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Cytoplasmic γ -Actin Expression in Diverse Animal Models of Muscular Dystrophy

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Abstract

We recently showed that cytoplasmic γ -actin (γ_{cyto} -actin) is dramatically elevated in striated muscle of dystrophin-deficient *mdx* mice. Here we demonstrate that γ_{cyto} -actin is markedly increased in golden retriever muscular dystrophy (GRMD), which better recapitulates the dystrophinopathy phenotype in humans. γ_{cyto} -Actin was also elevated in muscle from α -sarcoglycan null mice, but not in several other dystrophic animal models, including mice deficient in β -sarcoglycan, α -dystrobrevin, laminin-2, or $\alpha 7$ integrin. Muscle from mice lacking dystrophin and utrophin also expressed elevated γ_{cyto} -actin, which was not restored to normal by transgenic overexpression of $\alpha 7$ integrin. However, γ_{cyto} -actin was further elevated in skeletal muscle from GRMD animals treated with the glucocorticoid prednisone at doses shown to improve the dystrophic phenotype and muscle function. These data suggest that elevated γ_{cyto} -actin is part of a compensatory cytoskeletal remodeling program that may partially stabilize dystrophic muscle in some cases where the dystrophin-glycoprotein complex is compromised.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe, X-linked, progressive muscle disease affecting 1 in every 3,500 male births. Mutations in the 2.5 million base pair DMD gene typically result in loss of the protein dystrophin [1]. Dystrophin functions as part of a larger oligomeric protein complex named the dystrophin-glycoprotein complex (DGC), which includes the dystroglycan subcomplex, the sarcoglycan/sarcospan subcomplex, dystrobrevins and syntrophins [2,3]. The DGC spans the sarcolemma and links the actin cytoskeleton with the extracellular matrix of myofibers [2,3].

We demonstrated that the DGC is required for strong mechanical coupling of costameric actin filaments to the sarcolemma and confirmed that sarcolemmal actin is exclusively comprised of the γ_{cyto} -actin isoform [4]. Transgenic expression of the dystrophin homolog utrophin restored the stable association of costameric actin with the sarcolemma [5]. Most recently, we

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demonstrated that γ_{cyto} -actin protein levels were elevated 10-fold in striated muscle from the dystrophin-deficient *mdx* mouse [6]. We hypothesized that elevated γ_{cyto} -actin levels may contribute to a compensatory remodeling of the dystrophin-deficient costameric cytoskeleton [6]. While studies of *mdx* mice have greatly advanced our understanding of dystrophinopathies in humans, there are a number of important pathological differences between dystrophin-deficient humans and mice. Furthermore, mutations in genes encoding other DGC components or associated proteins have been implicated in clinically distinct forms of muscular dystrophy [2,3]. Finally, the complexity of the costameric protein network supports the hypothesis that additional proteins may form distinct mechanical linkages parallel to the DGC γ_{cyto} -actin axis. Therefore, it is of interest to determine whether the increased γ_{cyto} -actin measured in *mdx* muscle [6] manifests in other animal models of dystrophy or is unique to the *mdx* mouse.

Here, we report that γ_{cyto} -actin was also dramatically increased in the GRMD canine model of DMD and in a mouse model of limb girdle muscular dystrophy 2D, but not in six additional mouse lines relevant to DGC function. Moreover, daily treatment of GRMD dogs with 2 mg/kg prednisone was previously shown to improve muscle function and overall phenotype [7] and is reported here to result in a further increase in γ_{cyto} -actin protein levels. We suggest that increased levels of γ_{cyto} -actin may participate in remodeling the costamere to partially reinforce the mechanically weakened dystrophin-deficient sarcolemma.

MATERIALS AND METHODS

Animals

C57BL/6J (6 or 16 weeks old), C57BL/10ScSn-DMD^{mdx}/J (16 weeks old), and C57BL/6J-Lama2^{dy} mice (6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice deficient for α -sarcoglycan, β -sarcoglycan, α -dystrobrevin or $\alpha 7$ integrin were described previously [8–11] and were analyzed at 14–16 weeks of age. Transgenic mice overexpressing $\alpha 7$ integrin [12] were bred onto *mdx*, and *mdx*/utrophin $-/-$ double knockout (*mdx*/utrn $-/-$) backgrounds and analyzed at 6 weeks of age. Muscle biopsies from control and GRMD dogs were described previously [7]. Where indicated, control and GRMD dogs were treated daily with 2 mg/kg from either 1 week to 6 months or 1 week to 2 months of age.

