

translational physiology

Drastic reduction of calsequestrin-like proteins and impaired calcium binding in dystrophic *mdx* muscle

KEVIN CULLIGAN, NIAMH BANVILLE, PAUL DOWLING, AND KAY OHLENDIECK

Department of Pharmacology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland

Received 29 August 2001; accepted in final form 2 November 2001

Culligan, Kevin, Niamh Banville, Paul Dowling, and Kay Ohlendieck. Drastic reduction of calsequestrin-like proteins and impaired calcium binding in dystrophic *mdx* muscle. *J Appl Physiol* 92: 435–445, 2002; 10.1152/jappphysiol.00903.2001.—Although the reduction in dystrophin-associated glycoproteins is the primary pathophysiological consequence of the deficiency in dystrophin, little is known about the secondary abnormalities leading to x-linked muscular dystrophy. As abnormal Ca^{2+} handling may be involved in myonecrosis, we investigated the fate of key Ca^{2+} regulatory membrane proteins in dystrophic *mdx* skeletal muscle membranes. Whereas the expression of the ryanodine receptor, the dihydropyridine receptor, the Ca^{2+} -ATPase, and calsequestrin was not affected, a drastic decline in calsequestrin-like proteins of 150–220 kDa was observed in dystrophic microsomes using one-dimensional immunoblotting, two-dimensional immunoblotting with isoelectric focusing, diagonal two-dimensional blotting technique, and immunoprecipitation. In analogy, overall Ca^{2+} binding was reduced in the sarcoplasmic reticulum of dystrophic muscle. The reduction in Ca^{2+} binding proteins might be directly involved in triggering impaired Ca^{2+} sequestration within the lumen of the sarcoplasmic reticulum. Thus disturbed sarcolemmal Ca^{2+} fluxes seem to influence overall Ca^{2+} homeostasis, resulting in distinct changes in the expression profile of a subset of Ca^{2+} handling proteins, which might be an important factor in the progressive functional decline of dystrophic muscle fibers.

calcium binding proteins; calcium homeostasis; calcium sequestration; muscular dystrophy; sarcoplasmic reticulum

THE DYSREGULATION OF Ca^{2+} handling has been recognized as playing a central role in rare neuromuscular disorders, such as malignant hyperthermia, central core disease, and Brody's disease (24, 35, 44). Abnormal Ca^{2+} homeostasis has also been described in more frequent disorders of skeletal and heart muscle cells, i.e., alcoholic myopathy (63) and dilated cardiomyopathy (41). In the case of the most commonly inherited

neuromuscular disorder, Duchenne muscular dystrophy (17), there is still debate about whether cytosolic accumulation of calcium ions and the accompanied Ca^{2+} -induced proteolysis (70) and/or altered developmental programming of regenerating myofibers (10) represents one of the major pathophysiological pathways leading to skeletal muscle fiber destruction (11). Conflicting reports have been published on the extent of perturbation of overall Ca^{2+} homeostasis in dystrophin-deficient muscle cells (28). Although Steinhardt and co-workers (2, 3, 19, 33, 68, 69) have postulated that ion flux through Ca^{2+} leak channels is responsible for an elevated Ca^{2+} -dependent net degradation of muscle proteins in dystrophic muscle cells, results from several other research groups do not agree with the idea of a drastic increase in total Ca^{2+} levels (12, 26, 32, 37, 64). An explanation for the contradictory findings could be that the cytosolic Ca^{2+} overload is not global but restricted to subsarcolemmal domains in dystrophic muscle fibers (48, 49). In addition, abnormal Ca^{2+} homeostasis in mitochondria might also be involved in the muscular degeneration process (65). Independent of the total extent and exact microdomain localization of the initial Ca^{2+} disturbance, even small changes in Ca^{2+} cycling might trigger a cascade of modifications in ion-regulatory membrane complexes. To determine whether protein degradation and/or compensatory up- or downregulation of individual elements of the Ca^{2+} handling apparatus occurs in dystrophin-deficient muscle cells, we have analyzed key components involved in the regulation of excitation-contraction coupling.

The motoneuron-induced sarcolemmal depolarization is sensed by transverse-tubular sensors and is physiologically coupled to the activation of the contractile apparatus via the Ca^{2+} release system of the sarcoplasmic reticulum (SR) (50, 54). Two well-character-

Address for reprint requests and other correspondence: K. Ohlendieck, Dept. of Pharmacology, Univ. College Dublin, Belfield, Dublin 4, Ireland (E-mail: kay.ohlendieck@ucd.ie).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

ized Ca^{2+} -channel complexes represent the central elements of this excitation-contraction coupling process: the voltage-sensing dihydropyridine receptor (8) and the ryanodine receptor (RyR) Ca^{2+} release channel (20). In skeletal muscle, both receptors directly interact during signal transduction at the triad junction (42), whereas, in developing skeletal muscle and cardiac fibers, a Ca^{2+} -induced Ca^{2+} release mechanism appears to be the dominant process (7). The skeletal muscle dihydropyridine receptor consists of the principal α_{1S} -subunit, which contains the voltage-sensing domain and the pore-forming structures, as well as the auxiliary subunits $\alpha_2\delta$, β , and γ , which have important regulatory functions (30). The Ca^{2+} release channel complex of the junctional SR is formed by the tetrameric RyR structure (66). Triadin and several not yet biochemically characterized junctional proteins are auxiliary components (22, 25, 72). After contraction, the Ca^{2+} -ATPase of the longitudinal tubules and terminal cisternae provides a rapid re-uptake mechanism for the removal of calcium ions during muscle relaxation (46, 66). Ca^{2+} sequestration within the SR lumen is mediated by various Ca^{2+} binding proteins, such as calsequestrin, sarcalumenin, and calreticulin (40, 52, 57). As a high-capacity ion-binding protein, calsequestrin has a central position in Ca^{2+} homeostasis (45). It has been shown to be an important endogenous regulator of the Ca^{2+} release channel (58). In contrast to calsequestrin of 63 kDa, the exact function of several calsequestrin-like proteins (CLPs) of higher molecular mass is not well understood (5, 47).

Although earlier studies (18, 39, 53, 62, 71) have analyzed the dystrophic chicken and merosin-deficient *dy* mouse, no comprehensive study has addressed the

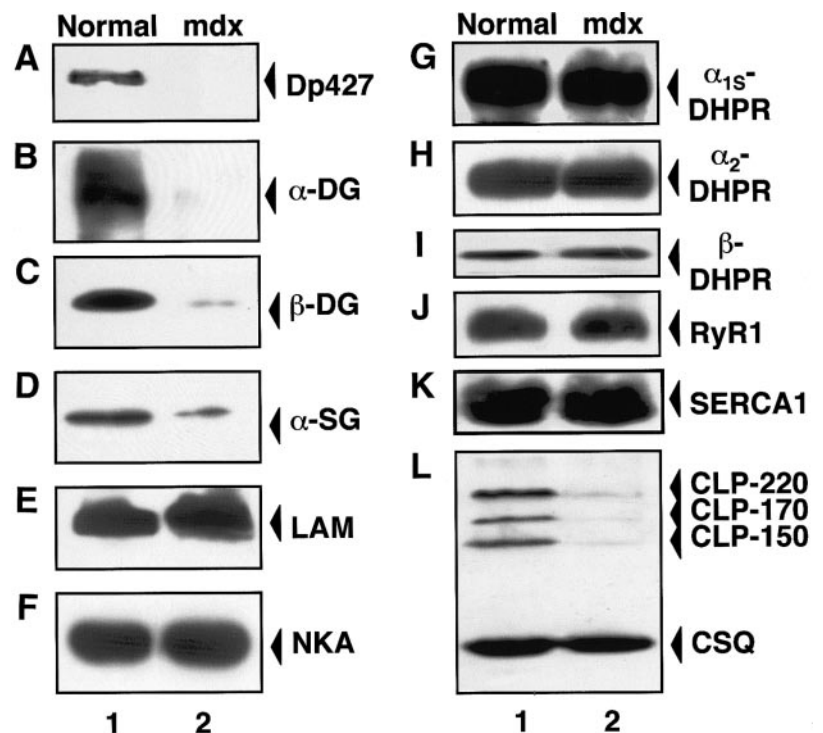
status of key Ca^{2+} regulatory membrane proteins in an established animal model of x-linked Duchenne muscular dystrophy such as the *mdx* mouse. Leg and torso *mdx* skeletal muscle fibers do not exhibit all of the observed pathobiochemical changes as seen in muscle specimens from patients afflicted with Duchenne muscular dystrophy (17). However, they do exhibit segmental necrosis (67), stretch-induced injury (43), increased susceptibility to osmotic shock (51), alterations of excitation-contraction coupling (15), and a drastic decrease in dystrophin-associated glycoproteins (13, 59), making them a suitable disease model (4). It was, therefore, of interest to determine potential secondary changes in Ca^{2+} regulatory proteins in *mdx* muscle preparations.

