

Subproteomics analysis of Ca²⁺-binding proteins demonstrates decreased calsequestrin expression in dystrophic mouse skeletal muscle

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Duchenne muscular dystrophy represents one of the most common hereditary diseases. Abnormal ion handling is believed to render dystrophin-deficient muscle fibres more susceptible to necrosis. Although a reduced Ca²⁺ buffering capacity has been shown to exist in the dystrophic sarcoplasmic reticulum, surprisingly no changes in the abundance of the main luminal Ca²⁺ reservoir protein calsequestrin have been observed in microsomal preparations. To address this unexpected finding and eliminate potential technical artefacts of subcellular fractionation protocols, we employed a comparative subproteomics approach with total mouse skeletal muscle extracts. Immunoblotting, mass spectrometry and labelling of the entire muscle protein complement with the cationic carbocyanine dye 'Stains-All' was performed in order to evaluate the fate of major Ca²⁺-binding proteins in dystrophin-deficient skeletal muscle fibres. In contrast to a relatively comparable expression pattern of the main protein population in normal vs. dystrophic fibres, our

analysis showed that the expression of key Ca²⁺-binding proteins of the luminal sarcoplasmic reticulum is drastically reduced. This included the main terminal cisternae constituent, calsequestrin, and the previously implicated Ca²⁺-shuttle element, sarcalumenin. In contrast, the 'Stains-All'-positive protein spot, representing the cytosolic Ca²⁺-binding component, calmodulin, was not changed in dystrophin-deficient fibres. The reduced 2D 'Stains-All' pattern of luminal Ca²⁺-binding proteins in mdx preparations supports the calcium hypothesis of muscular dystrophy. The previously described impaired Ca²⁺ buffering capacity of the dystrophic sarcoplasmic reticulum is probably caused by a reduction in luminal Ca²⁺-binding proteins, including calsequestrin.

Keywords: calsequestrin; mdx; mouse skeletal muscle; muscular dystrophy; sarcalumenin.

Duchenne muscular dystrophy is a lethal genetic disease of childhood that affects approximately 1 in 3500 live males at birth, making it the most frequent neuromuscular disorder in humans [1]. Since the pioneering discovery of the *DMD* gene encoding the membrane cytoskeletal protein, dystrophin [2], and the biochemical identification of a dystrophin-associated surface glycoprotein complex [3], a variety of promising therapeutic strategies have been suggested to counteract the muscle-wasting symptoms associated with X-linked muscular dystrophy [4]. This includes pharmacological intervention [5–8], myoblast transfer [9] and stem cell therapy [10,11], as well as gene therapy [12–15]. However, to date no therapeutic approach has been developed that provides a long-lasting abolishment of progressive muscle wasting in humans. Gene therapy is associated with serious immunological deficiencies, and the success of cell-based

therapies is hindered by a lack of the efficient introduction of sufficient amounts of dystrophin-positive muscle precursor cells into bulk tissue. Biological approaches, such as the up-regulation of utrophin [16] or inhibition of myostatin [8], may not result in long-term improvement because of difficulties with the regeneration of dystrophin-deficient fibres [5]. This array of biomedical problems suggests that it would be worthwhile studying alternative approaches.

To overcome the potential problems associated with drug-, cell- or gene-based therapy approaches, and in order to unravel new pathophysiological factors, the application of high-throughput analyses, such as microarray technology or proteomics screening, might unearth new targets in the treatment of muscular dystrophy [17]. Expression profiling to define the molecular steps involved in X-linked muscular dystrophy by Tkatchenko *et al.* [18] and Chen *et al.* [19] suggests that, besides other destructive mechanisms, abnormal ion handling triggers an altered developmental programming in degenerating and regenerating fibres. This agrees with the calcium hypothesis of muscular dystrophy [20–22]. Deficiency in the Dp427 isoform of dystrophin results in the reduction of a specific subset of sarcolemmal glycoproteins [23,24]. The lack of the surface membrane-stabilizing dystrophin–glycoprotein complex causes the loss of a proper *trans*-sarcolemmal linkage between the actin membrane cytoskeleton and the

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Abbreviations: ECL, enhanced chemiluminescence; IPG, immobilized pH gradient.

