sub>0</sub> during the second (LC2) and first (LC1) lengthening contractions, expressed as a percentage of P<sub>0</sub> during LC1. Force deficits following two lengthening contractions were calculated as the difference between P<sub>0</sub> measured 1 minute after LC2 and P<sub>0</sub> during LC1, expressed as a percentage of P<sub>0</sub> during LC1 (6).
ACKNOWLEDGEMENTS

We thank Robert Maniker for excellent technical assistance, the University of Michigan transgenic animal model core, and Dr Stanley Froehner for providing antibodies to α1-syntrophin and α-dystrobrevin-2. This work was supported by grants from the Muscular Dystrophy Association (USA) and the National Institutes of Health, AR40864 (to J.S.C.).

REFERENCES


