Comparative analysis of Dp427-deficient mdx tissues shows that the milder dystrophic phenotype of extraocular and toe muscle fibres is associated with a persistent expression of β-dystroglycan

Paul Dowling, James Lohan, Kay Ohlendieck[1)]
Department of Biology, National University of Ireland, Maynooth, County Kildare/Ireland

Received October 23, 2002
Received in revised version January 22, 2003
Accepted January 22, 2003

Dystrophin – dystroglycan – mdx – muscular dystrophy – calcium handling

The cell biological hypothesis of Duchenne muscular dystrophy assumes that deficiency in the membrane cytoskeletal element dystrophin triggers a loss in surface glycoproteins, such as β-dystroglycan, thereby rendering the sarcolemmal membrane more susceptible to micro-rupturing. Secondary changes in ion homeostasis, such as increased cytosolic Ca^{2+} levels and impaired luminal Ca^{2+} buffering, eventually lead to Ca^{2+}-induced myonecrosis. However, individual muscle groups exhibit a graded pathological response during the natural time course of x-linked muscular dystrophy. The absence of the dystrophin isofom Dp427 does not necessarily result in a severe dystrophic phenotype in all muscle groups. In the dystrophic mdx animal model, extraocular and toe muscles are not as severely affected as limb muscles. Here, we show that the relative expression and sarcolemmal localization of the central trans-sarcolemmal linker of the dystrophin-glycoprotein complex, β-dystroglycan, is preserved in mdx extraocular and toe fibres by means of two-dimensional immunoblotting and immunofluorescence microscopy. Thus, with respect to improving myology diagnostics, the relative expression levels of β-dystroglycan appear to represent reliable markers for the severity of secondary changes in dystrophin-deficient fibres. Immunoblotting and enzyme assays revealed that mdx toe muscle fibres exhibit an increased expression and activity of the sarcoplasmic reticulum Ca^{2+}-ATPase. Chemical crosslinking studies demonstrated impaired calsequestrin oligomerization in mdx gastrocnemius muscle indicating that abnormal calsequestrin clustering is involved in reduced Ca^{2+} buffering of the dystrophic sarcoplasmic reticulum. Previous studies have mostly attributed the sparing of certain mdx fibres to the special protective properties of small-diameter fibres. Our study suggests that the rescue of dystrophin-associated glycoproteins, and possibly the increased removal of cytosolic Ca^{2+} ions, might also play an important role in protecting muscle cells from necrotic changes.

Introduction

Duchenne muscular dystrophy represents the most common inherited neuromuscular disorder and the most prevalent and lethal gender-specific genetic disease of childhood (Emery, 2002). An established animal model of x-linked muscular dystrophy is the mdx mouse (Bulfield et al., 1984), which is missing the dystrophin isoform Dp427 due to a point mutation (Sicinski et al., 1989). Although not a perfect replica of the human pathology, this dystrophic animal model exhibits many cellular and molecular abnormalities seen in x-linked human muscular dystrophy. Skeletal muscle fibres from mdx mice exhibit elevated levels of serum creatine kinase (Bulfield et al., 1984), are more susceptible to osmotic shock (Menke and Jockusch, 1991), exhibit segmental necrosis (Torres and Duchen, 1987), show stretch-induced injury (Lynch et al., 2000), as well as abnormal excitation-contraction coupling (DeLuca et al., 2001) and a drastic reduction in dystrophin-associated glycoproteins (Culligan et al., 2001). This makes it a suitable disease model (Allamand and Campbell, 2000), widely employed for testing novel therapeutic strategies such as myoblast transfer therapy (Partridge and Davies, 1995), gene therapy (Ahmad et al., 2000; Hartigan-O'Connor and Chamberlain, 2000; Wang et al., 2000) and pharmacological intervention (Badalamente and Stracher, 2000; Tidball and Spencer, 2000; Bogdanovich et al., 2002). In addition, mdx muscle is used to evaluate pathophysiological hypotheses by studying the cell

[1)] Dr. Kay Ohlendieck, Department of Biology, National University of Ireland, Maynooth, Co.Kildare/Ireland, e-mail: kay.ohlendieck@may.ie, Fax: +353 1 708 3845.

0171-9335/03/82/05-222 $15.00/0

biological effects following genetic modifications (Wehling et al., 2001).

With a view to developing new treatment strategies for muscular dystrophies, medical problems commonly associated with myoblast and gene transfer therapy are an insufficient therapeutic effect, immune rejection and cytotoxic side effects. To overcome these biomedical obstacles, an alternative approach to establish novel therapeutic targets is the examination of naturally protected skeletal muscle fibres (Porter, 1998; Andrade et al., 2000). This might help to better understand compensatory mechanisms counteracting the pathophysiology of Duchenne muscular dystrophy and could thus lead to the discovery of novel pathways or receptor classes involved in the endogenous elimination of myonecrosis. Two muscle groups which are deficient in the dystrophin isoform Dp427, but only show a mild dystrophic phenotype, are extraocular and toe muscles from the mdx animal model (Matsumura et al., 1992). While the sparing of muscle fibres is at least partially due to the special protective properties of fast-twitching small-diameter fibres (Karpati et al., 1988; Andrade et al., 2000), the up-regulation of utrophin is also implicated as a compensatory mechanism which rescues the sarcolemmal integrity in these unique cells (Porter et al., 1998). The absence of the membrane cytoskeletal component dystrophin triggers a drastic reduction in several crucial sarcolemmal glycoproteins (Ohlendieck and Campbell, 1991) named dystroglycan, sarcoglycans, dystrobrevin, syntrophin and sarcospan (Culligan et al., 1998). In mdx toe muscles these dystrophin-associated proteins are not as severely affected as in dystrophic mdx leg muscles (Dowling et al., 2002). Since the disintegration of the dystrophin-glycoprotein complex is postulated to interrupt the trans-sarcolemmal linkage between the actin membrane cytoskeleton and the extracellular matrix element laminin, thereby rendering muscle fibres more susceptible to necrosis (Ohlendieck, 1996; Cohn and Campbell, 2000), the rescue of dystrophin-associated glycoproteins may play a key role in preventing the dystrophic phenotype.

Ion flux through Ca^{2+}-leak channels appears to be responsible for an elevated Ca^{2+}-dependent net degradation of muscle proteins in dystrophic muscle cells (Alderton and Steinhardt, 2000). Although conflicting reports have been published on the extent of perturbation of Ca^{2+} homeostasis in dystrophic fibres (Gillis, 1996; Culligan and Ohlendieck, 2002), recent studies have established that the cytosolic Ca^{2+} overload in dystrophin-deficient muscle cells is not global but restricted to the subsarcolemmal domain (Mallouk et al., 2000). In addition, abnormal Ca^{2+} cycling through the lumen of the sarcoplasmic reticulum (Culligan et al., 2002) and mitochondria (Robert et al., 2001) is also involved in the muscular degeneration process. In dystrophic mdx leg skeletal muscle, the expression of the four main Ca^{2+}-regulatory elements of the excitation-contraction-relaxation cycle, i.e. the voltage-sensing dihydropyridine receptor, the ryanodine receptor Ca^{2+}-release channel, the Ca^{2+}-binding protein calsequestrin and the sarcoplasmic reticulum Ca^{2+}-ATPase, is not modified (Culligan et al., 2002). It was therefore of interest to study whether a compensatory change in the relative abundance of these key ion-regulatory muscle proteins exists in naturally protected mdx toe and extraocular fibres, as compared to more severely affected dystrophic mdx leg muscles. Our analysis indicates that a selective up-regulation of the sarcoplasmic reticulum Ca^{2+} pump might be involved in the fast removal of excess cytosolic Ca^{2+} ions thereby reducing the detrimental effects of myonecrosis in mdx toe fibres. These findings might have important implications for the future design of therapeutic approaches to treat x-linked muscular dystrophy, since the elimination of Ca^{2+}-dependent proteolysis appears to be a promising new way to counteract dystrophic changes in muscle cells (Badalamente and Stracher, 2000).