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extracellular matrix component laminin [25]. This, in turn, renders the sarcolemma more susceptible to microrupturing [26]. Probably, the introduction of Ca^{2+} leak channels during the natural process of surface membrane resealing triggers increased cytosolic Ca^{2+} levels in dystrophin-deficient muscle fibres [27]. Increased cytosolic Ca^{2+} levels contribute to enhanced protease activity, resulting in muscle degeneration [28].

In addition to disturbed cytosolic Ca^{2+} levels, the Ca^{2+} buffering capacity of the dystrophic sarcoplasmic reticulum is also significantly impaired [29]. The pathophysiological consequence of a reduced Ca^{2+} -binding capacity of the sarcoplasmic reticulum is an amplification of the elevated free cytosolic Ca^{2+} levels in muscular dystrophy, thereby accelerating the Ca^{2+} -dependent proteolysis of muscle proteins [20–22]. Recent studies suggest that this is partially caused by a reduction in the minor Ca^{2+} -binding protein, sarcalumenin [30], and possibly because of an altered oligomerization status of the major luminal Ca^{2+} reservoir element, calsequestrin [31]. Surprisingly, immunoblotting of calsequestrin revealed no changes in the abundance of the 63 kDa molecular mass monomer in normal vs. dystrophic microsomes [29]. As subcellular fractionation protocols may distort comparative immunoblotting data, it was of interest to re-examine the fate of calsequestrin by studying the entire complement of key Ca^{2+} -binding elements in dystrophin-deficient skeletal muscle fibres. Because the carbocyanide dye 'Stains-All' represents an established biochemical tool to reproducibly visualize Ca^{2+} -binding proteins following electrophoretic separation [32], we combined the 2D gel technique, dye binding and mass spectrometry to identify 'Stains-All'-labelled muscle proteins and thereby determine, reliably, changes in their expression levels in muscular dystrophy. This approach identified 11 major dye-positive elements in normal fibres and a reduction in eight of these protein species in mdx fibres, including the 63 kDa molecular mass spot representing the calsequestrin monomer. Thus, in addition to our previous observation that minor Ca^{2+} -binding elements, such as sarcalumenin [30], and the calsequestrin-like proteins CLP-150, CLP-170 and CLP-220 [29], are affected in dystrophin-deficient fibres, this study demonstrates that the main luminal Ca^{2+} -binding protein, calsequestrin, is also greatly reduced in mdx skeletal muscles. Hence, impaired Ca^{2+} buffering of the dystrophic sarcoplasmic reticulum appears to be caused by the abnormal expression of the main luminal Ca^{2+} -binding protein species.

Experimental procedures

Materials

Electrophoresis grade chemicals, the PhastGel protein silver staining kit, the PhastGel Coomassie Blue R-350 staining kit and immobilized pH gradient (IPG) strips of pH 3–10 (linear) and IPG buffer of pH 3–10 were obtained from Amersham Biosciences (Little Chalfont, Bucks., UK). Sequencing grade-modified trypsin was from Promega (Madison, WI, USA). C-18 Zip-Tips for desalting were purchased from Millipore Ireland B.V. (Carrigtwohill, Co. Cork, Ireland). All chemicals used for MALDI-ToF mass spectrometry were obtained from Sigma Chemical

Company (Poole, Dorset, UK), with the exception of acetonitrile (Amersham Biosciences) and the α -cyano-4-hydroxycinnamic acid matrix kit (Laserbiolabs, Sophia-Antipolis, France). Protease inhibitors were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Chemiluminescence substrates were obtained from Perbio Science UK (Tattenhall, Cheshire, UK). Primary antibodies were from Affinity Bioreagents (Golden, CO, USA; mAb VIIIID₁₂ to calsequestrin, mAb XIIC4 to sarcalumenin, mAb IIIH11 to the fast SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+} ATPase, mAb IIID5 to the α_1 -subunit of the dihydropyridine receptor, and pAb to calreticulin), (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK; mAb DYS-2 to the C-terminus of the dystrophin isoform Dp427), Sigma Chemical Company (mAb 6D4 to calmodulin) and Upstate Biotechnology (Lake Placid, NY, USA; mAb C464.6 to the α_1 -subunit of the Na^+/K^+ ATPase and mAb VIA₄₁ to α -dystroglycan). Peroxidase-conjugated secondary antibodies were obtained from Chemicon International (Temecula, CA, USA). Protran nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany). All other chemicals used were of analytical grade and purchased from Sigma Chemical Company.