Antibodies

Affinity purified polyclonal antibodies and monoclonal antibodies to γ_{cyto} -actin were previously characterized [6]. Antibodies to sarcomeric α -actin were from Sigma (St. Louis, MO). Peroxidase-labeled secondary antibodies against rabbit and mouse immunoglobulins were from Chemicon International, Inc. (Temecula, Ca) and Roche (Indianapolis, IN), respectively.

Muscle extracts and DNaseI chromatography

Frozen skeletal muscle was pulverized in a liquid nitrogen-cooled mortar and pestle, extracted in Tris-buffered saline and enriched for soluble monomeric actin by DNaseI affinity chromatography as described [6].

SDS-PAGE and western blot analysis

SDS-polyacrylamide gel electrophoresis, transfer to nitrocellulose and western blotting were performed as described [6].

Quantitative western blot analysis

γ_{cyto} -Actin immunoreactivity was detected from western blots by autoradiography using ^{125}I -labeled goat anti-mouse IgG as secondary (PerkinElmer, Boston, MA) and quantified

densitometrically as previously described [6]. Statistical comparisons between groups were performed by ANOVA followed by a non-paired Tukey post hoc test to determine significance and data are reported as the mean $\pm$ SD.

RESULTS AND DISCUSSION

To determine whether γ_{cyto} -actin is dramatically elevated in other dystrophin-deficient animals besides the *mdx* mouse, DNaseI-enriched muscle extracts from control and GRMD dogs were compared for γ_{cyto} -actin immunoreactivity by western blot analysis (Fig. 1A). In blind trials, all GRMD specimens were distinguished from controls based on increased γ_{cyto} -actin immunoreactivity (Fig. 1A). Quantitative western blot analysis (Fig. 1B) reported a 15-fold elevation in γ_{cyto} -actin levels of GRMD muscle, which was significantly different from control canine muscle.

Treatment of both human DMD patients and GRMD dogs with the glucocorticoid prednisone has been shown to slow disease progression and produce short-term functional improvements in dystrophic muscle [7,13–15]. To determine if prednisone treatment affected the levels of γ_{cyto} -actin in skeletal muscle from dystrophin-deficient dogs, four controls and four GRMD dogs were administered daily treatment with 2 mg/kg prednisone from one week to six months of age. Two additional GRMD dogs were treated with 2 mg/kg prednisone from one week to two months of age. While prednisone did not affect γ_{cyto} -actin levels in control skeletal muscle, γ_{cyto} -actin levels in prednisone-treated GRMD dogs were significantly increased compared to untreated GRMD dogs (Figs. 1A and B). There was no difference in cytoplasmic γ_{cyto} -actin levels between the GRMD dogs treated with prednisone for two or six months. The pooled data indicate that γ_{cyto} -actin protein levels in skeletal muscle from prednisone-treated GRMD dogs were increased 20-fold over skeletal muscle from control dogs (Fig. 1B).

γ_{cyto} -Actin levels were also evaluated in seven additional mouse models of dystrophy relevant to DGC function. α - and β -sarcoglycans are integral subunits of the sarcoglycan-sarcospan complex that are thought to stabilize the interactions of β -dystroglycan with dystrophin and α -dystroglycan [16]. Mutations in α - and β -sarcoglycan cause forms of limb-girdle muscular dystrophy in humans and progressive muscular dystrophy when knocked out in mice [16]. Surprisingly, quantitative western blot analysis reported significantly elevated γ_{cyto} -actin levels in DNaseI-enriched muscle extracts from α -sarcoglycan null mice but not from β -sarcoglycan null animals (Fig. 2). While the molecular basis for this difference is presently unclear, both human patients and mouse models ablated for different sarcoglycan subunits present with distinct molecular, cellular and/or tissue phenotypes [9,16,17]. Consistent with the improved phenotype and significantly elevated γ -actin in prednisone-treated GRMD muscle compared to untreated GRMD muscle (Fig. 1), the greater elevation of γ -actin levels in α -sarcoglycan null muscle correlated with their less severe phenotype as compared to β -sarcoglycan deficient animals [9].