Materials and methods

Materials

Protease inhibitors and acrylamide stock solutions were obtained from Boehringer Mannheim (Lewis, East Sussex, UK). Immobilon-P nitrocellulose membranes were from Millipore Corporation (Bedford, MA). Chemiluminescence substrates were purchased from Perbio Science UK Ltd. (Tattenhall, Cheshire, UK). Superfrost Plus positively charged microscope slides were from Menzel-Gläser (Braunschweig, Germany). All other chemicals used were of analytical grade and purchased from Sigma Chemical Company (Poole, Dorset, UK).

Antibodies

Primary antibodies were purchased from Novocastra Laboratories Ltd., Newcastle upon Tyne, UK (mAb NCL-43 against β-dystroglycan and mAb DYS-2 to the carboxy-terminus of the dystrophin isoform Dp427), Affinity Bioreagents, Golden, CO, USA (mAb $VIIID1_2$ to fast calsequestrin; mAb IIH11 to the fast SERCA1 isoform of Ca^{2+}-ATPase; and mAb 20A to the α_2-subunit of the dihydropyridine receptor), and Upstate Biotechnology, Lake Placid, NY, USA (mAb $VIA4_1$ to α-dystroglycan) and Sigma Chemical Company (pAb to laminin). Fluorescein-, rhodamine- and peroxidase-conjugated secondary antibodies were obtained from Boehringer Mannheim.

Animals

Dystrophin-deficient male *mdx/y* mice, which are missing the Dp427 dystrophin isoform due to a point mutation in exon 23 (Sicinski et al., 1989), were obtained from Jackson Laboratory (Bar Harbor, Maine, USA) through the Biomedical Facility of the National University of Ireland, Dublin. In order to compare the relative expression levels of β-dystroglycan in normal and mdx gastrocnemius, extraocular fibres and toe muscles, tissue specimens were prepared for both cell biological and biochemical analyses. For immunofluorescence microscopy, muscle specimens were carefully dissected and immediately quick-frozen in liquid nitrogen-cooled isopentane, transported on dry ice and stored at −70 °C prior to cryosectioning. For immunoblot analysis, skeletal muscle samples were quick-frozen in liquid nitrogen, transported in a container with dry ice and then stored at −70 °C prior to homogenization.

Immunofluorescence microscopy

For the comparative immuno-decoration of normal and mdx muscle tissue sections, 12-μm cryosections were prepared using a standard cryostat (Microm, Heidelberg, Germany) and mounted on Superfrost Plus positively charged microscope slides. Fixation, blocking, incubation with primary antibodies, washing steps, incubation with secondary antibodies, as well as image acquisition were performed by established methodology (Culligan et al., 2001). For double-immunostaining procedures, a mixture of the appropriate primary antibodies was applied to tissue sections for 1 h at 37 °C, cryosections were washed, and then separately incubated for 30 min each with the appropriate secondary antibodies, i.e. a fluorescein- or rhodamine-conjugated probe. Incubation with the DNA-binding dye diamidino-phenyindole (DAPI) was used to label nuclei.

Membrane preparation

In order to compare the relative expression levels of β-dystroglycan in normal and mdx leg muscle, extraocular muscle and toe muscle by immunoblotting, crude microsomal membrane vesicles were isolated

from tissue homogenates by an established protocol at 0 – 4 °C (Glover et al., 2002) in the presence of a protease inhibitor cocktail (0.2 mM Pefabloc, 1.4 µM pepstatin A, 0.3 µM E-64, 1 µM leupeptin, 1 mM EDTA, and 0.5 µM soybean trypsin inhibitor) (Murray and Ohlendieck, 1997). Microsomal pellets were resuspended at a protein concentration of 10 mg/ml and used immediately for gel electrophoretic separation and immunoblot analysis. The protein concentration of isolated membrane vesicles was determined by the method of Bradford (1976), using bovine serum albumin as a standard. Comparative enzyme assays to determine Ca^{2+}-ATPase activity were performed by standard methods, recently optimized in our laboratory for the analysis of muscle membrane samples (Culligan et al., 2002). A direct colorimetric assay procedure using a malachite green-molybdate-polyvinyl alcohol mixed reagent was employed. Ca^{2+}-ATPase activity was calculated by comparison of measurements with a potassium dihydrogen phosphate standard graph.

Gel electrophoresis and immunoblot analysis

Because only restricted amounts of microsomal membranes could be obtained from extraocular and toe muscle fibres, a one- and two-dimensional Mini-MP3 electrophoresis system and mini blotting cell from Bio-Rad Laboratories (Hempel Hempstead, Herts., UK) was used. For the one-dimensional gel electrophoretic separation of microsomal muscle proteins, sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions was carried out by standard methodology (Laemmli, 1970) using 5% or 7% resolving gels and 20 µg protein per lane (Dowling et al., 2002). Two-dimensional electrophoresis was carried out by a technique recently optimized for the analysis of muscle membrane proteins (Froemming and Ohlendieck, 2001). For blotting experiments, separated microsomal muscle proteins were electrophoretically transferred for 1 h at 100 V onto nitrocellulose membranes by the method of Towbin et al. (1979). Membrane blocking, incubation with primary antibodies, washing steps, incubation with peroxidase-conjugated secondary antibodies, visualization of immunodecorated protein bands and densitometric scanning of developed immunoblots were carried out as previously described in detail (Harmon et al., 2001). Densitometric scanning of enhanced chemiluminescence blots was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA, USA) with ImageQuant V3.0 software.

Results

An established feature of dystrophin-deficient skeletal muscle fibres is a drastic reduction in various surface glycoproteins (Ohlendieck and Campbell, 1991). Necrotic changes, however, do not appear to occur in all mutant muscle fibre species to the same extent. It was therefore of interest to study the fate of a key dystrophin-associated glycoprotein, β-dystroglycan, in muscle tissues that are relatively unaffected, such as mdx extraocular fibres (Porter, 1998) and mdx toe fibres (Dowling et al., 2002). The relative expression level of β-dystroglycan was studied in normal and mdx gastrocnemius, extraocular and toe membranes using two-dimensional immunoblotting (Fig. 1) and immunofluorescence microscopy (Fig. 2). Since abnormal Ca^{2+} homeostasis might be involved in rendering dystrophin-deficient muscle fibres more susceptible to necrosis (Culligan and Ohlendieck, 2002), the biochemical and cell biological status of important Ca^{2+}-handling proteins was determined (Figs. 3 – 5).

Preserved β-dystroglycan expression in mdx extraocular and toe muscle fibres

Prior to our comparative immunoblot analysis of β-dystroglycan expression in gastrocnemius, extraocular and toe fibres, the mutant status of the mdx animals had to be established. All three mdx tissues clearly exhibited a lack in the dystrophin protein band of apparent 427 kDa (not shown). Standard one-dimensional immunoblotting confirmed the previously shown dramatic reduction in β-dystroglycan in dystrophin-deficient mdx gastrocnemius membrane preparations (not shown). To complement these findings by the more discriminatory technique of two-dimensional gel electrophoresis, muscle membranes were separated in the first dimension by isolectric focusing and in the second dimension by SDS polyacrylamide gel electrophoresis. The four two-dimensional immunoblots shown in Figure 1C – F revealed that both the relative expression and isoelectric point of β-dystroglycan is not affected in dystrophin-deficient extraocular and toe fibres. In contrast, the expression of β-dystroglycan was drastically reduced in mdx gastrocnemius membranes (Fig. 1A, B).