Preparation of total muscle extracts

For the comparative gel electrophoretic analysis of normal vs. dystrophic skeletal muscle fibres, total extracts of the muscle protein complement were prepared from 9-week-old normal control C57BL/10 mice and age-matched mdx mice of the Dmd^{mdx} strain (Jackson Laboratory, Bar Harbor, ME, USA). One gram of fresh tissue was quick-frozen in liquid nitrogen and ground into fine powder using a pestle and mortar. Subsequently the muscle tissue powder was resuspended in 5 mL of ice-cold buffer A [0.175 M Tris/HCl, pH 8.8, 5% (w/v) SDS, 15% (v/v) glycerol, 0.3 M dithiothreitol]. To avoid protein degradation, the solution was supplemented with a freshly prepared protease inhibitor cocktail (0.2 mM pefabloc, 1.4 μM pepstatin, 0.15 μM aprotinin, 0.3 μM E-64, 1 μM leupeptin, 0.5 mM soybean trypsin inhibitor and 1 mM EDTA) [33]. In order to eliminate excessive viscosity of the extract as a result of DNA, 2 μL of DNase I (200 units) was added per 100 μL of buffer [30]. Following filtration through two layers of miracloth and the addition of four volumes of ice-cold 100% (v/v) acetone, the tissue homogenate was mixed by vortexing and then incubated for 1 h at -20°C to precipitate the total protein fraction. The suspension was centrifuged at 5000 *g* for 15 min. The resulting protein pellet was washed in 20 mL of ice-cold 80% (v/v) acetone and thoroughly broken up by vortexing and sonication. The centrifugation and washing step was repeated once and the final protein precipitate collected by centrifugation and resuspended in 1 mL of buffer B [9.5 M urea, 4% (w/v) CHAPS, 0.5% (w/v) ampholytes, pH 3–10, and 100 mM dithiothreitol] by gentle pipetting and vortexing. After incubation for 3 h at room temperature (whereby samples were vortexed every 10 min for 5 s), the suspension was centrifuged at 4°C in an Eppendorf 5417R centrifuge (Eppendorf, Hamburg, Germany) for 20 min at 20 000 *g* and then subjected to isoelectric focusing.

Gel electrophoretic separation for muscle proteomics

As only limited technical information exists on the specific identification of skeletal muscle proteins by proteomics analysis [34,35], we followed the general practical recommendations of Westermeier & Naven [36] for our MS-based proteomics approach. Isoelectric focusing was performed using an IPGphor focusing system from Amersham Biosciences, with 13 cm IPG strips of pH 3–10 (linear) and 50 μ A per strip, as previously described in detail [37]. Total muscle protein extracts were diluted in the above described buffer A [complemented with 0.05% (w/v) bromphenol blue as a tracking dye] to achieve a final protein concentration of 50 μ g of protein per IEF strip for silver staining, hot Coomassie staining, 'Stains-All' labelling or immunoblotting. The following running conditions were used: 60 min at 100 V, 60 min at 500 V, 60 min at 1000 V, and a final step of 150 min at 8000 V. Separation in the second dimension was performed with a 12% (w/v) resolving gel using the Protean Xi-II Cell from Bio-Rad Laboratories (Hemel Hempstead, Herts., UK) [33].

Protein visualization for muscle proteomics

For hot Coomassie staining, PhastGel Coomassie Blue R-350 tablets were used. The staining solution consisted of one PhastGel blue tablet that had been dissolved in 1.6 L of 10% (v/v) acetic acid to give a 0.025% (w/v) dye staining solution. The dye-containing solution was heated to 90 °C and carefully poured over the 2D gel in a stainless steel tray. The tray was then placed on top of a hot plate and the temperature maintained at 90 °C for 5 min to aid the staining of protein spots. The tray was then placed on a laboratory shaker for a further 10 min at room temperature. Destaining was achieved by placing gels in a 10% (v/v) acetic acid solution and slow agitation overnight. Excess Coomassie dye was soaked up by filter paper presented in the destaining solution. Gels were processed immediately for mass spectrometric analysis or stored in a plastic folder with 10 mL of a 1% (v/v) acetic acid solution and were stored at 4 °C until further usage. For silver staining, the PhastGel protein silver staining kit was used (omitting glutaraldehyde from the sensitizing solution and formaldehyde from the silver staining solution to allow for compatibility) to identify protein spots by MALDI-ToF MS. Densitometric scanning of Coomassie- or silver-stained gels was performed on a Molecular Dynamics 300S computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA) with IMAGEQUANT V3.0 software. Major Ca²⁺-binding proteins were identified by labelling with the cationic carbocyanine dye, 'Stains-All', according to the method of Campbell *et al.* [32]. Following the second dimension electrophoretic separation, gels were washed for 1 h in 25% (v/v) isopropanol, the solution changed and incubation continued overnight to remove excess SDS. Following three subsequent washes for 1 h each in 25% (v/v) isopropanol, the gels were immersed in 'Stains-All' solution [0.005% (w/v) 'Stains-All' dye, 15 mM Tris/HCl; pH 8.8, 10% (v/v) formamide, 25% (v/v) isopropanol], the container sealed with a lid and placed overnight in a black plastic bag on an orbital shaker. For optimum staining, the 'Stains-All' solution was prepared 2 weeks prior to use and

