Talin, vinculin and DRP (utrophin) concentrations are increased at mdx myotendinous junctions following onset of necrosis

Douglas J. Law1*, David L. Allen1 and James G. Tidball1,2

1Department of Physiological Science and 2Jerry Lewis Neuromuscular Research Center, University of California, 405 Hilgard Avenue, Los Angeles, CA 90024-1527, USA

*Author for correspondence at present address: School of Biological Sciences, University of Missouri, 5100 Rockhill Rd, Kansas City, MO 64110-2499, USA

SUMMARY

Duchenne muscular dystrophy (DMD) and the myopathy seen in the mdx mouse both result from absence of the protein dystrophin. Structural similarities between dystrophin and other cytoskeletal proteins, its enrichment at myotendinous junctions, and its indirect association with laminin mediated by a transmembrane glycoprotein complex suggest that one of dystrophin’s functions in normal muscle is to form one of the links between the actin cytoskeleton and the extracellular matrix. Unlike Duchenne muscular dystrophy patients, mdx mice suffer only transient muscle necrosis, and are able to regenerate damaged muscle tissue. The present study tests the hypothesis that mdx mice partially compensate for dystrophin’s absence by upregulating one or more dystrophin-independent mechanisms of cytoskeleton-membrane association. Quantitative analysis of immunoblots of adult mdx muscle samples showed an increase of approximately 200% for vinculin and talin, cytoskeletal proteins that mediate thin filament-membrane interactions at myotendinous junctions. Blots also showed an increase (143%) in the dystrophin-related protein called utrophin, another myotendinous junction constituent, which may be able to substitute for dystrophin directly. Muscle samples from 2-week-old animals, a period immediately preceding the onset of muscle necrosis, showed no significant differences in protein concentration between mdx and controls. Quantitative analyses of confocal images of myotendinous junctions from mdx and control muscles show significantly higher concentrations of talin and vinculin at the myotendinous junctions of mdx muscle. These findings indicate that mdx mice may compensate in part for the absence of dystrophin by increased expression of other molecules that subsume dystrophin’s mechanical function.

Key words: dystrophin, myotendinous junction, talin, vinculin, utrophin

INTRODUCTION

Dystrophin, the protein absent in Duchenne muscular dystrophy (DMD) (Hoffman et al., 1987a), is thought to function as a structural link between the muscle cell cytoskeleton and the extracellular matrix. This expectation is supported by the structural similarities between dystrophin and the cytoskeletal proteins α-actinin and spectrin (Byers et al., 1989; Hammonds, 1987; Koenig et al., 1988). Also, dystrophin is located subjacent to the muscle cell membrane (Arahata et al., 1988; Cullen et al., 1990; Watkins et al., 1988), and biochemical studies have shown binding affinity between dystrophin’s N-terminal domain and actin (Hemmings et al., 1992; Way et al., 1992), and between part of its C-terminal domain and a transmembrane glycoprotein complex (Suzuki et al., 1992). One subunit of that dystrophin-associated glycoprotein (DAG) complex can bind to the basement membrane component laminin (Ervasti and Campbell, 1993; Ibraghimov-Beskrovnaya et al., 1992), thereby completing the transmembrane association of structural proteins.

Dystrophin is also absent in the mdx mouse (Hoffman et al., 1987). However, unlike the progressive muscle wasting seen in DMD, mdx muscle suffers only a transient period of degeneration at 3-4 weeks of age, followed by nearly complete regeneration and restoration of function (Anderson et al., 1988; Coulton et al., 1988; Cullen and Jaros, 1988). This regeneration suggests that mdx mice are able to compensate for dystrophin’s absence, possibly by upregulating the expression of one or more proteins that can function similarly to dystrophin. For example, the dystrophin-related protein (DRP) utrophin (Love et al., 1989), an autosomal gene product that is extremely similar in primary structure to dystrophin (Tinsley et al., 1992), can bind to the DAG complex and may substitute for dystrophin in some mdx tissue (Matsumura et al., 1992).