MATERIALS AND METHODS

Dystrophic animal model. Eight-wk-old normal control and dystrophic *mdx* mice (Jackson Laboratory, Bar Harbor, MN) were comparatively analyzed in this study. The *mdx* mouse lacks the dystrophin isoform Dp427 due to a point mutation in exon 23, making it a suitable animal model for studying potential pathophysiological changes in x-linked muscular dystrophy (4). Although not a perfect replica of the human disease process, this mouse model exhibits many of the muscular degeneration processes seen in Duchenne muscular dystrophy (6, 59). To establish the mutant status of the *mdx* mice used in this study, microsomal membranes and muscle cryosections were analyzed for the dystrophin isoform Dp427 by immunoblotting (see Fig. 1A) and immunofluorescence microscopy (see Fig. 6D), respectively.

Reagents. Protease inhibitors and acrylamide stock solutions were purchased from Boehringer Mannheim (Lewis, East Sussex, UK). Western blotting chemiluminescence substrates and Slide-A-Lyzer Mini Dialysis MWCO-10000 cas-

Fig. 1. Immunoblot analysis of Ca^{2+} regulatory membrane proteins in dystrophic *mdx* skeletal muscle. Shown are immunoblots decorated with antibodies to the Dp427 isoform of dystrophin (A), α -dystroglycan (DG; α -DG; B), β -DG (C), α -sarcoglycan (α -SG; D), laminin (LAM; E), the α -subunit of the Na^+ - K^+ -ATPase (NKA; F), the α_{1S} -subunit of the voltage-sensing dihydropyridine receptor (DHPR; α_{1S} -DHPR; G), the α_2 -subunit of the transverse-tubular DHPR (α_2 -DHPR; H), the β -subunit of the DHPR (β -DHPR; I), the ryanodine receptor (RyR)1 isoform of the RyR Ca^{2+} release channel of the sarcoplasmic reticulum (J), the fast-twitch isoform of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA)1 (K), and calsequestrin (CSQ) of apparent 63 kDa and the 3 CSQ-like proteins (CLP) of 150 kDa (CLP-150), 170 kDa (CLP-170), and 220 kDa (CLP-220) (L). Lanes 1 and 2 represent microsomal membranes isolated from normal control and dystrophic *mdx* skeletal muscle, respectively. The position of immunodecorated protein bands is marked by arrow heads.



ettes were obtained from Pierce & Warriner (Chester, Cheshire, UK). Ecoscint-A scintillation fluid was from National Diagnostics (Hull, UK). Immobilon-P nitrocellulose sheets were from Millipore (Bedford, MA). Reagents for isoelectric focusing (impedance pneumograph pH 3–10 strips and impedance pneumograph pH 3–10 buffer systems), as well as $^{45}\text{CaCl}_2$, were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Superfrost Plus positively charged microscope slides were from Menzel Gläser (Braunschweig, Germany), and Kodacolor Gold 400ASA VR film was obtained from Eastman Kodak (Rochester, NY). All other chemicals, including those for membrane isolation, electrophoresis, and blotting, were of analytic grade and purchased from Sigma Chemical (Poole, Dorset, UK).

Antibodies. Characterization of established antibodies to the dystrophin-glycoprotein complex and ion-regulatory muscle proteins was performed as previously described (13, 21). Monoclonal antibody (MAb) IIID5 against the α_1 -subunit of the dihydropyridine receptor and MAb VD2₁ to the β -subunit of the dihydropyridine receptor were a generous gift from Dr. Kevin P. Campbell (University of Iowa, Iowa City, IA). A polyclonal antibody to α -sarcoglycan was raised by four monthly injections of a peptide representing the last 15 residues of the carboxy-terminus using a standard immunization protocol (13). Peptides had been synthesized and coupled to keyhole limpet hemocyanin carrier by Research Genetics (Huntington, AL). Antisera to junctin were a generous gift of Dr. Steve Cala (Wayne State University, Detroit, MI) (29). Commercially available primary antibodies were from Novocastra Laboratories (Newcastle upon Tyne, UK; MAb NCL-43 against β -dystroglycan, MAb DYS-1 to the Dp427 rod domain), Upstate Biotechnology (Lake Placid, NY; MAb VIA4₁ to α -dystroglycan, MAb c464.6 to the α -subunit of the Na^+ - K^+ -ATPase), Affinity Bioreagents [Golden, CO; MAb VIIID1₂ to calsequestrin, MAb 20A to the α_2 -subunit of the dihydropyridine receptor, MAb IIH11 to the fast-twitch sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA)1 isoform of the SR Ca^{2+} -ATPase], and Sigma Chemical (MAb 34C to the RyR1 isoform of the RyR Ca^{2+} release channel). Peroxidase- or fluorescein-conjugated secondary antibodies were purchased from Boehringer Mannheim.

Isolation of microsomal membranes. For gel electrophoretic and immunoblot analysis, as well as $^{45}\text{CaCl}_2$ binding and Ca^{2+} -ATPase assays, microsomal membranes were isolated from normal control and *mdx* leg and back skeletal muscle homogenates by an established subcellular fractionation procedure (55). To minimize proteolytic degradation, all procedures were carried out at 4°C, and all buffers contained a protease inhibitor cocktail [0.3 μM *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), 0.2 mM pefabloc, 1.4 μM pepstatin, 0.15 μM aprotinin, 1 μM leupeptin, 0.5 μM soybean trypsin inhibitor, and 1 mM EDTA]. Final microsomal membrane pellets were resuspended at a protein concentration of 10 mg/ml and used immediately for gel electrophoretic separation, Ca^{2+} binding studies, and Ca^{2+} -ATPase assays.

Gel electrophoresis and immunoblotting. Gel electrophoretic separation using one-dimensional 5% (wt/vol) or 7% (wt/vol) resolving gels with a 5% (wt/vol) stacking gel in the presence of SDS and dithiothreitol was performed for 200 V·h employing a Mini-MP3 electrophoresis system from Bio-Rad Laboratories (Hempel Hempstead, Herts, UK), whereby 25 μg protein was loaded per well (23). The two-dimensional gel electrophoresis techniques used in this study have been previously optimized for the analysis of integral muscle membrane proteins in our laboratory. This includes diagonal nonreducing/reducing two-dimensional gel electrophoresis

for the determination of complex formation (47) and standard two-dimensional gels using isoelectric focusing in the first dimension and SDS polyacrylamide gel electrophoresis in the second dimension for the evaluation of isoelectric point (pI) values of individual protein isoforms (23). Immunodecoration of nitrocellulose replicas of polyacrylamide gels (21) was carried out by established procedures using the enhanced chemiluminescence technique (31). Densitometric scanning of enhanced chemiluminescence blots was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA) with ImageQuant V3.0 software.

Immunoprecipitation. Comparative immunoprecipitation experiments were performed as previously described (29). Microsomal membranes from both normal control and dystrophic *mdx* muscle were solubilized in a 1% (vol/vol) Tween-20-containing buffer (50 mM Tris·Cl; pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.3 μM E-64, 0.2 μM pefabloc), and precleared with a 1:1 slurry of protein A-Sepharose (Sigma Chemical) for 1 h at 4°C to allow removal of nonspecific binding proteins. Formation of the antigen-antibody complexes was then achieved by incubation of the supernatant with a 1:20 dilution of MAb VIIID1₂ to calsequestrin for 2 h at 4°C. Antigen-antibody complexes were removed by addition of an equal volume of a 1:1 slurry of protein A-Sepharose. The suspension was slowly rotated for 1 h at 4°C. After sedimentation and three washes with the above buffer, the antigen-antibody complexes were removed from the Sepharose beads by boiling in reducing electrophoresis buffer for 5 min. The eluted complexes were separated by standard SDS polyacrylamide gel electrophoresis and analyzed by immunoblot analysis.