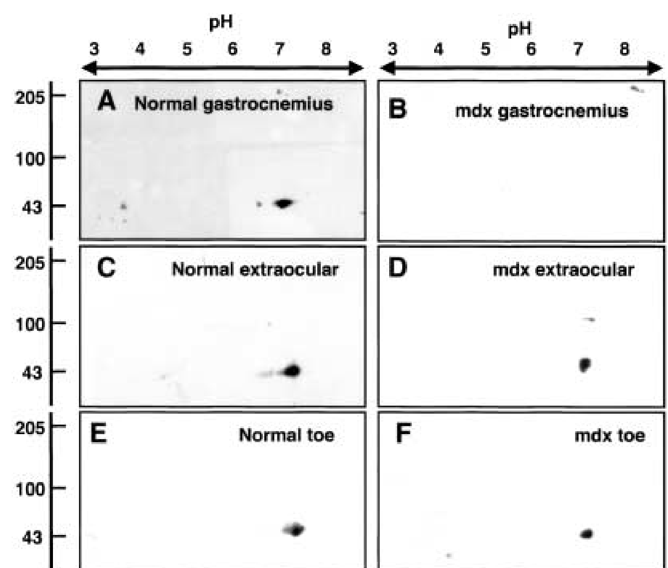
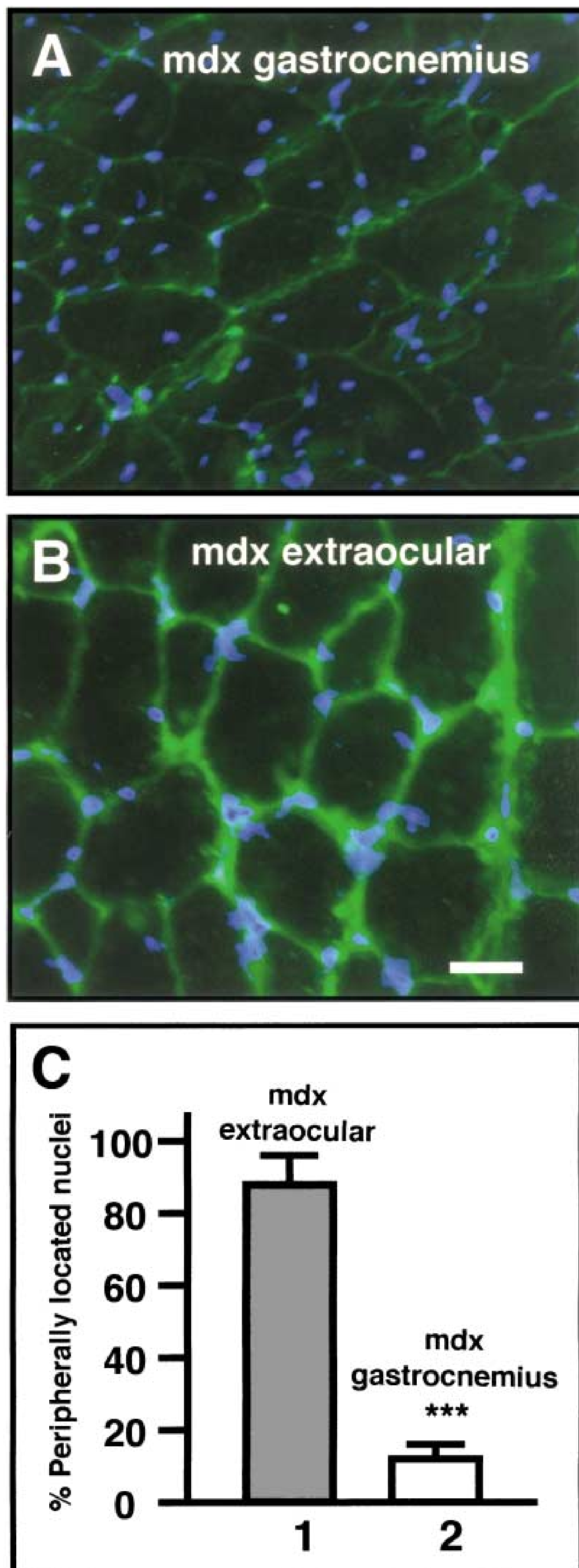


Fig. 1. Comparative two-dimensional immunoblot analysis of β-dystroglycan expression. Crude microsomal membranes from normal gastrocnemius (**A**), mdx gastrocnemius (**B**), normal extraocular (**C**), mdx extraocular (**D**), normal toe (**E**) and mdx toe (**F**) muscle homogenates were separated in the first dimension by isolelectric focusing and in the second dimension by SDS polyacrylamide gel electrophoresis. Identical two-dimensional blots were labeled with an antibody against 43-kDa β-dystroglycan. The pH values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated on the top and on the left of the panels, respectively.

Preserved sarcolemma localization of β-dystroglycan in mdx extraocular muscle fibres

Comparative immunofluorescence microscopy confirmed the sarcolemmal localization of β-dystroglycan in normal gastrocnemius muscle and its drastically reduced expression in the dystrophin-deficient mdx gastrocnemius muscle periphery (not shown). In analogy to the results of the comparative immunoblot analysis presented in Figure 1, immunofluorescence microscopy of transversely cut extraocular fibres demonstrated a preserved sarcolemmal localization of β-dystroglycan in mdx extraocular muscles (Fig. 2). Although fluorescein labeling is only a semi-quantitative tool, direct comparison of relative intensity levels indicate similar expression levels of β-dystro-



◀ **Fig. 2.** Peripheral nucleation and preserved β-dystroglycan expression in extraocular mdx fibres. Skeletal muscle specimens were taken from mdx gastrocnemius (**A**) and mdx extraocular (**B**) muscle fibres. Cryosections were labeled with an antibody to β-dystroglycan (β-DG) and the DNA-binding dye diamidino-phenyindole (DAPI). The direct comparison of the fluorescein labeling intensity of β-dystroglycan demonstrates the higher expression level of this dystrophin-associated glycoprotein in extraocular fibres, as compared to dystrophic leg muscle. DAPI staining revealed a high degree of central nucleation in mdx gastrocnemius sections and more peripheral nucleation in mdx extraocular fibres. Bar = 20 μm. (**C**) Graphical presentation of DAPI labelling patterns (n = 5; ***$P < 0.001$; unpaired t-test). In transverse cryosections from individual mdx mice, approximately 60 cells were evaluated for their DAPI staining pattern.

glycan in both normal and mdx fibres (not shown). In contrast to dystrophic gastrocnemius, the degree of central nucleation is less in extraocular mdx fibres (Fig. 2C). To illustrate this differing DAPI staining pattern and to show the higher expression levels of β-dystroglycan, representative gastrocnemius and extraocular fibres are shown in Figure 2A, B. This immunofluorescence micrograph clearly demonstrates the less intense green fluorescein labeling of the cell periphery and the higher degree of centrally located DAPI-stained nuclei in mdx gastrocnemius sections. Immunofluorescence labeling of toe fibres did not result in strong enough fluorescein staining patterns above background (not shown), so the cell biological analysis of mdx toe fibres could not be performed.