maintained in a blackened bottle. Gels were destained in 25% (v/v) isopropanol for 2 h to allow sufficient removal of excess dye from the gel. Coloured gels were scanned using an Epson Perfection 1200S colour scanner from Seiko Epson Corporation (Nagano, Japan).

Skeletal muscle proteomics

Excision of protein spots, trypsin digestion, and protein identification by mass spectrometric analysis using an Ettan MALDI-ToF Pro instrument from Amersham Biosciences was performed according to an established methodology [36]. Coomassie-stained spots of interest were excised from the gels using 1 mL pipette tips with their tops cut off. Gel plugs were placed into a presiliconized 1.5 mL plastic tube for destaining, desalting and washing steps. The remaining liquid above the gel plugs was removed and sufficient acetonitrile was added in order to cover the gel plugs. Following shrinkage of the gel plugs, acetonitrile was removed and the protein-containing gel pieces were rehydrated for 5 min with a minimal volume of 100 mM ammonium bicarbonate. An equal volume of acetonitrile was added and after 15 min of incubation the solution was removed from the gel plugs and the samples then dried down for 30 min using a Heto type vacuum centrifuge from Jouan Nordic A/S (Allerød, Denmark). Individual gel pieces were then rehydrated in digestion buffer (1 μ g of trypsin in 20 μ L of 50 mM ammonium bicarbonate) to cover the gel pieces. More digestion buffer was added if all the initial volume had been absorbed by the gel pieces. Exhaustive digestion was carried out overnight at 37 °C. After digestion, the samples were centrifuged at 12 000 *g* for 10 min using a model 5417R bench top centrifuge from Eppendorf. The supernatant was carefully removed from each sample and placed into clean and siliconized plastic tubes. Samples were stored at –70 °C until analysed by MS. For spectrometric analysis, mixtures of tryptic peptides from individual samples were desalted using Millipore C-18 Zip-Tips (Millipore) and eluted onto the sample plate with the matrix solution [10 mg·mL⁻¹ α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid (v/v)]. Mass spectra were recorded using the MALDI ToF instrument operating in the positive reflector mode at the following parameters: accelerating voltage 20 kV; and pulsed extraction: on (focus mass 2500). Internal calibration was performed using trypsin autolysis peaks at *m/z* 842.50 and *m/z* 2211.104. The mass spectra were analysed using MALDI evaluation software (Amersham Biosciences), and protein identification was achieved with the PMF Pro-Find search engine for peptide mass fingerprints.

Immunoblot analysis

Electrophoretically separated proteins were transferred onto Immobilon NC-pure nitrocellulose membranes, as previously described [38], and immunoblotting of gel replicas was carried out by the method of Bradd & Dunn [39]. The total muscle protein complement was transferred at 4 °C for 1 h at 100 V, whereby the efficiency of transfer was evaluated by Ponceau-S-Red staining of membranes, followed by destaining in 50 mM sodium phosphate, pH 7.4, 0.9% (w/v) NaCl [NaCl/P_i (PBS)]. Nitrocellulose sheets were blocked

for 1 h in 5% (w/v) fat-free milk powder in NaCl/P_i (PBS) and then incubated for 3 h at room temperature with primary antibody, appropriately diluted with blocking buffer. Nitrocellulose blots were subsequently washed twice for 10 min in blocking solution and then incubated with the appropriate dilution of a corresponding peroxidase-conjugated secondary antibody for 1 h at room temperature. The nitrocellulose membranes were washed twice for 10 min in blocking solution and then rinsed twice for 10 min with NaCl/P_i (PBS). Immunodecorated protein bands were visualized using the SuperSignal enhanced chemiluminescence (ECL) kit from Pierce & Warriner (Chester, Cheshire, UK). Densitometric scanning of ECL images was performed on a Molecular Dynamics 300S computing densitometer (Molecular Dynamics) with IMAGEQUANT V3.0 software.