Calcium-binding and calcium-ATPase assay. Comparative binding experiments using radiolabeled $^{45}\text{CaCl}_2$ and assays of Ca^{2+} -ATPase enzyme activity were performed by standard methods (60). For equilibrium dialysis, microsomal vesicles derived from normal control or dystrophic *mdx* muscle (0.1 mg protein) were placed in a Slide-A-Lyzer Mini Dialysis MWCO-10000 cassette system (0.25-ml volume) and dialyzed against 100 ml of 5 mM Tris·Cl, pH 7.5, 0.1 mM $^{45}\text{CaCl}_2$ (20,000 counts·min⁻¹·ml⁻¹) for 24 h at 4°C. Equal samples from both inside and outside the dialysis cassette were dissolved in 10 ml of Ecoscint-A scintillation fluid and were radioactivity counted in a standard scintillation counter. Specific Ca^{2+} binding per milligram protein could then be calculated from the increased radioactivity within the dialysis cassette. To determine potential differences in the Ca^{2+} -ATPase enzyme activity between normal and dystrophic microsomes, the direct colorimetric assay procedure using a malachite green-molybdate-polyvinyl alcohol mixed reagent (9) was employed (60). Ca^{2+} -ATPase activity was calculated by comparison of measurements with a potassium dihydrogen phosphate standard graph.

Immunofluorescence microscopy. For indirect immunofluorescence microscopy, the tibialis anterior muscle from normal control and dystrophic *mdx* mice was quick-frozen in liquid nitrogen-cooled isopentane and stored at -70°C before cryosectioning. Transverse sections of 12 μm thickness were prepared using a standard cryostat (Microm, Heidelberg, Germany), mounted on Superfrost Plus positively charged microscope slides and subsequently fixed, blocked, washed, hematoxylin-and-eosin stained, or immunolabeled with primary and secondary antibodies, and then photographed for documentation, as previously described (13).

RESULTS

Although the primary genetic defects leading to the various forms of muscular dystrophy have been identified, very little is known about the secondary abnormalities leading to myonecrosis. Studies over recent years have produced conflicting data with respect to changes in cytosolic Ca^{2+} levels, the postulated triggering factor in Ca^{2+} -induced muscle cell destruction. To determine novel potential factors involved in the pathophysiology of dystrophin-deficient muscle fibers, we have investigated the fate of key Ca^{2+} regulatory membrane proteins involved in the regulation of excitation-contraction coupling. A survey of triad and SR markers was carried out using one-dimensional immunoblotting with established antibodies (Fig. 1), followed by a more in-depth analysis of CLPs (Fig. 2), two different two-dimensional immunoblot techniques with

isoelectric focusing or diagonal gel separation (Fig. 3), immunoprecipitation (Fig. 4), Ca^{2+} binding and Ca^{2+} -ATPase assays of SR vesicles employing equilibrium dialysis and enzyme testing, respectively (Fig. 5), and comparative histological and immunofluorescence microscopy of normal and dystrophic muscle fibers (Fig. 6).

Characterization of *mdx* microsomes. Before analysis of the three main excitation-contraction coupling elements, i.e., the transverse-tubular voltage sensor, the Ca^{2+} release channel complex of the junctional SR, and the luminal Ca^{2+} reservoir complex, the dystrophic status of the *mdx* preparation was established. As illustrated in Fig. 1, A–D, microsomal membranes isolated from *mdx* skeletal muscle homogenates were completely deficient in the Dp427 isoform of dystrophin and exhibited a greatly reduced expression of the dystrophin-associated glycoproteins: α -dystroglycan, β -dystroglycan, and α -sarcoglycan. In analogy to dystrophic muscle from patients afflicted with Duchenne muscular dystrophy, this reduction in dystrophin-associated surface proteins is believed to play a central role in the pathomolecular process leading to myonecrosis (6, 59), making *mdx* microsomes a suitable model system for studying potential dystrophy-induced changes in Ca^{2+} handling proteins. In contrast to the dystroglycans and α -sarcoglycan, laminin, the extracellular protein linked to the dystrophin-glycoprotein complex via α -dystroglycan, was shown not to be reduced in dystrophic microsomes but exhibited a slightly increased expression (Fig. 1E). Relatively comparable levels of expression were found for an established surface marker, the Na^+ - K^+ -ATPase (Fig. 1F), demonstrating that the different expression levels of dystrophin and its associated glycoproteins in normal vs. *mdx* preparations are not an artifact of the subcellular fractionation procedure, differential proteolytic degradation, protein solubilization, electrophoretic separation, and/or the immunoblotting methodology.

Expression of Ca^{2+} regulatory proteins in *mdx* membranes. Immunoblotting with established antibodies to the principal α_{1S} -subunit of the dihydropyridine receptor and its auxiliary α_2 - and β -subunits revealed no drastic difference in their expression between normal and *mdx* microsomes (Fig. 1, G–I). The RyR1 isoform of the SR Ca^{2+} release channel and the fast SERCA1 isoform of the Ca^{2+} -ATPase were also found to exist at comparable levels in control and dystrophin-deficient membrane preparations (Fig. 1, J and K). Thus in contrast to the dramatic reduction in dystrophin-associated glycoproteins in *mdx* membranes (Fig. 1, B–D), the relative abundance of the two central elements of excitation-contraction coupling and the central ion pump responsible for muscle relaxation is not affected by dystrophic changes. The same was shown for the major Ca^{2+} binding protein of the terminal cisternae region, calsequestrin of apparent 63 kDa (Fig. 1L).

Reduced expression of CLPs in *mdx* membranes. In stark contrast to calsequestrin, three CLPs recognized by MAb VIID1₂, exhibited a greatly reduced expression in *mdx* preparations (Fig. 1L). These luminal SR

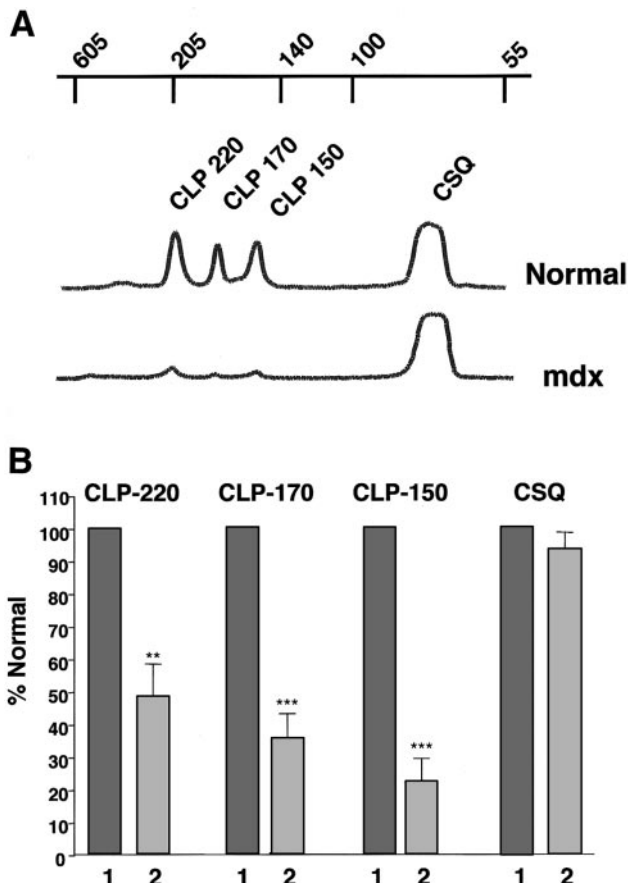


Fig. 2. Expression of CLPs in dystrophic *mdx* skeletal muscle. A: densitometric scans of representative immunoblots of normal control and dystrophic *mdx* membranes labeled with monoclonal antibody (MAb) VIID1₂ to CSQ and CLP demonstrating the greatly decreased expression of CLP-150, CLP-170, and CLP-220 in dystrophin-deficient microsomes. The relative position of molecular mass standards (in kDa) is indicated on top of the scans. B: statistical analysis using paired *t*-tests documents the significantly reduced relative density of all 3 CLPs in *mdx* muscle preparations ($n = 5$; ** $P < 0.01$; *** $P < 0.001$), whereas the expression of CSQ is comparable between normal and dystrophic membranes. Bars 1 and 2 represent microsomal membranes isolated from normal control and dystrophic *mdx* skeletal muscle, respectively. Error bars, SD.