Status of Ca^{2+}-handling proteins in mdx tissues

Abnormal Ca^{2+} homeostasis is implicated in secondary changes leading to muscle cell degeneration. The main ion-regulatory elements involved in the regulation of the excitation-contraction-relaxation cycle in skeletal muscle fibres are the voltage-sensing dihydropyridine receptor of the transverse tubules, the ryanodine receptor Ca^{2+}-release channel of the junctional sarcoplasmic reticulum, the main luminal Ca^{2+}-buffering protein calsequestrin of the terminal cisternae region and the SERCA isoforms of the sarcoplasmic reticulum Ca^{2+}-ATPases. As illustrated in the immunoblot analyses shown in Figure 3A, the relative expression level of the 63-kDa fast calsequestrin isoform is relatively comparable between normal and mdx membranes isolated from gastrocnemius, extraocular and toe muscles. The same result was found for the α_2-subunit of the transverse-tubular dihydropyridine receptor complex (not shown). Immunoblotting of extraocular and toe membranes with antibodies to the RyR1 isoform of the sarcoplasmic reticulum Ca^{2+}-release channel did not result in good enough labeling above background for a proper comparative study (not shown). The drastic reduction in calsequestrin-like proteins of approximately 150 to 220 kDa in mdx leg muscle has previously been documented (Culligan et al., 2002). Both, extraocular and toe muscles do not appear to express the same complement of the high-molecular-mass isoforms of the main Ca^{2+}-reservoir element to the same degree as gastrocnemius fibres (Fig. 3A). However, extraocular preparations clearly contain the CLP-220 isoform and this protein is drastically reduced in dystrophin-deficient membranes (Fig. 3A). It is not clear whether the CLP isoforms of calsequestrin represent a separate class of luminal Ca^{2+}-binding proteins or whether these peripheral proteins of the sarcoplamic reticulum are aggregates of the 63-kDa calsequestrin monomer (Cala et al., 1990).

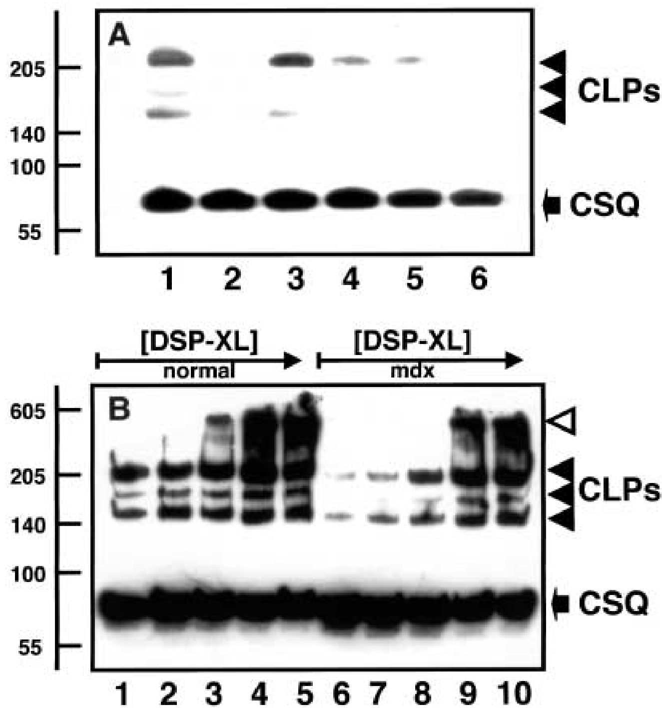


Fig. 3. Impaired oligomerization of calsequestrin in mdx muscle fibres. Blots were labeled with monoclonal antibody $VIIID1_2$ to calsequestrin. This antibody recognizes, in addition to the 63-kDa monomeric CSQ band, also three high-molecular-mass bands of approximately 150 kDa, 170 kDa and 220 kDa, termed calsequestrin-like proteins (CLPs). In panel (**A**), *lanes 1* to *6* represent membrane vesicles derived from normal gastrocnemius, mdx gastrocnemius, normal extraocular, mdx extraocular, normal toe and mdx toe muscle homogenates, respectively. Panel (**B**) shows the immunoblot analysis of chemically crosslinked gastrocnemius membranes. *Lanes 1* to *5* (normal) and *lanes 6* to *10* (mdx) represent 0, 12.5, 50, 100, and 200 μg dithio-bis-succinimidyl propionate (DSP) crosslinker (XL) per mg membrane protein, respectively. The positions of immuno-decorated proteins are marked by arrows. Molecular mass standards (in kDa) are indicated on the left.

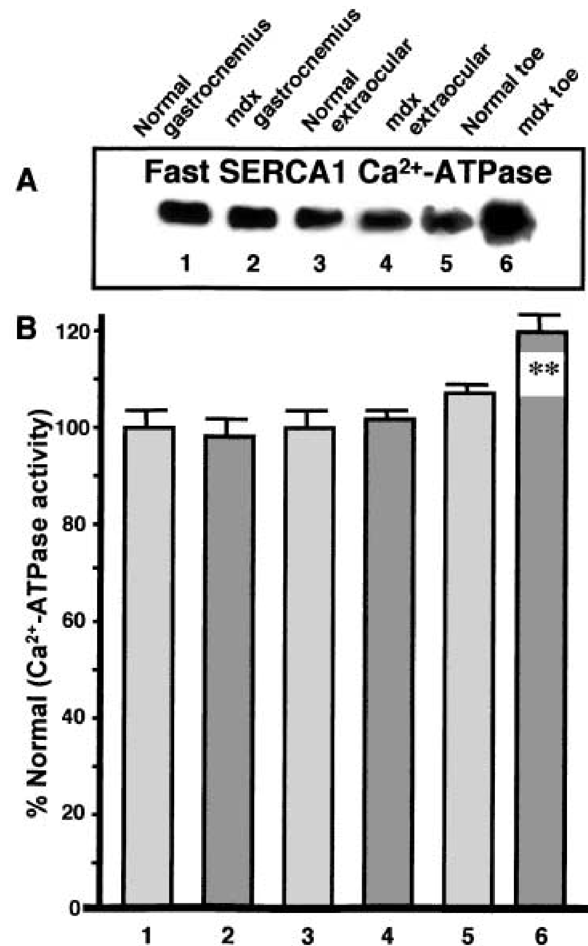


Fig. 4. Increased expression of the SERCA1 Ca^{2+}-ATPase and up-regulation of Ca^{2+}-ATPase enzyme activity in mdx toe muscle fibres. A blot was labeled with monoclonal antibody IIH11 against the fast-twitch SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (**A**). *Lanes 1* to *6* represent membrane vesicles derived from normal gastrocnemius, mdx gastrocnemius, normal extraocular, mdx extra-ocular, normal toe and mdx toe muscle homogenates, respectively. (**B**) Graphical representation of the Ca^{2+}-ATPase enzyme activity of normal and mdx membrane preparations ($n = 5$; $^{**}P < 0.01$; unpaired t-test). *Bars 1* to *6* represent membrane vesicles derived from normal gastrocnemius, mdx gastrocnemius, normal extraocular, mdx extra-ocular, normal toe and mdx toe muscle homogenates, respectively.

To address this question and in order to determine whether the reduction in high-molecular-mass calsequestrin isoforms is due to decreased expression or impaired oligomerization, we have performed a chemical crosslinking analysis of microsomal membranes. Figure 3B shows that in the absence of the crosslinker probe, normal microsomes exhibit 3 CLP bands, while mdx membranes exhibit a greatly reduced signal for these proteins. Incubation with increasing concentrations of the homo-bifunctional hydrophobic 1.2 nm probe dithio-bis-succinimydyl-propionate (DSP) causes the appearance of an immuno-decorated band with a very low electrophoretic mobility in normal membranes. Interestingly, in mdx preparations, the same crosslinker-induced effect is seen at slightly higher DSP concentrations. This suggests that the three CLP bands of approximately 150 kDa, 170 kDa and 220 kDa represent aggregates of the smaller calsequestrin monomer, rather than distinct isoforms. The DSP-inducible restoration of their appearance in mdx membranes indicates that the reduced expression of the high-molecular-mass species in dystrophic microsomes is most likely not due to a loss in individual isoforms, but because of impaired oligomerization of 63-kDa calsequestrin units. Since chemical crosslinking experiments require relatively large amounts of membrane vesicles for the optmization of the incubation conditions, this analysis could only be performed with the more abundant material from normal and mdx gastrocnemius muscle, and not with extraocular and toe preparations.

