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

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**The Dysregulation of Ca²⁺ Handling**

The dysregulation of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺-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 Ca²⁺ homeostasis in dystrophin-deficient muscle cells (28). Although Steinhardt and co-workers (2, 3, 19, 33, 68, 69) have postulated that ion flux through Ca²⁺ leak channels is responsible for an elevated Ca²⁺-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 Ca²⁺ levels (12, 26, 32, 37, 64). An explanation for the contradictory findings could be that the cytosolic Ca²⁺ overload is not global but restricted to subsarcolemmal domains in dystrophic muscle fibers (48, 49). In addition, abnormal Ca²⁺ 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 Ca²⁺ disturbance, even small changes in Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ release system of the sarcoplasmic reticulum (SR) (50, 54). Two well-character-
alyzed 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 \(\alpha_{1S}\)-subunit, which contains the voltage-sensing domain and the pore-forming structures, as well as the auxiliary subunits \(\alpha_\delta\), \(\beta\), and \(\gamma\), 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 pathobiocchemical 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-
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 $^{45}$CaCl$_2$, were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Superfrost Plus positively charged microscope slides were from Menzel Gla
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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-regular muscle proteins was performed as previously described (13, 21). Monoclonal antibody (Mab) IIIDS against the $\alpha_1$-subunit of the dihydropyridine receptor and Mab VD2; to the $\beta$-subunit of the dihydropyridine receptor were a generous gift from Dr. Kevin P. Campbell (University of Iowa, Iowa City, IA). A polyclonal antibody to o-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 junctional 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 $\beta$- dystroglycan, Mab DYS-1 to the Dp427 rod domain), Upstate Biotechnology (Lake Placid, NY); Mab VIA4, to o-dystroglycan, Mab e464.6 to the $\alpha$-subunit of the Na$^+$/K$^+$-ATPase, Affinity Bioreagents (Golden, CO); Mab VIID12 to calsequestrin; Mab 20A to the $\alpha_2$-subunit of the dihydropyridine receptor, Mab I11 to the fast-twitch sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA), Sigma Chemical (MAb 34C to the SR Ca$^{2+}$-ATPase), and Sigma Chemical (MAb 34C to the SR Ca$^{2+}$-ATPase (SERCA) isoform (13). Peptides had been synthesized and coupled to keyhole limpet hemocyanin carrier by Research Genetics (Huntington, AL). Antisera to junctional were a generous gift of Dr. Steve Cala (Wayne State University, Detroit, MI) (29).

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**Immunoprecipitation.** Comparative immunoprecipitation experiments were performed as previously described (29). Microsomal membranes from normal control and dystrophic mdx muscles 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 peffaboloc), 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 VIID12 to calsequestrin for 2 h at 4°C. Antibody-antigen 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 same 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 $^{45}$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 $^{45}$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 matrix 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\(_{15S}\)-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 VIIID12, exhibited a greatly reduced expression in mdx preparations (Fig. 1L). These luminal SR
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 -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 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.
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 respect to pI values, immunoblotting of standard two-dimensional gels with isoelectric focusing in the first dimension and reducing SDS-polyacrylamide 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 110-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. 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 $-2 \mu$mol Pi/mg protein $^{-1}$-min $^{-1}$. Error bars, SD.

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 $\mu$m.
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 \(-20\%\) decrease in the overall capacity to sequester calcium ions was demonstrated in dystrophin-deficient microsomal membranes. This pathophysiologically 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-

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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

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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 ([Ca\(^{2+}\)]\(_c\)) in dystrophic muscle fibers has been confirmed, influx of calcium ions trigger an increase in subsarcolemmal ion levels ([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 ([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 affected 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 \(\alpha_{S0}\)-subunit, \(\alpha_{2}\)-subunit, and \(\beta\)-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 Na\(^{+}\)/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 proteins, 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.

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