We propose the alternative hypothesis that regeneration of adult mdx muscle is associated with upregulation of proteins that form transmembrane assemblies that normally function in parallel with dystrophin. One such parallel assembly comprises vinculin, talin, integrin, and one of several integrin-binding extracellular matrix proteins (Burridge and Mangeat, 1984; Horwitz et al., 1986; Hynes, 1987). The DAG-dystrophin based assembly and integrin-talin based assembly are similar in that they are both concentrated at myotendinous junctions (MTJs), the principal sites of force transmission across the
muscle cell membrane (Bozyczko et al., 1989; Byers et al., 1991; Samitt and Bonilla, 1990; Shear and Bloch, 1985; Shimizu et al., 1989; Swasdison and Mayne, 1989; Tidball et al., 1986), and they both form links between the actin cytoskeleton and extracellular structural proteins. The present study tests the upregulation of this parallel mechanism for cytoskeleton-membrane association by answering the following questions: (1) does adult mdx muscle have more vinculin and talin than control muscle? (2) if so, is the increase in protein localized to the MTJ? and (3) if vinculin and talin are elevated, is this a general feature of mdx muscle at all ages or rather a change observed only following the cellular damage that results from dystrophin’s absence?

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies to vinculin and talin were purchased from Sigma (anti-human vinculin, clone hVIN-1; anti-chicken talin, clone 8d4). Polyclonal antiserum made in rabbit against a TrpE fusion protein consisting of the C-terminal domain of the dystrophin-related protein (DRP) was a gift from Dr Tejvir Khurana (Khurana et al., 1990). Affinity-purified polyclonal rabbit anti α-actinin, made against purified chicken gizzard α-actinin, was a gift from Dr Keith Burridge.

Immunoblots

Tibialis anterior muscles from one hindlimb of adult (11-month) or 2-week-old mdx and C57BL/10Snj control mice were dissected out fresh, and frozen immediately in liquid nitrogen. These ages were selected for analysis because they represent stages in the mdx pathophysiology before the onset of muscle necrosis and inflammation (2 weeks) or following complete regeneration of the muscle (11 months). Muscles undergoing necrosis were not analyzed because they contain a large volume fraction of inflammatory cells and necrotic fibers at that stage. C57BL/10Snj was used as control because it is the strain from which mdx mutants were derived. The mdx mutation is a point mutation in the dystrophin gene (Sicinski et al., 1989) and is unknown to affect any other gene. Samples were homogenized in SDS-PAGE sample buffer (Laemmli, 1970) without Bromphenol Blue, and soluble protein concentrations determined by UV spectrophotometry. Gels were loaded with 50 μg total protein per lane, and samples separated electrophoretically. Proteins were transferred to nitrocellulose (Burnette, 1981), and the transfer gels stained with Coomassie Blue to ensure uniform transfer. In addition, uniformity of sample loading was checked on identically-loaded gels that were subsequently stained with Coomassie Blue. Nitrocellulose blots were blocked for 2-14 hours in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% NaN3 (buffer A), with the addition of 0.2% gelatin, 0.05% Tween-20, and 3% nonfat dry milk. Blots were then overlaid for 90 minutes with antisera diluted in buffer A, as follows: anti-vinculin, 1:200; anti-talin, 1:40; anti-DRP, 1:250; anti α-actinin, 1:20. Blots were washed 6x 10 minutes in the above blocking buffer with the nonfat dry milk concentration reduced to 0.3%, and then overlaid 60-90 minutes with alkaline phosphatase-conjugated, species-specific secondary antibodies (Sigma, St Louis, MO) diluted 1:2000 in buffer A with 0.2% gelatin, 0.05% Tween-20, and 5% inactivated horse serum. After 6x 10 minute washes, bound antibodies were detected by incubating the blots with 0.3 mg/ml nitro blue tetrazolium and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M NaHCO3, 1.0 mM MgCl2, pH 9.8. Blots were quantified using a laser densitometer (Ultrascan, Pharmacia, Piscataway, NJ). Samples from 3 mdx and 3 control animals were run on the same gel. Integrated absorbencies for the immunostained bands in each lane were determined, and mean values for mdx and control muscles were compared for each blot using Student’s t-test.

Immunofluorescence microscopy

The tibialis anterior muscles that were not used to make samples for SDS-PAGE were dissected out of mdx and control animals, frozen in liquid isopentane cooled in liquid nitrogen, and stored at −80°C in isopentane. Samples were warmed to ~20°C in a cryostat, sectioned longitudinally at 10 μm thickness, and sections mounted on gelatin-coated slides. Sections were blocked for 1 hour in buffer A with 0.2% gelatin, 0.05% Tween-20, and 0.3% nonfat dry milk, rinsed twice for 2 minutes in buffer A, then incubated for 3 hours in anti-vinculin or anti-talin, diluted 1:20 in buffer A. Sections were rinsed 2x 2 minutes in buffer A, incubated for 30 minutes in the above blocking buffer, then rinsed twice more in buffer A. The sections were then incubated for 1 hour in TRITC-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL) diluted 1:250 in buffer A, then rinsed 3x 5 minutes in buffer A. Finally, sections were rinsed for 2 minutes in distilled water, mounted with aqueous medium, and photographed using an Olympus BH-2 microscope equipped with epifluorescence and Nomarski differential interference contrast. Controls consisted of sections processed identically, except buffer A was used in place of primary antibody.