Ca^{2+} reuptake is a critical step in muscle relaxation and changes in the relative expression of the sarcoplasmic reticulum Ca^{2+}-ATPases play a role in certain muscle pathologies such as Brody's disease and alcoholic myopathy. We therefore determined potential changes in the expression levels of the predominant Ca^{2+} pump isoform, the fast SERCA1 species, in normal and mdx gastrocnemius, extraocular and toe fibres. As shown in immunoblot analysis (Fig. 4A), the abundance of SERCA1 is relatively comparable in control and dystrophic gastrocnemius and extraocular muscle preparations, but increased in mdx toe microsomes. In contrast, laminin expression

is unaffected in all three mdx tissues as compared to normal muscles (not shown). To evaluate whether the increased SERCA1 abundance in mdx toe has a functional consequence, we determined the Ca^{2+}-ATPase enzyme activity in the six different muscle preparations. Membranes derived from homogenized mdx toe fibres exhibited a significant increase in Ca^{2+}-ATPase activity (Fig. 4B) indicating a potential adaptive response in these dystrophin-deficient fibres. Comparative immunofluorescence microscopy revealed that laminin and Ca^{2+}-ATPase localization is unaffected in mdx toe and extraocular fibres (Fig. 5). In contrast to the slightly increased labeling of the mdx fibre periphery for laminin, no drastic SERCA up-regulation in mdx fibres was observed (Fig. 5). Probably the differences in relative density of SERCA units are too small to be detected by immunofluorescence labeling.

Discussion

To address the issue of why the absence of the dystrophin Dp427 isoform does not necessarily result in a severe dystrophic phenotype, we have analysed two naturally protected muscle groups, the mdx extraocular fibres and mdx toe muscles (Matsumura et al., 1992), and have compared them to more severely affected cells from mdx leg muscles. The challenge with both extraocular and toe fibres is the restricted amount of tissue available for immunoblotting procedures. However, using miniature gel and transfer equipment, highly specific monoclonal antibodies and the enhanced chemiluminescence detection technique, it was possible to study the fate of β-dystroglycan in low-abundance mdx muscle tissues. The sparing of certain mdx fibres has previously been attributed to the special protective properties of small-diameter fibres (Karpati et al., 1988). Here we can show that both extraocular and toe mdx muscles exhibit a preserved expression of the critical trans-sarcolemmal linker element of the dystrophin-glycoprotein complex, β-dystroglycan of apparent 43 kDa. Since the disintegration of the dystrophin-associated glycoprotein complex is believed to play a central role in the secondary pathophysiology of several inherited muscular dystrophies (Campbell, 1995; Ohlendieck, 1996), the rescue of β-dystroglycan could be a relevant factor in the protective phenotype of mdx toe and

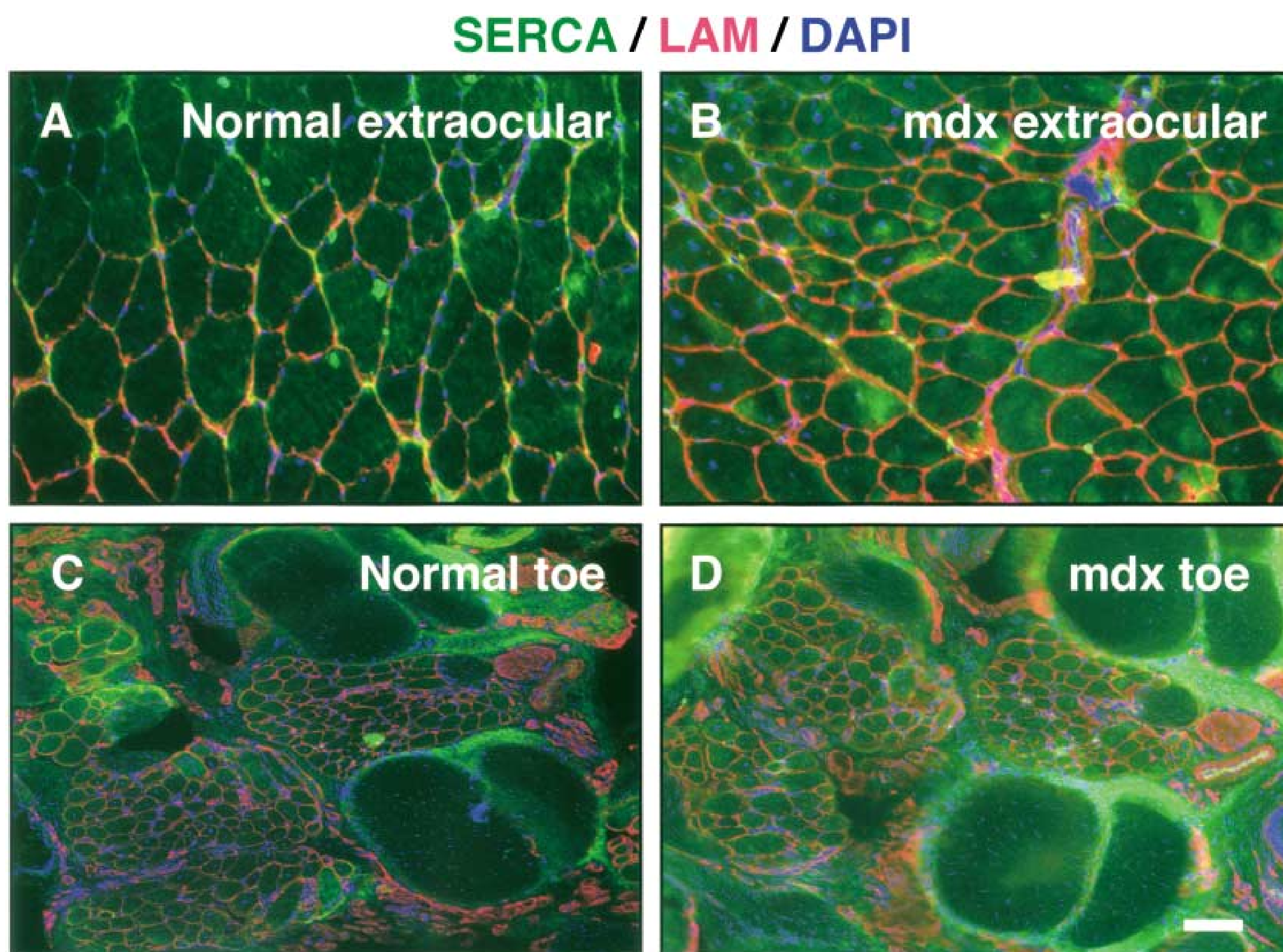


Fig. 5. Immunofluorescence localization of Ca^{2+}-ATPase in mdx muscle. Cryosections were labeled with antibodies to the fast-twitch SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (*green*) and laminin (*red*). Nuclei were stained with the DNA-binding dye diamidino-phenyindole DAPI (*blue*). Skeletal muscle specimens were taken from normal (**A, C**) and mdx (**B, D**) extraocular (**A, B**) and toe (**C, D**) muscle fibres. Laminin staining is almost exclusively found in the cellular periphery and labeling of the Ca^{2+}-ATPase occurred mostly in the internal structures of the fibres. Bar = 20 μm.

extraocular fibres. In addition, the increased removal of cytosolic Ca^{2+} ions from mdx toe fibres, via up-regulation of the SERCA1 Ca^{2+}-ATPase units, might also protect dystrophin-deficient muscle cells from Ca^{2+}-induced myonecrosis.