Results

In order to determine the fate of the terminal cisternae Ca²⁺-binding protein, calsequestrin, and related luminal sarcoplasmic reticulum elements in dystrophin-deficient skeletal muscle, we employed a comparative 2D gel electrophoretic approach for separating the entire protein complement of normal vs. dystrophic muscle fibres. Using a combination of MS-based proteomics, immunoblotting with mAbs and dye labelling with the cationic carbocyanine dye 'Stains-All', expression levels of the major muscle proteins involved in luminal Ca²⁺ cycling were evaluated.

Comparative 2D analysis of dystrophic muscle

As illustrated by the silver-stained 2D gels in Fig. 1, the comparative gel electrophoretic analysis of normal vs. dystrophic muscle extracts revealed no drastic differences in the overall protein spot pattern. However, because the separation of muscle proteins by IEF in the first dimension, and by SDS/PAGE in the second dimension, is hampered by a range of technical problems, the 2D spot pattern is not representative of the complete protein repertoire of skeletal muscle. Many integral proteins, low-molecular-mass peptides, highly basic or acidic components, very high-molecular-mass proteins and low-abundance species might be underrepresented by this methodology. As different proteins are stained to different degrees with the standard dyes employed in biochemistry, in certain cases proteins that are not visualized by the silver-staining procedure might be present in a gel. In addition, highly abundant muscle proteins, such as myosin or actin, distort the 2D pattern and often result in a streaky spot pattern. Therefore, silver-stained 2D patterns of muscle proteins probably overestimate the presence of soluble proteins and underestimate the expression of membrane-associated proteins. Despite these problems, the proteomics analysis of the protein complement of normal mouse skeletal muscle (Fig. 1A) vs. dystrophin-deficient mdx mouse skeletal muscle (Fig. 1B) can be used, in conjunction with the Swiss-Prot 2D data bank, to demonstrate the proper electrophoretic separation of muscle proteins prior to immunoblotting and dye-binding analysis. For the identification of proteins by MS, Coomassie-labelled protein spots were numbered and no major differences were apparent in normal controls