The relative amounts of vinculin and talin present at myotendinous junctions were estimated using digital analysis of confocal images of the above immunolabeled sections of mdx and control muscle. Sections were examined briefly using a Nikon Optiphot microscope equipped with conventional epifluorescence, to identify fields that included labeled MTJs. Confocal images of the MTJs were obtained with a Molecular Dynamics (Sunnyvale, CA) laser confocal microscope, using a ×60 oil-immersion objective with a numerical aperture of 1.4. Serial images of 1.0 μm thickness were examined, and the image halfway between the top and bottom of the labeled MTJ was chosen for quantitative analysis. This was done to eliminate possible variation in signal intensity due to the inclusion of images of the top or bottom surfaces of a muscle fiber. Three transects were drawn across the labeled MTJ interface, from the extracellular space into the interior of the muscle cell. The intensity of the fluorescent signal was measured at each pixel along a given transect, and the peak values were recorded. In addition, the intensity value at the intracellular end of each transect was recorded, to estimate background fluorescence intensity. Peak intensity values and background values were obtained for the MTJs of six muscle cells from each of three mdx mice, and an equal number of values obtained from three C57 control mice. This yielded a total of 54 peak values from mdx MTJs, and the same number from controls. All settings, including laser wattage and photomultiplier tube voltage, were identical for all specimens. Means of the peak values and the background values from the two groups of animals were compared using Student’s t-test. Statistical significance was assigned to differences in means for which P<0.05.

RESULTS

The specificities of the antibodies used in the present study are shown on western blots of normal adult mouse skeletal muscle samples (Fig. 1). The anti-vinculin labeled both the 117 kDa vinculin band, and a 135 kDa band that has been previously identified as meta-vinculin, a vinculin-related protein (Byrne et al., 1992). Anti-talin labeled a single band at 235 kDa, and anti-α-actinin a single band at 100 kDa. The anti-DRP labeled a doublet in the control muscle sample at approximately 400 kDa (Fig. 1b). The stronger, lower band is dystrophin, with which this polyclonal antiserum cross-reacts (Khurana et al., 1991), and the weaker, higher band is the DRP, which has been
Increased talin, vinculin and DRP in *mdx*

shown to migrate slightly slower than dystrophin in SDS-PAGE (Ohlendieck et al., 1991).

Blots of skeletal muscle samples from adult *mdx* and control animals (Fig. 2) showed increased concentrations of vinculin, talin, and DRP in the *mdx* muscle relative to control levels. There was no apparent difference in the concentration of α-actinin in the adult *mdx* samples relative to controls, indicating that the elevations in the other three proteins are specific, and not due to a more general upregulation of cytoskeletal protein expression in *mdx* muscle. Blots of muscle samples from 2-week-old mice showed no systematic differences between *mdx* and controls, regarding concentration of any of the above proteins (not shown). These observations were confirmed by quantitative comparisons of the immunoblots of 2-week and 11-month muscles, using scanning densitometry. The results (Table 1) indicate statistically-significant increases in the concentrations of vinculin (170%), meta-vinculin (311%), talin (218%), and DRP (143%) in muscles from adult *mdx* mice relative to control concentrations, and no significant differences in concentration of any of the proteins from the 2-week mice.

Frozen sections of adult *mdx* and control mouse hindlimb muscle were immunolabeled for talin and vinculin to determine if the increased amounts of these proteins seen in immunoblots were localized at the MTJ. The results of conventional fluorescence microscopy showed that MTJs were the most numerous and most intensely labeled structures for talin and vinculin in both C57 and *mdx* muscle (Figs 3 and 4). Sections processed by identical labeling procedures but from which primary antibodies were eliminated showed no MTJ labeling. Measurements of maximum fluorescence intensity at MTJs in single confocal images demonstrated a 42% increase in intensity in *mdx* muscles labeled for vinculin, and a 36% increase in talin, compared with the values obtained from control muscles (Table 2). The mean values for background fluorescence intensity from these samples were not significantly different, indicating that the differences in maximum MTJ signal are due to differences in the amount of antigen present in the confocal section, rather than to a non-specific increase in immunoreactivity of the *mdx* tissue compared to controls. The difference in the magnitude of the increases seen