The rapid advances in routine molecular analyses of patient samples has greatly improved the diagnosis of muscle diseases (Miller and Hoffman, 1994; Hoffman, 1999). However, since many abnormal proteins are muscle-specific isoforms, current protein tests still depend on relatively large amounts of muscle tissue taken by biopsy and can often not properly differentiate between the various forms of muscular dystrophy affecting similar proteins (Prior, 1995). With respect to improving the diagnostics of neuromuscular diseases, this study indicates that the relative expression levels of β-dystroglycan represent a reliable marker of the severity of secondary changes in dystrophin-deficient fibres. To avoid the potential misdiagnosis of muscular dystrophies with overlapping symptoms and to differentiate between differently affected muscle groups within one individual, the standardized two-dimensional mini blot screening with antibodies to β-dystroglycan could be helpful. In combination with molecular biological assays such as Southern blotting and the polymerase chain reaction, immunocytochemical evaluations of tissue cryosections and protein gel analysis, two-dimensional immunoblot analysis could be an extremely helpful tool in the development of a comprehensive biomedical algorithm for the rapid differential diagnosis of inherited muscular dystrophies that directly or indirectly affect the dystrophin-glycoprotein complex.

The mdx mouse does not exhibit all pathological features of severe Duchenne muscular dystrophy. Nevertheless, this natural mutant strain represents the most widely used animal model of x-linked muscular dystrophy (Watchko et al., 2002). Deficiency in the Dp427 isoform in mdx skeletal muscle is due to a stop codon mutation in exon 23 of the dystrophin gene (Sicinski et al., 1989) and the occurring of a dystrophic process in mdx muscle is evident by elevated creatine kinase levels (Bulfield et al., 1984). Although not a perfect replica of human x-linked muscular dystrophy, the mdx model can certainly be employed for detailed biochemical and cell biological studies on the molecular pathogenesis of dystrophin deficiency and to evaluate novel therapeutic strategies such as gene therapy. The reduction in dystrophin-associated glycoproteins is clearly the primary pathophysiological consequence of the deficiency in the Dp427 isoform (Campbell, 1995; Ohlendieck, 1996). However, it is not well understood what cascade of secondary abnormalities leads to muscle weakness. One potential pathobiochemical pathway involves abnormal Ca^{2+} handling (Alderton and Steinhardt, 2000; Culligan and Ohlendieck, 2002). A recent survey of the expression patterns of key Ca^{2+}-regulatory membrane proteins in dystrophic mdx leg muscle membranes revealed that the expression of the ryanodine receptor Ca^{2+}-release channel complex, the dihydropyridine receptor, the Ca^{2+}-ATPase and calsequestrin are not affected in muscular dystrophy. The only major difference between normal and mdx microsomes from leg muscle homogenates was a drastic decline in calsequestrin-like proteins (CLPs) of 150 to 220 kDa (Culligan et al., 2002). In analogy, the Ca^{2+}-binding capacity of the mdx sarcoplasmic reticulum was shown to be reduced by approximately 20%. Hence, the reduction in Ca^{2+}-binding proteins might be directly involved in triggering impaired Ca^{2+} sequestration within the lumen of the sarcoplasmic reticulum.

Based on the findings in this report, the hypothesis of impaired Ca^{2+} buffering being involved in muscular dystrophy should be revised slightly. Here we could show by chemical crosslinking analysis that the CLP elements of the terminal cisternae region appear to be aggregates of calsequestrin and not distinct high-molecular-mass isoforms. Native membrane-associated calsequestrin complexes were first stabilized by the introduction of small covalent linker molecules, followed then by the solubilization of the membrane structures and subsequent electrophoretic separation of crosslinker-stabilized complexes. Thus, the results of this analytical approach are of physiological significance and represent a well-established method of determining quaternary structures under native conditions (Wong, 1991). This indicates that impaired oligomerization of calsequestrin units might be a central factor of reduced Ca^{2+} binding in the dystrophic sarcoplasmic reticulum. The data agree with the previous finding that calsequestrin clusters exhibit positive co-operativity with respect to high-capacity Ca^{2+} binding (Tanaka et al., 1986). Direct protein-protein interactions play an integral part in regulating luminal Ca^{2+} concentrations. Upon ion binding to the carboxy-terminal region, Ca^{2+}-induced conformational changes trigger calsequestrin to fold into a more compact structure (MacLennan and Reithmeier, 1998; Wang et al., 1998). In addition, calsequestrin is proposed to act directly as an endogenous regulator of the junctional ryanodine receptor Ca^{2+}-release channel complex (Ohkura et al., 1998). The luminal Ca^{2+} concentration appears to influence the probability of Ca^{2+}-release channel opening and thereby modulates Ca^{2+}-flux rates from the luminal sarcoplasmic reticulum (Donoso et al., 1996). Since calsequestrin appears to be the central regulator of Ca^{2+} cycling through the sarcoplasmic reticulum and represents an essential mediator between excitation-contraction coupling and muscle relaxation, it is not surprising that impaired physiological functioning of this terminal cisternae element results in major tissue damage.

The calcium hypothesis of muscular dystrophy assumes a complex pathophysiological mechanism of abnormal Ca^{2+} handling by the surface membrane, the transverse tubules, the sarcoplasmic reticulum and mitochondria (Culligan and Ohlendieck, 2002). It is not clear to what extent individual subcellular domains contribute to overall muscle fibre destruction. It is, however, well established that dystrophin-deficient skeletal muscle membranes succumb to exercise-induced membrane ruptures more frequently than those of normal fibres (Clarke et al., 1993). Transient micro-ruptures allow for the insertion of Ca^{2+}-leak channels into the skeletal muscle periphery during the natural processes of cell membrane resealing. This in turn is believed to result in localized Ca^{2+} elevations, contributing to a cycle of enhanced protease activity and leak channel activation (Alderton and Steinhardt, 2000). However, the recent analysis of transgenic mdx muscles, that exhibit an over-expression of utrophin, questions the role of mechano-sensitive Ca^{2+}-channels in initiating the dystrophic process (Squire et al., 2002). Possibly, the pathophysiological modification of other ion-regulatory surface elements precedes the increased Ca^{2+}-leak channel activation, followed by downstream alterations of intracellular Ca^{2+} handling. The impaired clustering of calsequestrin units, as described in this report, may contribute to decreased luminal Ca^{2+} buffering. This might indirectly amplify the elevated free cytosolic Ca^{2+} concentrations, which is possibly limited to micro-domains near the sarcolemma (Mallouk et al., 2000). Hence, disturbed sarcolemmal Ca^{2+} fluxes appear to influence overall Ca^{2+} handling resulting in changes in the oligomerization of a subset of Ca^{2+}-

handling proteins which might be an important factor in the progressive functional decline of dystrophic muscle fibres. In this respect, the up-regulation of the sarcoplasmic reticulum Ca^{2+}-ATPase in mdx toe fibres could be a compensatory mechanism. Possibly the increased removal of cytosolic Ca^{2+} ions plays a role in the rescue of these dystrophin-deficient fibres, as diagrammatically summarized in Figure 6. Both, the up-regulation and extra-junctional expression of the autosomal dystrophin homologue utrophin (Tinsley and Davis, 1993; Tinsley et al., 1996; Rafael, 1998) and concomitant rescue of the dystrophin-associated glycoprotein complex, and the removal of pathophysiological Ca^{2+} levels in the cystosol might be factors involved in the protected phenotype of mdx extraocular and toe fibres.