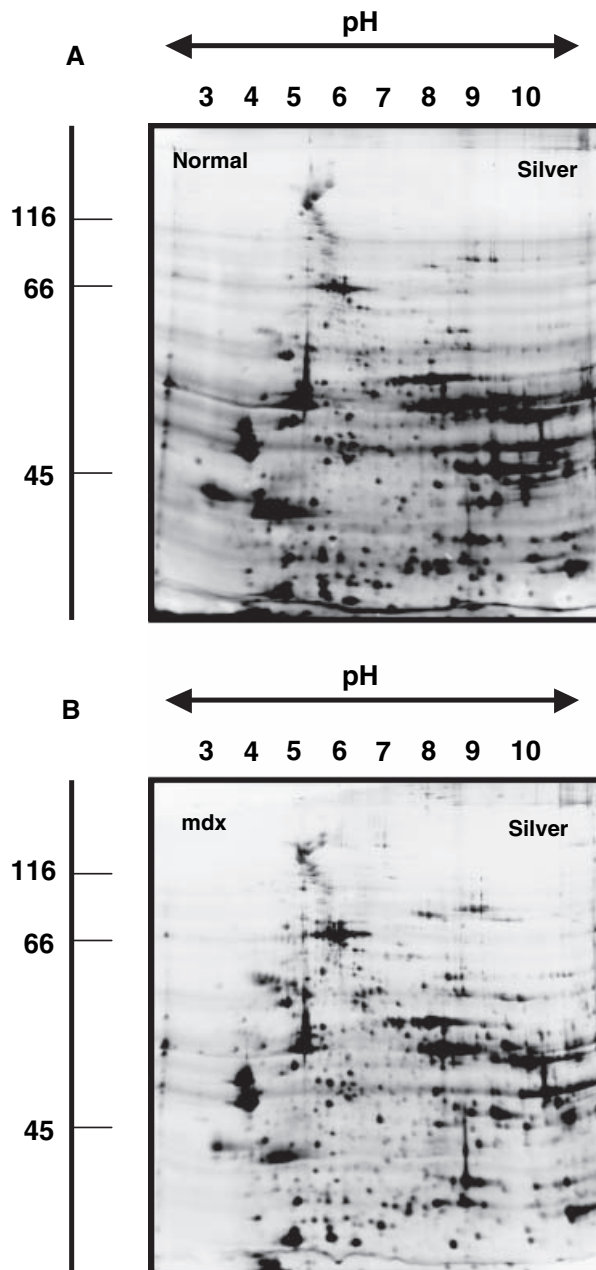


Fig. 1. 2D gel electrophoretic comparison between normal and mdx muscle extracts. Shown are silver-stained 2D gels of total protein extracts from normal (A) and dystrophic mdx (B) skeletal muscle. The pH values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated at the top and on the left of the panels, respectively.

(Fig. 2A) vs. mdx fibres (Fig. 2B). Table 1 summarizes positively identified protein species and lists their respective pI value and approximate molecular mass, as well as their accession number in the Swiss-Prot 2D data bank. Major muscle proteins representing the contractile apparatus and its regulatory components were located. This included myosin, actin, troponin and tropomyosin. Other abundant proteins, such as albumin, desmin, aldolase, carbonic anhydrase and triosephosphate isomerase, responded to

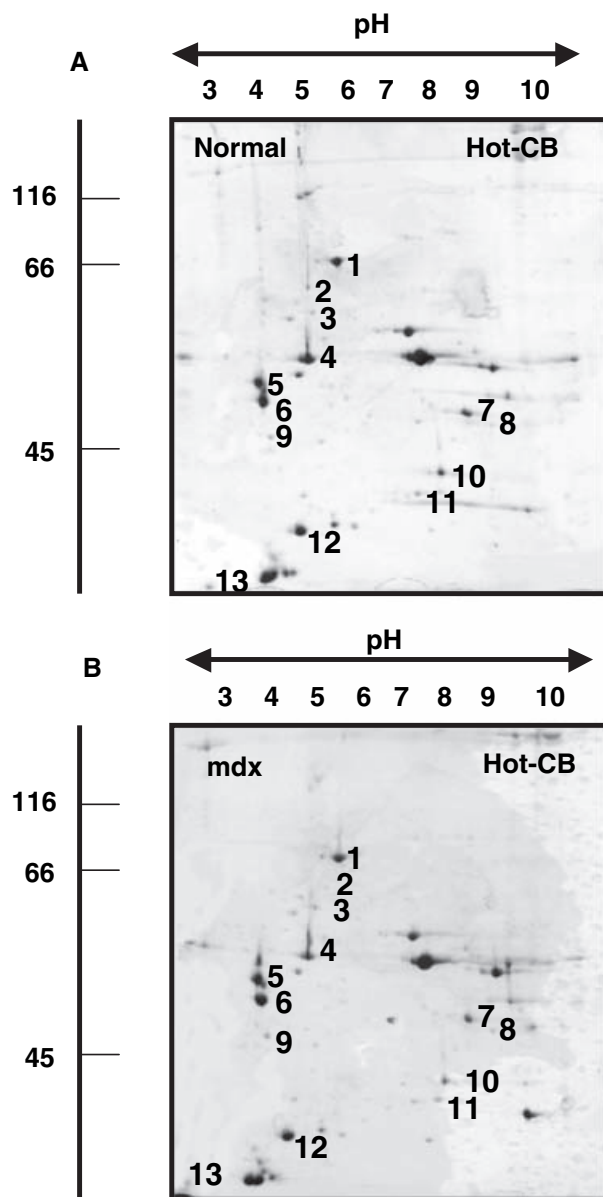


Fig. 2. Proteomics-based identification of major protein species in normal and mdx muscle extracts. Shown are Coomassie-stained 2D gels of total extracts from normal (A) and dystrophic mdx (B) skeletal muscle. Starting with the mass spectrometric analysis of 38 major protein spots, 13 spots were clearly identifiable. The results are listed in Table 1. The pH values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated at the top and on the left of the panels, respectively.

distinct 2D protein spots. A relatively muscle-specific enzyme, creatine kinase, was identified as a Coomassie-labelled spot and no major effect on its expression level was detectable (Fig. 2). Importantly, the initial proteomics approach clearly demonstrated that our 2D gel electrophoretic technique has sufficiently and reproducibly separated major protein species of skeletal muscle fibres. This result was an essential prerequisite for the subsequent subproteomics approach using antibodies and the 'Stains-All' dye,

because it showed that both the normal and dystrophic protein complement is properly represented on the 2D gels.