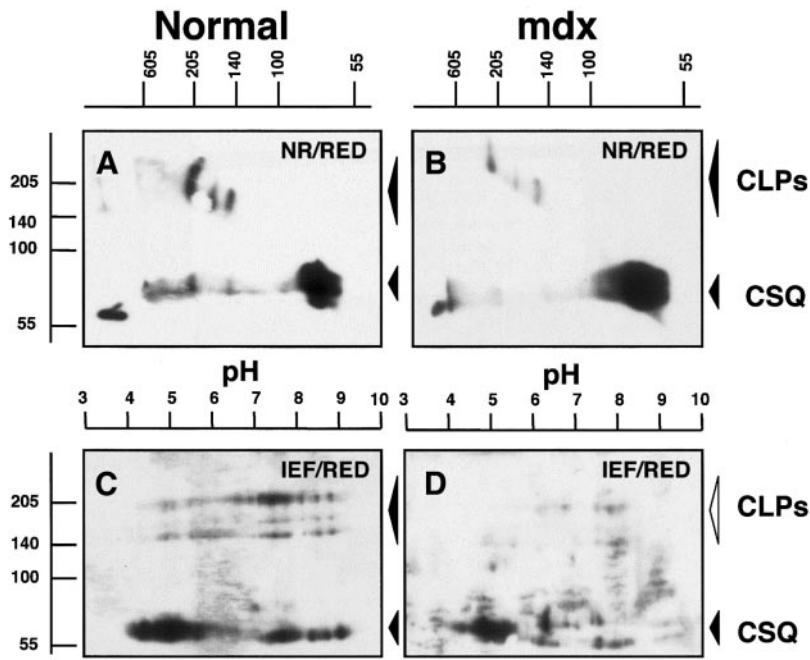


Fig. 3. Two-dimensional immunoblot analysis of CLPs in dystrophic *mdx* skeletal muscle. Shown are representative immunoblots using diagonal nonreducing/reducing 2-dimensional gel electrophoresis (NR/RED) for the determination of complex formation (A and B) and standard 2-dimensional gels using isoelectric focusing (pH 3–10) in the first dimension and reducing SDS polyacrylamide gel electrophoresis in the second dimension (IEF/RED) for the evaluation of isoelectric point values of individual protein isoforms (C and D). A and C: normal control microsomes; B and D: membranes isolated from dystrophic *mdx* muscle. Positions of immunodecorated protein bands are marked by arrowheads. Sizes of molecular mass standards (in kDa), as deduced from rat myofibrillar proteins, are indicated on the left and on the top of the blots.

proteins of ~150 kDa (CLP-150), 170 kDa (CLP-170), and 220 kDa (CLP-220) appear to be the only major excitation-contraction coupling proteins with different relative densities in dystrophic microsomes. Immunoblotting with antibodies to other minor Ca^{2+} regulatory proteins, such as junctin, calmodulin, sarcalumenin, 12-kDa FK506 binding protein, slow calsequestrin, triadin, the sarcolemmal Ca^{2+} -ATPase, the Na^{+}/Ca^{2+} exchanger, and the γ -dihydropyridine receptor, did not result in good enough immunodecoration for proper evaluation (not shown) and thus could not be studied further. As illustrated in the densitometric scans of representative immunoblots of CLPs (Fig. 2A) and the graphical representation of the relative abundance of CLP-150, CLP-170, and CLP-220 (Fig. 2B), the three CLPs are much less abundant in *mdx* membranes compared with normal control microsomes.

Diagonal two-dimensional immunoblot analysis of CLPs. To further investigate the status of CLPs in dystrophic microsomes, two different two-dimensional

immunoblotting techniques were employed. First, diagonal nonreducing/reducing two-dimensional gel electrophoresis (Fig. 3, A and B) and, second, isoelectric focusing in the first dimension and standard SDS polyacrylamide gel electrophoresis in the second dimension (Fig. 3, C and D). Diagonal two-dimensional gel electrophoresis was used to differentiate between calsequestrin of 63 kDa and its oligomeric structures on the one hand and the three CLPs of 150 kDa, 170 kDa, and 220 kDa on the other hand. Proteins remaining on the diagonal represent monomers. Immunodecorated species exhibiting distinct shifts to the left represent oligomeric forms of the protein situated on the diagonal line. Figure 3, A and B, clearly demonstrates that a subpopulation of the 63-kDa calsequestrin species exists in both normal control and dystrophic *mdx* membranes as high-molecular-mass clusters of >600 kDa. The protein spot representing calsequestrin monomers in two-dimensional gels is relatively broad (Fig. 3, A and B). This effect is most likely due to the uniform

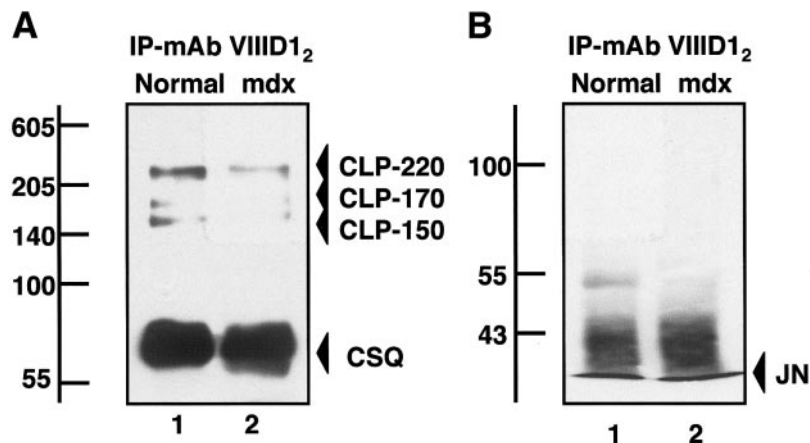
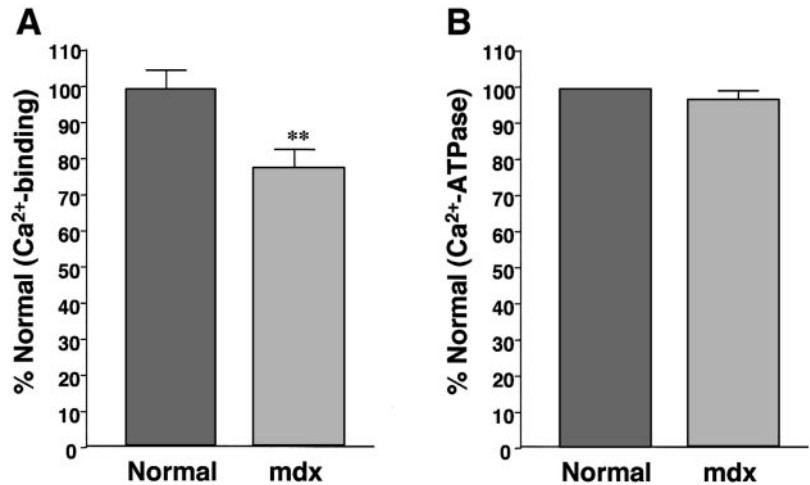


Fig. 4. Immunoprecipitation analysis of CLPs in dystrophic *mdx* skeletal muscle. Shown are immunoblots of solubilized membrane fractions immunoprecipitated (IP) with mAb VIIIID12 to CSQ of apparent 63 kDa and CLP-150, CLP-170, and CLP-220, which were subsequently immunolabeled with the same antibody to CSQ (A) or a polyclonal antibody to junctin (JN; B). Lanes 1 and 2 represent microsomal membranes isolated from normal control and dystrophic *mdx* skeletal muscle, respectively. Positions of immunodecorated protein bands are marked by arrowheads. Sizes of molecular mass standards (in kDa), as deduced from rat myofibrillar proteins, are indicated on the left.

Fig. 5. Analysis of the Ca^{2+} binding capacity and Ca^{2+} -ATPase activity of the sarcoplasmic reticulum from dystrophic *mdx* skeletal muscle. **A**: graphical representation of the Ca^{2+} binding properties of normal control and dystrophic *mdx* microsomes ($n = 5$; $**P < 0.01$; unpaired *t*-test). Ca^{2+} binding was determined using equilibrium dialysis, whereby normal mouse muscle microsomes bound ~ 170 nmol Ca^{2+} /mg protein. For comparison, **B** graphically represents the Ca^{2+} -ATPase enzyme activity of normal control and dystrophic *mdx* microsomes ($n = 8$). With the use of a paired *t*-test, no statistically significant difference was found, whereby normal mouse muscle microsomes exhibited an enzyme activity of ~ 2 $\mu\text{mol P}_i \cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$. Error bars, SD.



tube gel system used in the nonreducing first dimension, which generally does not concentrate protein samples as well as slab gels. In contrast to calsequestrin, CLP-150, CLP-170, and CLP-220 remain on the diagonal after nonreducing/reducing two-dimensional gel electrophoresis and do not appear to exist under nonreducing conditions as higher molecular mass structures. In analogy to the findings using one-dimensional immunoblotting, this two-dimensional technique confirms the greatly reduced expression of the three CLPs in dystrophic membranes (Fig. 3, A and B).