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**Table 1. Protein concentration, relative to 100% of values from C57 control samples (mean ± s.e.m.)**

<table>
<thead>
<tr>
<th>Protein</th>
<th>2-week C57</th>
<th>2-week <em>mdx</em></th>
<th>Adult C57</th>
<th>Adult <em>mdx</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinculin</td>
<td>100±19</td>
<td>86±14</td>
<td>100±18</td>
<td>270±5</td>
</tr>
<tr>
<td>Metavinculin</td>
<td>100±37</td>
<td>85±26</td>
<td>100±44</td>
<td>411±8</td>
</tr>
<tr>
<td>Talin</td>
<td>100±6</td>
<td>82±10</td>
<td>100±18</td>
<td>318±17</td>
</tr>
<tr>
<td>DRP</td>
<td>100±21</td>
<td>107±13</td>
<td>100±14</td>
<td>243±12</td>
</tr>
<tr>
<td>α-actinin</td>
<td>100±7</td>
<td>94±10</td>
<td>100±10</td>
<td>104±2</td>
</tr>
</tbody>
</table>

*P < 0.05.
n = 3.

**Table 2. Fluorescence intensity at MTJs in single confocal sections (mean ± s.e.m.; arbitrary units)**

<table>
<thead>
<tr>
<th>Protein</th>
<th>mdx <em>I</em>max</th>
<th>C57 <em>I</em>max</th>
<th>mdx <em>I</em>0</th>
<th>C57 <em>I</em>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-vinculin</td>
<td>142±6</td>
<td>100±8</td>
<td>85±9</td>
<td>100±8</td>
</tr>
<tr>
<td>Anti-talin</td>
<td>136±4</td>
<td>100±4</td>
<td>100±7</td>
<td>100±14</td>
</tr>
</tbody>
</table>

*I*max = maximum intensity.

*I*0 = background intensity.
n = 54.

*P < 0.05.
in the confocal data compared to those from densitometry of the blots likely resulted from differences in the conditions of binding between antibodies and antigens in the two methods, and thus cannot be interpreted.

**DISCUSSION**

Dystrophin deficiency in *mdx* and DMD muscle is the primary defect that gives rise to muscle fiber damage, characterized in early pathological stages by Z-band streaming, dilation of sarcoplasmic reticulum and myofibril disorganization (Cullen and Jaros, 1988; Torres and Duchen, 1987). This structural damage has been related to an increase in intracellular calcium concentrations resulting from calcium entry through membrane tears (Mokri and Engel, 1975) or leaky calcium channels (Turner et al., 1993). Supranormal calcium concentrations are expected to activate intracellular calcium-dependent proteases (calpains), which can then initiate much of the degenerative processes observed in dystrophin-deficient muscle (Bodeneister and Engel, 1978; Gorospe and Hoffman, 1992). If this general mechanism associating dystrophin deficiency with muscle cell death is true, then two possible hypotheses can be proposed for the mechanism by which *mdx* mice are able to recover from the onset of muscle necrosis: (1) *mdx* muscle compensates for dystrophin by increased expression of analogous proteins; or (2) *mdx* muscle arrests necrosis by controlling calpain-mediated proteolysis.

Results of previous studies (Man et al., 1991; Matsumura et al., 1992) have supported the first hypothesis by showing that DRP, a protein that is structurally similar to dystrophin, is present at elevated concentrations in *mdx* muscle. Strong evidence that DRP can ‘rescue’ dystrophin-deficient muscle fibers comes from Matsumura et al. (1992), who showed that the increase in DRP concentration in cardiac and small-caliber...
skeletal muscle fibers is accompanied by near-normal levels of expression of the dystrophin-associated glycoproteins (DAG). However, the increase in DRP seen in quadriceps muscles did not result in a similar preservation of control levels of DAG expression. Instead, as demonstrated in previous studies (Ervasti et al., 1990; Ohlendieck and Campbell, 1991), the DAG concentration was greatly reduced in the mdx hindlimb muscles. If the normal function of dystrophin requires its interaction with the DAGs, then the increase in DRP concentration in mdx muscle shown in the previous (Man et al., 1991; Matsumura et al., 1992) and current studies may be sufficient to compensate for dystrophin’s absence in the small-caliber and cardiac muscle fibers in mdx mice (Matsumura et al., 1992), but not in other mdx skeletal muscles.