α -Dystrobrevin is a cytoplasmic DGC component that binds dystrophin and the sarcoglycan complex. Ablation of α -dystrobrevin in mice results in a progressive myopathy with increased fiber necrosis and regeneration but without the compromised sarcolemmal integrity observed in dystrophin-deficient muscle [10]. Quantitative western blot analysis revealed no difference in γ_{cyto} -actin levels of DNaseI-enriched muscle extracts from control and α -dystrobrevin null mice (Fig. 3). Two-hybrid screens have identified synemin [18] and syncoilin [19,20] as α -dystrobrevin binding proteins. Both are structurally related to intermediate filament proteins and interact with the muscle-specific intermediate filament protein desmin [18–20]. Synemin also directly binds to α -actinin [21] and vinculin [22] to provide additional mechanical linkages between the DGC and muscle cytoskeleton. Thus, it appears that mechanical coupling of the

DGC to the intermediate filament cytoskeleton is specifically disrupted in α -dystrobrevin null muscle without effect on the dystrophin/ γ_{cyto} -actin linkage.

The dystrophic *dy/dy* mouse is deficient in laminin-2 [23], but shows normal sarcolemmal expression of the DGC [24], retention of costameric actin on peeled sarcolemma [4], and little evidence of sarcolemma disruption prevalent in *mdx* muscle [25]. While *dy/dy* mice exhibit substantial muscle cell necrosis and regeneration [26], we observed similar γ_{cyto} -actin levels in skeletal muscle from control and dystrophic *dy/dy* mice (Fig. 4A). Recent studies [27–29] indicate that the DGC and the laminin-binding $\alpha 7 \beta 1$ integrin perform overlapping functions in skeletal muscle. However, western blot analysis demonstrated that γ_{cyto} -actin was not altered in dystrophic $\alpha 7$ integrin null muscle compared to control (Fig. 4B). Finally, mice deficient in both dystrophin and utrophin (*mdx/utrn* $-/-$) present with a more severe DMD-like phenotype [30,31], which is partially rescued by transgenic overexpression of $\alpha 7$ integrin [12]. Therefore, we examined how the combined absence of dystrophin and utrophin as well as $\alpha 7$ integrin overexpression affected γ_{cyto} -actin protein levels. γ_{cyto} -Actin immunoreactivity was similarly increased in *mdx* and *mdx/utrn* $-/-$ muscle compared to control regardless of the level of $\alpha 7 \beta 1$ integrin expression (Fig. 4C). Taken together, the results of Figs 2–4 indicate that elevated γ_{cyto} -actin is neither unique to dystrophin deficiency, nor is it a feature common to all forms of skeletal myopathy including those directly involving the DGC.

The actin binding proteins γ -filamin, talin, vinculin, plectin and utrophin are all upregulated and/or redistributed to costameres in dystrophin-deficient muscle [4,5,32–34]. With the exception of utrophin, all of these proteins interact with integrins *in vivo* and $\alpha 7 \beta 1$ integrin is also upregulated in dystrophin-deficient muscle [35,36]. Therefore, we previously suggested that γ_{cyto} -actin levels may be elevated in dystrophin-deficient muscle as part of a compensatory remodeling program to fortify the weakened sarcolemmal cytoskeleton through recruitment of parallel mechanical linkages [6]. Because dystrophy persists, these parallel linkages are either not completely redundant with the DGC, or the compensatory upregulation/recruitment is not maximal. In support of the latter possibility, transgenic overexpression of $\alpha 7$ integrin has been shown to partially compensate for the combined deficiency of dystrophin and utrophin [12]. In support of the former possibility, we have shown that γ_{cyto} -actin protein levels were not altered in mice deficient in $\alpha 7$ integrin or in animals overexpressing $\alpha 7$ integrin on wild type, *mdx* or *mdx/utrn* $-/-$ double knockout backgrounds. We conclude that endogenous upregulation of γ_{cyto} -actin and $\alpha 7$ integrin are two factors that may limit the effectiveness of compensatory cytoskeletal remodeling in the dystrophin-deficient *mdx* mouse.