Two-dimensional isoelectric focusing analysis of CLPs. To determine potential changes in the isoform expression pattern of calsequestrin or CLPs with re-

spect to pI values, immunoblotting of standard two-dimensional gels with isoelectric focusing in the first dimension and reducing SDS polycrylamide gel electrophoresis in the second dimension was performed. As shown by one-dimensional immunoblotting and the above-described diagonal two-dimensional method, this gel system also demonstrated a drastic reduction in the expression levels of the three CLPs of ~ 150 kDa, 170 kDa, and 220 kDa (Fig. 3, C and D). In both normal control and dystrophic membranes, the main calsequestrin species of apparent 63 kDa migrated at a pI value around 5. In contrast to *mdx* preparations, normal microsomes also showed calsequestrin species at approximately pH 7–9. The calsequestrin species of

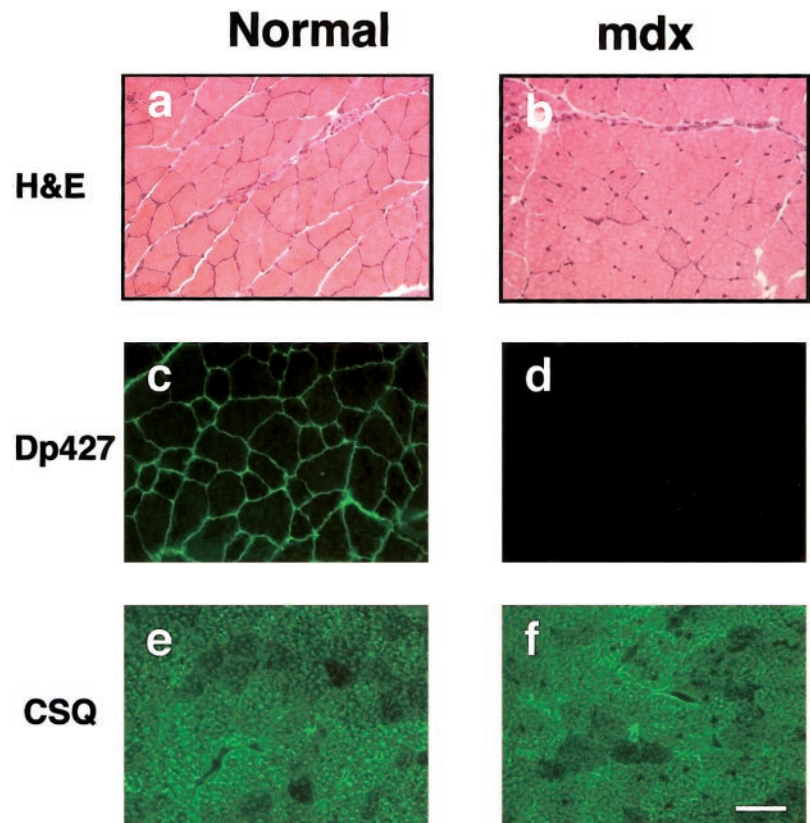


Fig. 6. Immunofluorescence localization of CSQ in dystrophic *mdx* skeletal muscle. Shown are transverse cryosections of normal control (A, C, E) and dystrophic *mdx* (B, D, F) skeletal muscle fibres, histologically stained with hematoxylin and eosin (H&E; A and B) and immunolabeled with antibodies to the Dp427 isoform of dystrophin (C and D) and CSQ (E and F). Many dystrophic fibers exhibited central nucleation (B) and a complete absence of dystrophin (D), establishing the mutant status of the *mdx* muscle used in this study. The different staining intensities of individual muscle cells for CSQ reflect the varying contents of this Ca^{2+} binding protein in fast-twitching vs. slower fibers (E and F), whereby no apparent difference was observed between normal and dystrophic tissues. Bar = 20 μm .

differing pI value probably represent differently phosphorylated protein species. This agrees with the idea that native calsequestrin exists as a mixture of non-phosphorylated and phosphorylated Ca^{2+} binding complexes. In microsomes from normal skeletal muscle, the major species of the three different CLPs exhibited an electrophoretic mobility at a pI value of ~ 7 – 8 . This is especially well illustrated for CLP-220 in Fig. 3C.

Immunoprecipitation analysis of CLPs. To confirm the findings from the one- and two-dimensional immunoblotting, immunoprecipitation experiments using MAb VIIID1₂ were performed (Fig. 4). This antibody not only recognizes both calsequestrin and CLPs in immunoblotting, but also precipitates these proteins as shown in Fig. 4A. Although the immunoprecipitation technique is not a reliable quantitative method due to potential variations in the interactions between antibodies and antigens in different starting materials, it can be employed in a semiquantitative approach. The immunoprecipitated fractions from normal control and *mdx* samples exhibited approximately equal amounts of the 63-kDa calsequestrin species (Fig. 4A) and the calsequestrin-binding protein named junctin (Fig. 4B). On the other hand, the CLPs were found at a lower relative concentration in the dystrophic fraction, which was especially well demonstrated for CLP-220 (Fig. 4A). It has been recently established that a subpopulation of calsequestrin exists in a heterogeneous triad complex closely associated with junctin, triadin, and the RyR Ca^{2+} release channel (29). The close neighborhood relationship between calsequestrin and junctin has been confirmed by our immunoprecipitation analysis, and this interaction does not appear to be changed in the dystrophic phenotype. Immunolabeling with antibodies to the RyR1 isoform of the Ca^{2+} release channel and the junctional marker triadin did not reveal the presence of these components in mouse muscle complexes immunoprecipitated by MAb VIIID1₂. Possibly these large triadic membrane complexes disintegrate during the subcellular fractionation and electrophoretic separation procedure, and/or the relative abundance of triadin and the RyR is too low to be detected by immunoblotting.

Reduced Ca^{2+} binding of dystrophic SR. As shown in Fig. 5A, Ca^{2+} binding assays of SR vesicles showed that Ca^{2+} binding was significantly reduced in *mdx* preparations compared with normal control membranes. With the use of equilibrium dialysis, an $\sim 20\%$ decrease in the overall capacity to sequester calcium ions was demonstrated in dystrophin-deficient microsomal membranes. This pathophysiological finding of disturbed luminal Ca^{2+} homeostasis agrees with the results from our immunoblot analysis of CLPs. Possibly the reduction in the Ca^{2+} binding proteins CLP-150, CLP-170, and CLP-220 causes a decreased capacity of the dystrophic SR to function as a Ca^{2+} reservoir. For comparison, no statistically significant difference in the Ca^{2+} -ATPase enzyme activity was determined (Fig. 5B). Thus, although earlier studies have shown impaired maximum velocity of Ca^{2+} uptake (36), the

total Ca^{2+} -ATPase activity does not seem to be impaired in dystrophic skeletal muscle fibers.

Immunofluorescence localization of calsequestrin in dystrophic muscle. Because immunoblotting and immunoprecipitation studies revealed a reduced expression of CLPs in *mdx* membranes, immunofluorescence microscopy was used to establish whether labeling with MAb VIIID1₂ detects any potential changes in the localization and/or abundance of the antigens recognized by this probe. Comparative histological staining with hematoxylin and eosin of normal and *mdx* muscle fibers clearly demonstrated the dystrophic phenotype of the mouse mutant (Fig. 6, A and B). While transverse cryosections of normal muscle fibers showed peripheral nucleation exclusively, large numbers of *mdx* muscle cells clearly exhibited central nucleation, thus demonstrating abnormal degeneration-regeneration cycles in these fibers. Immunofluorescence microscopy with an antibody to the Dp427 isoform of dystrophin showed staining of the cellular periphery in normal muscle and a complete absence of this membrane cytoskeletal protein in *mdx* fibers (Fig. 6, C and D), thus establishing the mutant status of the animal model employed in this study. Immunolabeling with antibody VIIID1₂ to calsequestrin and the CLPs did not reveal a drastic difference in the localization pattern or the relative intensity of the immunofluorescence signal (Fig. 6, E and F). Probably, this technique does not reflect minor changes in the abundance and/or recognizes the calsequestrin isoform of 63 kDa better than the CLPs, CLP-150, CLP-170, and CLP-220. However, fiber-type-specific differences in the distribution of calsequestrin were recognized by this antibody, staining fast-twitching fibers more intensely than slower fibers (Fig. 6, E and F). No difference in the distribution of fast and slow fibers was detected by this method between normal control and dystrophic *mdx* preparations.