We propose that the increased concentration of vinculin and talin, demonstrated here, also may be a mechanism by which mdx mice can compensate for the absence of dystrophin. Vinculin and talin are well-characterized components of the protein complex that anchors actin-containing stress fibers to the cell membrane at focal adhesions of cultured fibroblasts (Beckerle and Yeh, 1990; Otto, 1990). Vinculin associates indirectly with actin, probably via the actin-binding protein tensin (Bockholt et al., 1992; Davis et al., 1991). Vinculin also binds to talin (Burridge and Mangeat, 1984), which in turn associates with members of the integrin family of extracellular matrix receptors (Horwitz et al., 1986). Thus, this mechanism is analogous to the dystrophin-based mechanism, in that they both provide links between the actin cytoskeleton and extracellular matrix. Both mechanisms are normally present at MTJs (Samitt and Bonilla, 1990; Shear and Bloch, 1985; Shimizu et al., 1989; Tidball et al., 1986), so it is likely that they function in parallel in normal muscle. The two mechanisms may not be completely independent, as indicated by a recent study showing binding affinity between dystrophin and talin in vitro (Senter et al., 1993). However, in the absence of dystrophin, talin and vinculin could retain the ability to link the actin cytoskeleton with the membrane, through integrins.

Our results showing no difference in vinculin or talin concentrations in muscles from 2-week-old mice indicate that the vinculin-talin mechanism has not yet been upregulated at this stage in the mouse’s life. This indicates that the compensation is not a direct response to the absence of dystrophin, because dystrophin is also absent in those young animals, and shows that the increased concentration of talin and vinculin is not constitutive in mdx, but rather is a condition that develops later in mdx pathology. It may be a response either to the cellular injury that begins at about 3 weeks, or to part of the process of muscle regeneration. It is also noteworthy that this compensatory mechanism does not exist in DMD muscle. On the contrary, vinculin concentration in DMD muscle is 50% lower than in controls (Minetti et al., 1992). These findings suggest that increased expression of vinculin and talin may be necessary to enable successful regeneration in mdx muscle, while DMD muscle, without such a compensatory mechanism, undergoes progressive necrosis.

Although the data presented here support the hypothesis that increased concentrations of talin and vinculin may compensate for dystrophin’s absence from mdx muscle, the compensation is not complete, because intracellular Ca²⁺ concentrations remain elevated in adult mdx mice (Turner et al., 1988). This suggests that talin and vinculin may correct for deficiencies in cytoskeleton associations with the membrane in dystrophic muscle, without compensating for dystrophin deficiency causing increased intracellular calcium concentration. Thus, any pathology resulting from elevated calcium concentration in dystrophic muscle must be controlled by an additional mechanism.

Recent investigations that have examined potential mechanisms by which calcium-dependent proteolysis in dystrophin-deficient muscle can be controlled have focused on two possibilities: (1) calcium may be buffered in mdx muscle (Gailly et al., 1993); or (2) calpains may be inhibited (Spencer and Tidball, 1992). Current evidence offers some support for elevated calcium buffering by parvalbumin, which is expressed at higher levels in mdx muscle than in controls (Gailly et al., 1993). In spite of this, parvalbumin concentrations remain lower in mdx muscle (Sano et al., 1990), so the role of parvalbumin in mdx pathology remains unclear. Other calcium-binding proteins, such as calmodulin, may be more important in this Ca²⁺ buffering role. Calmodulin concentrations are elevated in dystrophic hamster muscle (Klamut et al., 1987), although this dystrophy does not result from dystrophin deficiency. Calmodulin levels have not yet been analyzed in mdx muscle.

The possibility that calcium-dependent necrosis may be controlled by regulating calpain activity has been supported by observations that leupeptin, an inhibitor of calpain and other thiol proteases, can inhibit proteolysis in mdx muscle (Turner et al., 1993). Furthermore, recent measurements of calpain concentration and activity in adult mdx and control muscle show that although calpain is present in higher concentrations in mdx muscle, the net Ca²⁺-dependent proteolytic activity of mdx muscle extracts is lower than in controls (Spencer and Tidball, 1992). Thus, inhibition of calpain activity may be an additional means by which mdx muscle arrests the necrotic process.

We conclude that mdx muscle can compensate for some structural defects associated with dystrophin deficiency by increasing the concentration of proteins that serve analogous functions in mediating cytoskeletal-membrane associations. However, these analogous proteins are not capable of compensating for the defect that gives rise to increased intracellular calcium. Further studies of the functional domains of dystrophin may help determine whether the region of the molecule that mediates cytoskeletal-membrane associations is distinct from that involved in control of intracellular calcium concentrations.

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