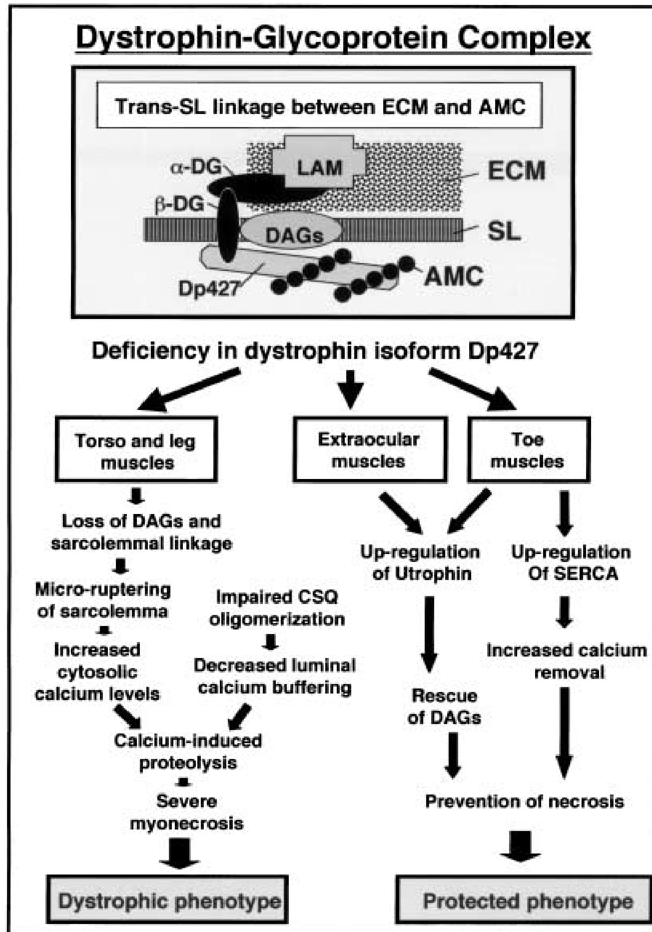


Fig. 6. Flow diagram of possible pathways that differentiate between dystrophic and protected phenotypes in dystrophin-deficient muscle fibre sub-types. The drastic reduction in dystrophin-associated glycoproteins is clearly the primary pathophysiological consequence of the deficiency in the Dp427 dystrophin isoform. Loss of the trans-sarcolemmal linkage between the extracellular matrix component laminin and the sub-sarcolemmal actin membrane cytoskeleton renders the muscle periphery more susceptible to micro-rupturing. During the membrane resealing process, Ca^{2+}-leak channels are introduced into the plasmalemma triggering the influx of Ca^{2+} ions into the cytosol. Together with an impaired Ca^{2+}-buffering capacity of the sarcoplasmic reticulum, this results in Ca^{2+}-induced proteolysis eventually causing muscle weakness in almost all torso and leg muscles. In contrast, the up-regulation and extra-junctional expression of utrophin appears to rescue the sarcolemmal expression of dystrophin-associated glycoproteins in mdx extraocular and toe muscles thereby preventing the same degree of muscle degeneration in these Dp427-deficient muscle fibres. The up-regulation of the sarcoplasmic reticulum Ca^{2+}-ATPase in mdx toe fibres might also play a role in establishing a protected phenotype. Abbreviations used: AMC, actin membrane cytoskeleton; CSQ, calsequestrin; DAG, dystrophin-associated glycoproteins; DG, dystroglycan; ECM, extracellular matrix; LAM, laminin; SERCA, sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase; SL sarcolemma.

Acknowledgements. Research was supported by project grants from Enterprise Ireland, Dublin (SC/2000/386), the Irish Health Research Board (HRB-RP01/2001), Muscular Dystrophy Ireland and the European Commission (RTN2 – 2001 – 00337).

References

Ahmad, A., Brinson, M., Hodges, B. L., Chamberlain, J. S., Amalfitano, A. (2000): Mdx mice inducibly expressing dystrophin provide insights into the potential of gene therapy for Duchenne muscular dystrophy. Hum. Mol. Genet. **9**, 2507 – 2515.

Alderton, J. M., Steinhardt, R. A. (2000): Calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. J. Biol. Chem. **275**, 9452 – 9460.

Allamand, V., Campbell, K. P. (2000): Animal models for muscular dystrophy: valuable tools for the development of therapies. Hum. Mol. Genet. **9**, 2459 – 2467.

Andrade, F. H., Porter, J. D., Kaminski, H. J. (2000): Eye muscle sparing by the muscular dystrophies: lessons to be learned? Microsc. Res. Tech. **48**, 192 – 203.

Badalamente, W. A., Stracher, A. (2000): Delay of muscle degeneration and necrosis in mdx mice by calpain inhibition. Muscle Nerve **23**, 106 – 111.

Bogdanovich, S., Krag, T. O. B., Barton, E. R., Morris, L. D., Whittemore, L. A., Ahima, R. S., Khurana, T. S. (2002) Functional improvement of dystrophic muscle by myostatin blockade. Nature **420**, 418 – 421.

Bradford, M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248 – 254.

Bulfield, G., Siller, W. G., Wight, P. A. L., Moore, K. J. (1984): X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc. Natl. Acad. Sci. USA **81**, 1189 – 1192.

Cala, S. E., Scott, B. T., Jones, L. R. (1990): Intralumenal sarcoplasmic reticulum Ca^{2+}-binding proteins. Semin. Cell Biol. **1**, 265 – 275.

Campbell, K. P. (1995): Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. Cell **80**, 675 – 679.

Clarke, M. S., Khakee, R., McNeil, P. L. (1993): Loss of cytoplasmic basic fibroblast growth factor from physiologically wounded myofibers of normal and dystrophic muscle. J. Cell Sci. **106**, 121 – 133.

Cohn, R. D., Campbell, K. P. (2000): Molecular basis of muscular dystrophies. Muscle Nerve **23**, 1456 – 1471.

Culligan, K., Mackey, A., Finn, D., Maguire, P. B., Ohlendieck, K. (1998): Role of dystrophin isoforms and associated glycoproteins in muscular dystrophy (review). Int. J. Mol. Med. **2**, 639 – 648.

Culligan, K., Glover, L., Dowling, P., Ohlendieck, K. (2001): Brain dystrophin-glycoprotein complex: Persistent expression of beta-dystroglycan, impaired oligomerization of Dp71 and up-regulation of utrophins in animal models of muscular dystrophy. BMC Cell Biol. **2**, 2 (http://www.biomedcentral.com/1471 – 2121/2).

Culligan, K., Banville, N., Dowling, P., Ohlendieck, K. (2002): Drastic reduction of calsequestrin-like proteins and impaired calcium binding in dystrophic mdx muscle. J. Appl. Physiol. **92**, 435 – 445.

Culligan, K., Ohlendieck, K. (2002) Abnormal calcium handling in muscular dystrophy. Bas. Appl. Myol. **12**, 147 – 157.

DeLuca, A., Pierno, S., Liantonio, A., Cetrone, M., Camerino, C., Simonetti, S., Papadia, F., Camerino, D. C. (2001): Alteration of excitation-contraction coupling mechanism in extensor digitorum

longus muscle fibres of dystrophic mdx mouse and potential efficacy of taurine. Br. J. Pharmacol. **132**, 1047 – 1054.

Donoso, P., Beltran, M., Hidalgo, C. (1996) Luminal pH regulated calcium release kinetics in sarcoplasmic reticulum vesicles. Biochemistry **35**, 13419 – 13425.