2D 'Stains-All' analysis of dystrophic muscle

The cationic carbocyanine dye 'Stains-All' was used to determine potential changes in the expression of major Ca²⁺-binding proteins in dystrophic fibres. A comparison between the selective dye labelling of protein spots in Fig. 3 showed that 11 main protein spots are recognized in normal fibres and that eight of these species are greatly reduced in mdx preparations. This clearly indicates a drastic effect of the deficiency in dystrophin on the expression of Ca²⁺-binding proteins. The relatively unique combination of the pI value and molecular mass of individual 2D spots can be useful in the initial identification of proteins. However, owing to the abnormal electrophoretic mobility of certain proteins, their 2D position does not necessarily match the isoelectric point or molecular mass taken from their amino acid sequence. In such cases, immunoblotting, as presented below in Figs 4 and 5, can clarify potential ambiguities. While the 'Stains-All'-labelled spot no. 10, with a relative molecular mass of 60 kDa and an acidic pI value, clearly represented the calsequestrin monomer of apparent 63 kDa, the 90 kDa protein spot no. 5 was shown to be sarcalumenin, whose monomer exhibits an apparent molecular mass of 160 kDa (Fig. 3). Spot no. 11 was identified as calmodulin. The mass spectrometric screening of tryptic peptides following 'Stains-All' labelling did not result in suitable mass spectra for the proper identification of Ca²⁺-binding proteins (data not shown). The analysis of 'Stains-All' labelled spot no. 8, using a corresponding Coomassie-labelled gel plug, resulted in the identification of the transcription cofactor vestigial-like protein 2 (UniProt AC: Q8BGW8; UniProt ID: VGL2_MOUSE). This cofactor of the transcription enhancer factor TEF-1 appears to be a new component of the myogenic programme that promotes muscle differentiation [40]. As a result of the overlap with other major muscle protein species, the screening of corresponding gel plugs from Coomassie gels did not result in mass spectra from Ca²⁺-binding proteins. Therefore, immunoblotting was employed to confirm the calsequestrin protein spot identified by 'Stains-All' labelling.

Immunoblot analysis of key Ca²⁺-binding proteins

In order to avoid potential technical problems associated with the comparative immunoblotting of subcellular fractions, we employed, in this study, total muscle extracts exclusively. As the full-length dystrophin isoform of 427 kDa does not enter 2D gels owing to its extremely large size, we initially used 1D immunoblotting to confirm the mutant status of the mdx fibres. As illustrated in Fig. 4A, the Dp427 isoform of dystrophin was completely absent from mdx skeletal muscle preparations. A representative member of the dystrophin-associated glycoprotein complex, α -dystroglycan, was reduced in dystrophin-deficient fibres (Fig. 4B). This agrees with previous studies [24]. In contrast, the expression of major ion-regulatory muscle components, such as the Na⁺/K⁺ ATPase, the SERCA1 isoform of the sarcoplasmic reticulum Ca²⁺ ATPase, and the α_1 -subunit of the dihydropyridine receptor, were not

Table 1. Representative muscle protein species identified by MS-based proteomics.

Spot no.	Protein species ^a	Sequence coverage (%)	Molecular mass (kDa)	Isoelectric point (pI)	2D Swiss-Prot accession no.
1	Serum albumin precursor	26.0	70.73	5.8	P07725
2	Actin (alpha 1) fragment	22.5	42.38	5.2	P99041
3	Desmin	9.2	53.54	5.2	P31001
4	Actin (alpha 1)	22.5	42.38	5.2	P99041
5	Tropomyosin (2, beta)	21.5	32.93	4.7	P58774
6	Tropomyosin (1, alpha)	33.1	32.75	4.7	P58771
7	Creatine kinase	30.2	43.26	6.6	P07310
8	Aldolase (1, isoform A)	15.9	39.79	8.8	Q9CP09
9	Actin (alpha 1) fragment	14.6	42.38	5.2	P99041
10	Carbonic anhydrase	15.0	29.63	6.9	P16015
11	Triosephosphate isomerase	19.7	27.04	6.9	P17751
12	Myosin (A1 light chain)	42.0	20.69	5.0	P05977
13	Troponin (C2 fast)	28.1	18.15	4.1	P20801