As shown for *mdx* mice [6], γ_{cyto} -actin was significantly elevated in skeletal muscle of the GRMD model of dystrophin-deficiency. Utrophin is upregulated in both animals [37–40] suggesting that a similar compensatory remodeling program is activated in dystrophin-deficient mice and dogs. However, GRMD dogs suffer a severe and rapidly progressing muscle disease with premature lethality more closely resembling DMD in humans while *mdx* mice exhibit a milder phenotype with no dramatic reduction in lifespan [3]. Thus, it appears that compensatory cytoskeletal remodeling is not as effective in dystrophin-deficient dogs compared to mice, which may be due to the greater mechanical stresses imposed on the sarcolemma of larger animals with correspondingly larger myofibers [30,41]. More interestingly, we demonstrated that daily treatment with prednisone correlated with a significant elevation in γ_{cyto} -actin levels in GRMD skeletal muscle as compared to muscle from non-treated GRMD dogs. These prednisone dosages were previously shown to improve dystrophic histopathology in GRMD dogs and increased isometric extensor forces towards normal [7]. Prednisone treatment also resulted in decreased fetal myosin heavy chain expression indicating reduced regeneration in treated versus non-treated GRMD dogs [7]. Our current results suggest that pharmacologically-induced upregulation of γ_{cyto} -actin correlates with improvement of the dystrophin-deficient phenotype. Furthermore, studies investigating

the mechanism by which prednisone increases γ_{cyto} -actin in dystrophin-deficient skeletal muscle may have clinical relevance.

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Figure 1. γ_{cyto} -Actin levels in dystrophin-deficient GRMD skeletal muscle

(A) Western blots loaded with DNaseI –enriched muscle extracts from untreated control (Con), GRMD, and prednisone-treated control or GRMD animals were stained for γ_{cyto} -actin and α -actin. (B) Quantitation of γ -actin immunoreactivity (OD, arbitrary units) in DNaseI eluates from muscle in control ($n = 4$), GRMD ($n=7$), prednisone-treated control ($n=4$), and prednisone-treated GRMD animals ($n=6$). γ_{cyto} -Actin was significantly elevated in GRMD muscle compared to control ($p<0.0005$) and in muscle from prednisone-treated GRMD compared to untreated GRMD animals ($p<0.05$).

Figure 2. γ cyto-Actin levels in α - and β -sarcoglycan null muscle

(A) Western blots loaded with DNaseI-enriched muscle extracts from 14–16 week-old control, dystrophin-deficient *mdx*, α -sarcoglycan deficient (*asg* $^{-/-}$) and β -sarcoglycan deficient (*bsg* $^{-/-}$) mice stained for γ - and α -actin. (B) Quantitation of γ cyto-actin immunoreactivity (OD, arbitrary units) in control ($n=3$), *mdx* ($n=3$), *asg* $^{-/-}$ ($n=4$) and *bsg* $^{-/-}$ ($n=4$) muscle where the asterisks indicate a significant difference from control ($p < 0.0005$).

Figure 3. γ cyto-Actin levels in α -dystrobrevin null muscle

(A) Western blots loaded with DNaseI-enriched muscle extracts from 14–16 week-old dystrophin-deficient *mdx*, control, and α -dystrobrevin deficient (*adbn*^{-/-}) mice stained for γ - and α -actin. (B) Quantitation of γ cyto-actin immunoreactivity (OD, arbitrary units) in *mdx* (n=3), control (n=3), and *adbn*^{-/-} (n=3) muscle where the asterisk indicates significance between *mdx* and the other groups ($p < 0.0000005$).

Figure 4. γ_{cyto} -Actin levels in other dystrophic animal models

(A) α - and γ_{cyto} -actin in DNaseI-enriched muscle extracts of 6 week-old control (Con) and laminin-2 deficient *dy/dy* mice. (B) α - and γ_{cyto} -actin in DNaseI-enriched muscle extracts of 14–16 week-old control (Con) and $\alpha 7$ integrin deficient ($\alpha 7^{-/-}$) mice. (C), α - and γ_{cyto} -actin in DNaseI-enriched muscle extracts of 6 week-old control, *mdx*, *mdx/utrn*^{-/-}, and mice overexpressing $\alpha 7$ integrin ($\alpha 7$ BX2) on *mdx*, *mdx/utrn*^{-/-} and control backgrounds.