Dowling, P., Culligan, K., Ohlendieck, K. (2002): Distal mdx muscle groups exhibiting up-regulation of utrophin and rescue of dystrophin-associated glycoproteins exemplify a protected phenotype in muscular dystrophy. Naturwissenschaften **89**, 75 – 88.

Emery, A. E. (2002): The muscular dystrophies. Lancet **359**, 687 – 695.

Froemming, G. R., Ohlendieck, K. (2001): The native dihydropyridine receptor exists as a supramolecular complex in skeletal muscle. Cell. Mol. Life Sci. **58**, 312 – 320.

Gillis, J. M. (1996): Membrane abnormalities and Ca homeostasis in muscles of the mdx mouse, an animal model of the Duchenne muscular dystrophy: a review. Acta Physiol. Scand. **156**, 397 – 406.

Glover, L., Quinn, S., Ryan, M., Pette, D., Ohlendieck, K. (2002): Supramolecular calsequestrin complex. Eur. J. Biochem. **269**, 4607 – 4616.

Harmon, S., Froemming, G. R., Leisner, E., Pette, D., Ohlendieck, K. (2001): Low-frequency stimulation of fast muscle affects the abundance of Ca^{2+}-ATPase but not its oligomeric status. J. Appl. Physiol. **90**, 371 – 379.

Hartigan-O'Connor, D., Chamberlain, J. S. (2000): Developments in gene therapy for muscular dystrophy. Microsc. Res. Tech. **48**, 223 – 238.

Hoffman, E. P. (1999): Muscular dystrophy: identification and use of genes for diagnostics and therapeutics. Arch. Pathol. Lab. Med. **123**, 1050 – 1052.

Karpati, G., Carpenter, S., Precott, S. (1988): Small caliber skeletal muscle fibres do not suffer necrosis in mdx mouse dystrophy. Muscle Nerve **11**, 795 – 803.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680 – 685.

Lynch, G. S., Rafael, J. A., Chamberlain, J. S., Faulkner, J. A. (2000): Contraction-induced injury to single permeabilized muscle fibers from mdx, transgenic mdx, and control mice. Am. J. Physiol. Cell Physiol. **279**, C1290 – C1294.

MacLennan, D. H., Reithmeier, R. A. (1998): Ion tamers. Nat. Struct. Biol. **5**, 409 – 411.

Mallouk, N., Jacquemond, V., Allard, B. (2000): Elevated subsarcolemmal Ca^{2+} in mdx mouse skeletal muscle fibres detected with Ca^{2+}-activated K^{+} channels. Proc. Natl. Acad. Sci. USA **97**, 4950 – 4955.

Matsumura, K., Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Campbell, K. P. (1992): Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. Nature **360**, 588 – 591.

Menke, A., Jockusch, H. (1991): Decreased osmotic stability of dystrophin-less muscle cells from mdx mouse. Nature **349**, 69 – 71.

Miller, R. G., Hoffman, E. P. (1994): Molecular diagnosis and modern management of Duchenne muscular dystrophy. Neurol. Clin. **12**, 699 – 725.

Murray, B. E., Ohlendieck, K. (1997): Cross-linking analysis of the ryanodine receptor and α_1-dihydropyridine receptor in rabbit skeletal muscle. Biochem. J. **324**, 689 – 696.

Ohkura, M., Furukawa, K., Fujimori, H., Kuruma, A., Kawano, S., Hiraoka, M., Kuniyasu, A., Nakayama, H., Ohizumi, Y. (1998): Dual regulation of the skeletal muscle ryanodine receptor by triadin and calsequestrin. Biochemistry **37**, 12987 – 12993.

Ohlendieck, K. (1996): Towards an understanding of the dystrophin-glycoprotein complex: linkage between the extracellular matrix and the subsarcolemmal membrane cytoskeleton. Eur. J. Cell Biol. **69**, 1 – 10.

Ohlendieck, K., Campbell, K. P. (1991): Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. J. Cell Biol. **115**, 1685 – 1694.

Partridge, T. A., Davies, K. E. (1995): Myoblast-based gene therapy. Br. Med. Bull. **51**, 123 – 137.

Porter, J. D. (1998): Extraocular muscle sparing in muscular dystrophy: a critical evaluation of potential protective mechanisms. Neuromusc. Disord. **8**, 198 – 203.

Porter, J. D., Rafael, J. A., Ragusa, R. J., Brueckner, J. K., Trickett, J. I., Davies, K. E. (1998): The sparing of extraocular muscle in dystrophinopathies is lost in mice lacking utrophin and dystrophin. J. Cell Sci. **111**, 1801 – 1811.

Prior, T. W. (1995): Perspectives and molecular diagnosis of Duchenne and Becker muscular dystrophies. Clin. Lab. Med. **15**, 927 – 941.

Rafael, J. A., Tinsley, J. M., Potter, A. C., Deconinck, A. E., Davies, K. E. (1998): Skeletal muscle-specific expression of utrophin transgene rescues utrophin-dystrophin deficient mice. Nature Genet. **19**, 79 – 82.

Robert, V., Massimino, M. L., Tosello, V., Marsault, R., Cantini, M., Sorrentino, V., Pozzan, T. (2001): Alteration in calcium handling at the subcellular level in mdx myotubes. J. Biol. Chem. **276**, 4647 – 4651.

Sicinski, P., Geng, Y., Ryder-Cook, A. S., Barnard, E. A., Darlison, M. G., Barnard, P. G. (1989): The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science **244**, 1578 – 1580.

Squire, S., Raymackers, J. M., Vandebrouck, C., Potter, A., Tinsley, J., Fischer, R., Gillis, J. M., Davies, K. E. (2002) Prevention of pathology in mdx mice by expression of utrophin: analysis using an inducible transgenic expression system. Hum. Mol. Genet. **11**, 3333 – 3344.

Tanaka, M., Ozawa, T., Maurer, A., Cortese, J. D., Fleischer, S. (1986): Apparent cooperativity of Ca^{2+} binding associated with crystallization of Ca^{2+}-binding protein from sarcoplasmic reticulum. Arch. Biochem. Biophys. **251**, 369 – 378.

Tidball, J. G., Spencer, M. J. (2000): Calpains and muscular dystrophies. Int. J. Biochem. Cell Biol. **32**, 1 – 5.

Tinsley, J. M., Davies, K. E. (1993): Utrophin: a potential replacement for dystrophin? Neuromusc. Disord. **3**, 537 – 539.

Tinsley, J. M., Potter, A. C., Phelps, S. R., Fisher, R., Trickett, J. I., Davies, K. E. (1996): Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature **384**, 349 – 353.

Torres, L. F. B., Duchen, L. W. (1987): The mutant mdx: inherited myopathy in the mouse. Brain **110**, 269 – 299.

Towbin, H., Staehelin, T., Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**, 4350 – 4354.

Wang, B., Li, J., Xiao, X. (2000): Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy. Proc. Natl. Acad. Sci. USA **97**, 13714 – 13719.

Wang, S., Trumble, W. R., Liao, H., Wesson, C. R., Dunker, A. K., Kang, C. H. (1998): Crystal structure of calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum. Nat. Struct. Biol. **5**, 476 – 483.

Watchko, J. F., O'Day, T. L., Hoffman, E. P. (2002): Functional characteristics of dystrophic muscle: insights from animal models J. Appl. Physiol. **93**, 407 – 417.

Wehling, M., Spencer, M. J., Tidball, J. G. (2001) A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. J. Cell Biol. **155**, 123 – 131.

Wong, S. S. (1991): Cross-linking of membrane proteins. Chemistry of protein conjugation and cross-linking. CRC Press, Boca Raton, FL, pp. 233 – 243.