DISCUSSION

Despite the fact that primary genetic defects have been identified for Duchenne muscular dystrophy and related muscular disorders (1) and an enormous amount of clinical data has been gathered about muscle weakness in these inherited diseases (17), the molecular processes leading to muscle cell destruction have not been adequately revealed. Deficiency in the membrane cytoskeletal protein dystrophin has been shown to be the underlying genetic cause for muscle weakness (38). In normal muscle, dystrophin is proposed to act as a molecular anchor that mediates a transsarcolemmal linkage between the extracellular matrix component laminin and the actin membrane cytoskeleton (6, 59). In dystrophic fibers, the absence of dystrophin causes the disintegration of a surface membrane complex consisting of a variety of sarcolemmal-associated proteins named dystroglycans, sarcoglycans, sarcospan, dystrobrevins, and syntrophins (14). It is not clear how reduced expression levels of these dystrophin-associated proteins trigger secondary ab-

normalities in muscular dystrophy. However, in an effort to develop treatments for inherited neuromuscular disorders, a better understanding of these secondary molecular and cellular mechanisms that lead to contractile failure is essential.

The secondary molecular mechanisms underlying skeletal muscle fiber necrosis are the link between a specific mutation in the human Duchenne muscular dystrophy gene on the one hand and end-stage myonecrosis on the other hand. Thus it is essential to analyze the pathobiochemical processes that impair normal muscle function in an effort to understand the overall molecular pathogenesis of muscular dystrophy. In this study, we have determined potential changes in the expression pattern of Ca^{2+} regulatory components involved in excitation-contraction coupling, because variations in ion homeostasis might trigger directly proteolytic degradation and/or compensatory changes in protein expression within cells (10, 70). This report clearly shows that the three CLPs, termed CLP-150, CLP-170, and CLP-220, are greatly reduced in their expression in microsomal vesicles isolated from dystrophin-deficient skeletal muscle homogenates. The finding that the overall Ca^{2+} binding capacity of SR vesicles derived from *mdx* muscle fibers is reduced by 20% agrees with this result.

On the other hand, the impaired luminal Ca^{2+} buffering might not be exclusively based on changes in the

expression of CLPs. First, under normal resting conditions, probably not all ion-binding sites are occupied in the luminal Ca^{2+} reservoir complex of the terminal cisternae region (5). Second, calsequestrin of apparent 63 kDa is a much more abundant Ca^{2+} binding protein compared with all other Ca^{2+} buffering elements in the SR, including the CLPs (45). It is, therefore, possible that even small changes in the 63-kDa calsequestrin isoform might account, at least partially, for the observed decrease in the overall Ca^{2+} binding capacity of the SR from dystrophic *mdx* skeletal muscle fibers. On the other hand, if a very large fraction of calsequestrin binding sites is not occupied in muscle fibers at rest, then a moderate reduction in Ca^{2+} binding might be without any functional significance. However, it is unlikely that a reduction in one-fifth of the buffering capacity does not influence overall Ca^{2+} cycling patterns.

Both the pathobiochemical status of the CLPs and the pathophysiological status of the luminal Ca^{2+} reservoir complex suggest that dystrophic skeletal muscle fibers exhibit abnormal ion homeostasis. The idea that changes in Ca^{2+} handling play an important role in the degeneration process of dystrophin-deficient fibers is confirmed by the analysis of myotubes from transgenic *mdx* mice expressing dystrophin (16). Whereas dystrophic fibers show higher resting levels of subsarcolemmal free calcium ions (48), which in turn appears to

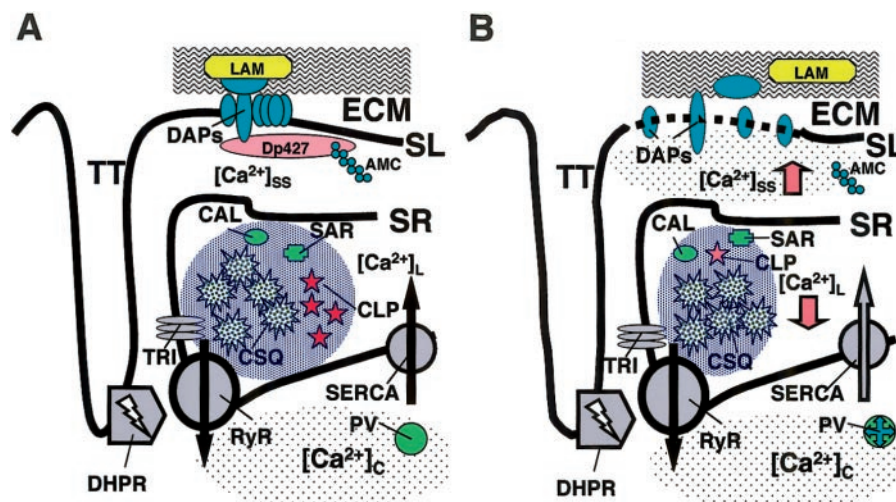


Fig. 7. Diagrammatic representation of the possible involvement of CLPs in the molecular pathogenesis of muscular dystrophy. Based on the analysis of Ca^{2+} regulatory membrane proteins in dystrophin-deficient *mdx* muscle preparations presented in this report, the model diagrams comparatively illustrate apparent differences in Ca^{2+} handling in normal (A) and dystrophic (B) skeletal muscle fibers. A major difference between normal and dystrophic fibers appears to be a drastic reduction in CLPs in the lumen of the sarcoplasmic reticulum (SR). The primary deficiency in the dystrophin isoform Dp427 triggers a reduction in sarcolemmal (SL) dystrophin-associated proteins (DAPs), which in turn may impair the linkage between the subsarcolemmal actin cytoskeleton (AMC) and the extracellular matrix (ECM) component LAM. Although no massive change in the overall cytosolic Ca^{2+} levels ($[\text{Ca}^{2+}]_c$) in dystrophic muscle fibers has been confirmed, influx of calcium ions trigger an increase in subsarcolemmal ion levels ($[\text{Ca}^{2+}]_{ss}$), causing increased Ca^{2+} -dependent proteolysis. Variations in the subsarcolemmal Ca^{2+} level is accompanied by a distinct reduction in CLPs. This then could result in impaired Ca^{2+} sequestration within the lumen ($[\text{Ca}^{2+}]_L$) of the SR, thereby contributing to the molecular pathogenesis of muscular dystrophy. In addition, impaired functioning of the SERCA and/or an increased turnover of the fast-twitch Ca^{2+} binding protein parvalbumin (PV) might be involved in abnormal ion handling. Other key components of Ca^{2+} homeostasis and excitation-contraction coupling are also indicated in the diagram: the transverse-tubular (TT) DHPR, the RyR Ca^{2+} release channel, sarcalumenin (SAR), triadin (TRI), and calreticulin (CAL).

increase Ca^{2+} -dependent proteolysis (3), restoration of dystrophin results in normal resting Ca^{2+} levels and Ca^{2+} leak channel activity (16). Hence the stabilization of the sarcolemmal membrane cytoskeleton via restoration of dystrophin in transgenic *mdx* mice strongly indicates that abnormal intracellular Ca^{2+} levels participate in the pathophysiological mechanisms of muscular dystrophy (2). In agreement with this hypothesis, we show here that a possible central factor in the abnormal ion homeostasis of dystrophic cells is abnormal Ca^{2+} binding in the lumen of the SR.

In addition to the reduced expression of Ca^{2+} binding proteins of the terminal cisternae region in dystrophin-deficient skeletal muscle fibers, previous studies on dystrophic muscle have shown that the SR Ca^{2+} -ATPase, calsequestrin, the dihydropyridine receptor, and the cytoplasmic Ca^{2+} binding protein parvalbumin might be affected in the disease process (27, 36, 56, 61). The analysis of muscle specimens from patients afflicted with Duchenne muscular dystrophy using the cationic carbocyanine dye Stains-All indicated a reduced expression of the 63-kDa calsequestrin band (56). Our immunoblot analysis with the highly specific MAb VIIIID1₂ to calsequestrin did not confirm this finding with respect to *mdx* muscle preparations. The previous finding that mRNA levels of the dihydropyridine receptor are affected in *mdx* muscle fibers (61) does not seem to be reflected on the protein level. Immunoblotting with antibodies to the α_{1S} -subunit, α_2 -subunit, and β -subunit of this transverse-tubular receptor did not reveal any drastic changes in its expression profile in dystrophic microsomes. In addition to reduced Ca^{2+} binding in dystrophic muscle presented here, the SERCA Ca^{2+} -ATPase isoform was shown to be functionally altered in muscular dystrophy (36). Although we can show here that neither the relative abundance nor the total enzyme activity of this pump protein is altered, the previous biochemical analysis suggests that the maximum velocity of Ca^{2+} uptake is impaired in dystrophic *mdx* fibers (36). In addition, findings on the major cytoplasmic Ca^{2+} binding element parvalbumin in dystrophic fast-twitch fibers (27) suggest that overall Ca^{2+} handling is reorganized in *mdx* muscle. The fact that parvalbumin protein expression is not changed in muscular dystrophy (34), but an upregulation of parvalbumin mRNA levels is observed (27), implies increased turnover rates for this protein. Thus changes in both luminal and cytosolic Ca^{2+} buffering and Ca^{2+} uptake into the SR might contribute to pathophysiological Ca^{2+} cycling in dystrophin-deficient fibers. Additional unknown factors are the dystrophic status of the sarcolemmal calmodulin-dependent Ca^{2+} -ATPase and the surface $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Potential abnormalities in these Ca^{2+} handling components might also play a role in altered ion homeostasis in *mdx* muscle fibers.

In conclusion, as diagrammatically shown in Fig. 7, the reduction in CLPs represents a major difference between normal and dystrophic skeletal muscle fibers. The deficiency in the dystrophin isoform Dp427 leads primarily to a reduction in dystrophin-associated pro-

teins, such as the sarcoglycans, dystroglycans and sarcospan. This in turn may directly trigger destruction of the plasmalemmal integrity by weakening the link between the subsarcolemmal actin cytoskeleton and the extracellular matrix component laminin. Influx of calcium ions causes an increase in intracellular levels, and that could be an important factor in the Ca^{2+} -induced myonecrosis. As demonstrated in this study, these changes in the subsarcolemmal Ca^{2+} level are accompanied by a distinct reduction in Ca^{2+} binding proteins. The pathophysiological consequence of this variation in protein expression is impaired Ca^{2+} sequestration within the lumen of the SR. Hence, the lack of the dystrophin-glycoprotein complex may trigger disturbed surface Ca^{2+} fluxes, which then influence downstream Ca^{2+} handling, thereby resulting in distinct changes in the expression profile of a subset of key Ca^{2+} handling proteins. This might explain one of the important steps in the molecular pathogenesis of muscular dystrophy.

We thank Drs. Kevin P. Campbell (University of Iowa, Iowa City, IA) and Steve Cala (Wayne State University, Detroit, MI) for supplying us with antibodies.

Research was supported by project grants from the European Commission (FMRX-CT960032 and RTN2-2001-00337), Enterprise Ireland (SC/2000/386), and the Irish Health Research Board (HRB-01/01).

REFERENCES

1. **Ahn AH and Kunkel LM.** The structural and functional diversity of dystrophin. *Nat Genet* 3: 283–291, 1993.
2. **Alderton JM and Steinhardt RA.** How calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. *Trends Cardiovasc Med* 10: 268–272, 2000.
3. **Alderton JM and Steinhardt RA.** 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, 2000.
4. **Allamand V and Campbell KP.** Animal models for muscular dystrophy: valuable tools for the development of therapies. *Hum Mol Genet* 9: 2459–2467, 2000.
5. **Cala SE, Scott BT, and Jones LR.** Intraluminal sarcoplasmic reticulum Ca^{2+} -binding proteins. *Semin Cell Biol* 1: 265–275, 1990.
6. **Campbell KP.** Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. *Cell* 80: 675–679, 1995.
7. **Cannell MB and Soeller C.** Sparks of interest in cardiac excitation-contraction coupling. *Trends Pharmacol Sci* 19: 16–20, 1998.
8. **Catterall WA.** Structure and function of voltage-gated ion channels. *Annu Rev Biochem* 64: 493–532, 1995.
9. **Chan KM, Delfert D, and Junger KD.** A direct colorimetric assay for Ca^{2+} -stimulated ATPase activity. *Anal Biochem* 157: 375–380, 1986.
10. **Chen YW, Zhao P, Borup R, and Hoffman EP.** Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology. *J Cell Biol* 151: 1321–1336, 2000.
11. **Cohn RD and Campbell KP.** Molecular basis of muscular dystrophies. *Muscle Nerve* 23: 1456–1471, 2000.
12. **Collet C, Allard B, Tourneur Y, and Jacquemond V.** Intracellular calcium signals measured with indo-1 in isolated skeletal muscle fibres from control and *mdx* mice. *J Physiol* 520: 417–429, 1999.
13. **Culligan K, Glover L, Dowling P, and Ohlendieck K.** Brain dystrophin-glycoprotein complex: persistent expression of β -dystroglycan, impaired oligomerisation of Dp71 and up-regulation

- of utrophins in animal models of muscular dystrophy. *BMC Cell Biol* 2: 2, 2001.
14. **Culligan K, Mackey A, Finn D, Maguire PB, and Ohlendieck K.** Role of dystrophin isoforms and associated glycoproteins in muscular dystrophy. *Int J Mol Med* 2: 639–648, 1998.
 15. **De Luca A, Pierno S, Liantonio A, Cetrone M, Camerino C, Simonetti S, Papadia F, and Camerino DC.** 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, 2001.
 16. **Denetclaw WF, Hopf FW, Cox GA, Chamberlain JS, and Steinhardt RA.** Myotubes from transgenic mdx mice expressing full-length dystrophin show normal calcium regulation. *Mol Biol Cell* 5: 1159–1167, 1994.
 17. **Engel AG, Yamamoto M, and Fischbeck KH.** Dystrophinopathies. In: *Myology, Basic and Clinical* (2nd Ed.), edited by Engel AG, Yamamoto M, and Fischbeck KM. New York: McGraw-Hill, 1994, p. 1133–1187.
 18. **Ettienne EM, Swartz K, and Singer RH.** Increased turnover of proteins from the sarcoplasmic reticulum of dystrophic chicken muscle cells in tissue culture. *J Biol Chem* 256: 6408–6412, 1981.
 19. **Fong PY, Turner PR, Denetclaw WF, and Steinhardt RA.** Increased activity of calcium leak channels in myotubes of Duchenne human and mdx mouse origin. *Science* 250: 673–676, 1990.
 20. **Franzini-Armstrong C and Protasi F.** Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiol Rev* 77: 699–729, 1997.
 21. **Froemming GR, Murray BE, Harmon S, Pette D, and Ohlendieck K.** Comparative analysis of the isoform expression pattern of Ca²⁺-regulatory membrane proteins in fast-twitch, slow-twitch, cardiac, neonatal and chronic low-frequency stimulated muscle fibres. *Biochim Biophys Acta* 1466: 151–168, 2000.
 22. **Froemming GR, Murray BE, and Ohlendieck K.** Self-aggregation of triadin in the sarcoplasmic reticulum of rabbit skeletal muscle. *Biochim Biophys Acta* 1418: 197–205, 1999.
 23. **Froemming GR and Ohlendieck K.** The native dihydropyridine receptor exists as a supramolecular complex in skeletal muscle. *Cell Mol Life Sci* 58: 312–320, 2001.
 24. **Froemming GR and Ohlendieck K.** Role of ion-regulatory membrane proteins in inherited muscle diseases. *Front Biosci* 6: D65–D74, 2001.
 25. **Froemming GR, Pette D, and Ohlendieck K.** The 90 kDa junctional sarcoplasmic reticulum protein forms an integral part of a supramolecular triad complex in skeletal muscle. *Biochem Biophys Res Commun* 261: 603–609, 1999.
 26. **Gailly P, Boland B, Himpens B, Casteels R, and Gillis JM.** Critical evaluation of cytosolic calcium determination in resting muscle fibres from normal and dystrophic (mdx) mice. *Cell Calcium* 14: 473–483, 1993.
 27. **Gailly P, Hermans E, Octave JN, and Gillis JM.** Specific increase in genetic expression of parvalbumin in fast skeletal muscle of mdx mice. *FEBS Lett* 326: 272–274, 1993.
 28. **Gillis JM.** 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, 1996.
 29. **Glover L, Culligan K, Cala S, Mulvey C, and Ohlendieck K.** Calsequestrin binds to monomeric and complexed forms of key calcium-handling proteins in native sarcoplasmic reticulum membranes from rabbit skeletal muscle. *Biochim Biophys Acta* 1515: 120–132, 2001.
 30. **Gurnett CA and Campbell KP.** Transmembrane auxiliary subunits of voltage-dependent ion channels. *J Biol Chem* 271: 27975–27978, 1996.
 31. **Harmon S, Froemming GR, Leisner E, Pette D, and Ohlendieck K.** Selected contribution: Low-frequency stimulation of fast muscle affects the abundance of Ca²⁺-ATPase but not its oligomeric status. *J Appl Physiol* 90: 371–379, 2001.
 32. **Head SI.** Membrane potential, resting calcium and calcium transients in isolated muscle fibres from normal and dystrophic mice. *J Physiol* 469: 11–19, 1993.
 33. **Hopf FW, Turner PR, Denetclaw WF, Reddy P, and Steinhardt RA.** A critical evaluation of resting intracellular free calcium regulation in dystrophic mdx muscle. *Am J Physiol Cell Physiol* 271: C1325–C1339, 1996.
 34. **Jockusch H, Friedrich G, and Zippel M.** Serum parvalbumin, an indicator of muscle disease in murine dystrophy and myotonia. *Muscle Nerve* 13: 551–555, 1990.
 35. **Jurkat-Rott K, McCarthy T, and Lehmann-Horn F.** Genetics and pathogenesis of malignant hyperthermia. *Muscle Nerve* 23: 4–17, 2000.
 36. **Kargacin ME and Kargacin GJ.** The sarcoplasmic reticulum calcium pump is functionally altered in dystrophic muscle. *Biochim Biophys Acta* 1290: 4–8, 1996.
 37. **Khammari A, Perea Y, Baudet S, and Noireaud J.** In situ study of the sarcoplasmic reticulum function in control and mdx mouse diaphragm muscle. *Can J Physiol Pharmacol* 76: 1161–1165, 1998.
 38. **Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, and Kunkel LM.** Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* 50: 509–517, 1987.
 39. **Leberer E, Hartner KT, and Pette D.** Postnatal development of Ca²⁺-sequestration by the sarcoplasmic reticulum of fast and slow muscles in normal and dystrophic mice. *Eur J Biochem* 174: 247–253, 1988.
 40. **Leberer E, Timms BG, Campbell KP, and MacLennan DH.** Purification, calcium binding properties, and ultrastructural localization of the 53,000- and 160,000 (sarcalumenin)-dalton glycoproteins of the sarcoplasmic reticulum. *J Biol Chem* 265: 10118–10124, 1990.
 41. **Lennon N and Ohlendieck K.** Impaired Ca²⁺-sequestration in dilated cardiomyopathy. *Int J Mol Med* 7: 131–141, 2001.
 42. **Leong P and MacLennan DH.** Complex interactions between skeletal muscle ryanodine receptor and dihydropyridine receptor proteins. *Biochem Cell Biol* 76: 681–694, 1998.
 43. **Lynch GS, Rafael JA, Chamberlain JS, and Faulkner JA.** Contraction-induced injury to single permeabilized muscle fibers from mdx, transgenic mdx, and control mice. *Am J Physiol Cell Physiol* 279: C1290–C1294, 2000.
 44. **MacLennan DH.** Ca²⁺ signalling and muscle disease. *Eur J Biochem* 267: 5291–5297, 2000.
 45. **MacLennan DH and Reithmeier RA.** Ion tamers. *Nat Struct Biol* 5: 409–411, 1998.
 46. **MacLennan DH, Rice WJ, and Green NM.** The mechanism of Ca²⁺ transport by sarco(endo)plasmic reticulum Ca²⁺-ATPases. *J Biol Chem* 272: 28815–28818, 1997.
 47. **Maguire PB, Briggs FN, Lennon N, and Ohlendieck K.** Oligomerisation is an intrinsic property of calsequestrin in normal and stimulated canine skeletal muscle. *Biochem Biophys Res Commun* 240: 721–727, 1997.
 48. **Mallouk N, Jacquemond V, and Allard B.** Elevated subsarcolemmal Ca²⁺ in mdx mouse skeletal muscle fibers detected with Ca²⁺-activated K⁺ channels. *Proc Natl Acad Sci USA* 97: 4950–4955, 2000.
 49. **McCarter GC and Steinhardt RA.** Increased activity of calcium leak channels caused by proteolysis near sarcolemmal ruptures. *J Membr Biol* 176: 169–174, 2000.
 50. **Melzer W, Herrmann-Frank A, and Lüttgau HC.** The role of Ca²⁺ ions in excitation-contraction coupling of skeletal muscle fibres. *Biochim Biophys Acta* 1241: 59–116, 1995.
 51. **Menke A and Jockusch H.** Decreased osmotic stability of dystrophin-less muscle cells from mdx mouse. *Nature* 349: 69–71, 1991.
 52. **Michalak M, Mariani P, and Opas M.** Calreticulin, a multifunctional Ca²⁺ binding chaperone of the endoplasmic reticulum. *Biochem Cell Biol* 76: 779–785, 1998.
 53. **Moro G, Saborido A, Delgado J, Molano F, and Megias A.** Dihydropyridine receptors in transverse tubules from normal and dystrophic chicken skeletal muscle. *J Muscle Res Cell Motil* 16: 529–542, 1995.
 54. **Murray BE, Froemming GR, Maguire PB, and Ohlendieck K.** Excitation-contraction-relaxation cycle: role of Ca²⁺-regula-

- tory membrane proteins in normal, stimulated and pathological skeletal muscle fibres. *Int J Mol Med* 1: 677–697, 1998.
55. **Murray BE and Ohlendieck K.** Cross-linking analysis of the ryanodine receptor and α_1 -dihydropyridine receptor in rabbit skeletal muscle triads. *Biochem J* 324: 689–696, 1997.
 56. **Niebroj-Dobosz I, Kornguth S, Schutta H, Siegel FL, and Hausmanowa-Petrusewicz I.** Proteins of muscle subcellular fractions in Duchenne progressive muscular dystrophy stained with “stains-all” cationic carbocyanine dye and with Coomassie Blue. *Muscle Nerve* 12: 273–280, 1989.
 57. **Nori A, Gola E, Tosato S, Cantini M, and Volpe P.** Targeting of calsequestrin to sarcoplasmic reticulum after deletions of its acidic carboxy terminus. *Am J Physiol Cell Physiol* 277: C974–C981, 1999.
 58. **Ohkura M, Furukawa K, Fujimori H, Kuruma A, Kawano S, Hiraoka M, Kuniyasu A, Nakayama H, and Ohizumi Y.** Dual regulation of the skeletal muscle ryanodine receptor by triadin and calsequestrin. *Biochemistry* 37: 12987–12993, 1998.
 59. **Ohlendieck K.** 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, 1996.
 60. **Ohlendieck K.** Extraction of membrane proteins. *Methods Mol Biol* 59: 293–304, 1996.
 61. **Pereon Y, Dettbarn C, Navarro J, Noireaud J, and Palade PT.** Dihydropyridine receptor gene expression in skeletal muscle from mdx and control mice. *Biochim Biophys Acta* 1362: 201–207, 1997.
 62. **Pessah IN and Schiedt MJ.** Early over-expression of low-affinity [3 H]ryanodine receptor sites in heavy sarcoplasmic reticulum fraction from dystrophic chicken pectoralis major. *Biochim Biophys Acta* 1023: 98–106, 1990.
 63. **Preedy VR, Patel VB, Reilly ME, Richardson PJ, Falkous G, and Mantle D.** Oxidants, antioxidants and alcohol: implications for skeletal and cardiac muscle. *Front Biosci* 4: E58–E66, 1999.
 64. **Pressmar J, Brinkmeier H, Seewald MJ, Naumann T, and Rudel R.** Intracellular Ca^{2+} concentrations are not elevated in resting cultured muscle from Duchenne (DMD) patients and in MDX mouse muscle fibres. *Pflugers Arch* 426: 499–505, 1994.
 65. **Robert V, Massimino ML, Tosello V, Marsault R, Cantini M, Sorrentino V, and Pozzan T.** Alteration in calcium handling at the subcellular level in mdx myotubes. *J Biol Chem* 276: 4647–4651, 2001.
 66. **Stokes DL and Wagenknecht T.** Calcium transport across the sarcoplasmic reticulum: structure and function of Ca^{2+} -ATPase and the ryanodine receptor. *Eur J Biochem* 267: 5274–5279, 2000.
 67. **Torres LFB and Duchen LW.** The mutant mdx: inherited myopathy in the mouse. *Brain* 110: 269–299, 1987.
 68. **Turner PR, Fong PY, Denetclaw WF, and Steinhardt RA.** Increased calcium influx in dystrophic muscle. *J Cell Biol* 115: 1701–1712, 1991.
 69. **Turner PR, Schultz R, Ganguly B, and Steinhardt RA.** Proteolysis results in altered leak channel kinetics and elevated free calcium in mdx muscle. *J Membr Biol* 133: 243–251, 1993.
 70. **Turner PR, Westwood T, Regen CM, and Steinhardt RA.** Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. *Nature* 335: 735–738, 1988.
 71. **Yap JL and MacLennan DH.** Characterization of the adenosinetriphosphatase and calsequestrin isolated from sarcoplasmic reticulum of normal and dystrophic chickens. *Can J Biochem* 54: 670–673, 1976.
 72. **Zorzato F, Anderson C, Ohlendieck K, Froemming GR, Guerrini R, and Treves S.** Identification of a novel 45 kDa protein (JP-45) from rabbit sarcoplasmic reticulum junctional face membrane. *Biochem J* 351: 537–543, 2000.