^a All certainty hits of protein species generated by the ProFound search engine for peptide mass fingerprinting were matched against the publicly available search engine Mascot (<http://www.matrixscience.com>).

affected in mdx muscle (Fig. 4C,D,E). Immunoblotting with mAb VIIID1₂ to calsequestrin revealed a drastic reduction in this Ca²⁺-binding protein (Fig. 4F), and this finding agrees with the reduced 'Stains-All' labelling of the calsequestrin spot region (Fig. 3B). Interestingly, the minor Ca²⁺-binding protein, calreticulin, which exists in mature skeletal muscle fibres at a relatively low abundance, does not seem to be affected by the deficiency in dystrophin (Fig. 4G).

Nitrocellulose replicas of the 2D gels shown in Figs 1 and 2 were used for the immunoblot analysis of calsequestrin. In contrast to the unchanged expression levels of the Na⁺/K⁺ ATPase (Fig. 5A) and calmodulin (Fig. 5C), the two luminal Ca²⁺ reservoir elements of the sarcoplasmic reticulum – calsequestrin (Fig. 5D) and sarcalumenin (Fig. 5E) – were shown to be drastically reduced in mdx preparations. This finding agrees with both the 2D 'Stains-All' analysis of Fig. 3 and the 1D immunoblotting of Fig. 4. As the full-length Dp427 isoform of dystrophin does not enter the second dimension of conventional 2D gels, the expression level of α -dystroglycan was employed to demonstrate the dystrophic phenotype by 2D immunoblotting. As illustrated in Fig. 5B, this dystrophin-associated glycoprotein is severely affected in its abundance in mdx skeletal muscle. Thus, in contrast to previous microsomal studies that have indicated a preservation of calsequestrin in muscular dystrophy, here we can show, by 2D analysis of total extracts, that the expression of this important luminal Ca²⁺-binding protein is changed in an established animal model of dystrophinopathy.

Discussion

Muscular dystrophy refers to a group of hereditary diseases characterized by progressive degeneration of skeletal muscles [17]. As abnormal ion-handling may play a crucial role in fibre destruction [20–22], and in order to better understand the overall impact of the primary genetic abnormality in dystrophin, we have performed a subproteomics analysis of mdx muscle extracts. As reviewed by Watchko *et al.* [41]

and Durbeej & Campbell [42], spontaneously occurring or genetically engineered animal models of neuromuscular diseases are an indispensable tool in modern myology research. They are employed for studying the molecular and cellular factors leading to necrotic changes and in evaluating new treatment strategies, such as gene therapy or stem cell therapy [11]. Although the dystrophin isoform Dp427 is absent in skeletal muscle fibres from mdx mice as the result of a point mutation [43], mdx mice do not represent a perfect replica of the human pathology seen in dystrophinopathies [1]. Nevertheless, the mdx animal model exhibits many molecular and cellular abnormalities seen in Duchenne muscular dystrophy [41], making it a suitable system for studying the effect of the loss of the dystrophin–glycoprotein complex.

The 2D 'Stains-All' and immunoblotting analysis performed here revealed a substantial loss of key Ca²⁺-binding elements in dystrophin-deficient mdx skeletal muscle fibres. In contrast to previous studies that have shown a persistent expression of calsequestrin in mdx microsomes [29], the analysis of total muscle extracts clearly showed a reduction of this luminal constituent in dystrophic fibres. Although other abundant ion-regulatory proteins, such as the fast SERCA1 isoform of the sarcoplasmic reticulum Ca²⁺ ATPase and the α_1 subunit of the transverse-tubular dihydropyridine receptor, are not affected in muscular dystrophy, the essential Ca²⁺-binding proteins calsequestrin and the previously implicated sarcalumenin [30] are greatly reduced. This shows that proteomics-based approaches can overcome potential problems associated with the conventional analysis of muscle microsomes. Although subcellular fractionation protocols are widely employed, it is important to stress that this standard biochemical technique may introduce artefacts, making the proper quantification of comparative immunoblotting data occasionally difficult.

As differential centrifugation is based upon the differences in the sedimentation rate of biological particles of different density and size, a muscle homogenate containing membrane vesicles, intact organelles and structural fragments of the contractile apparatus can be divided into

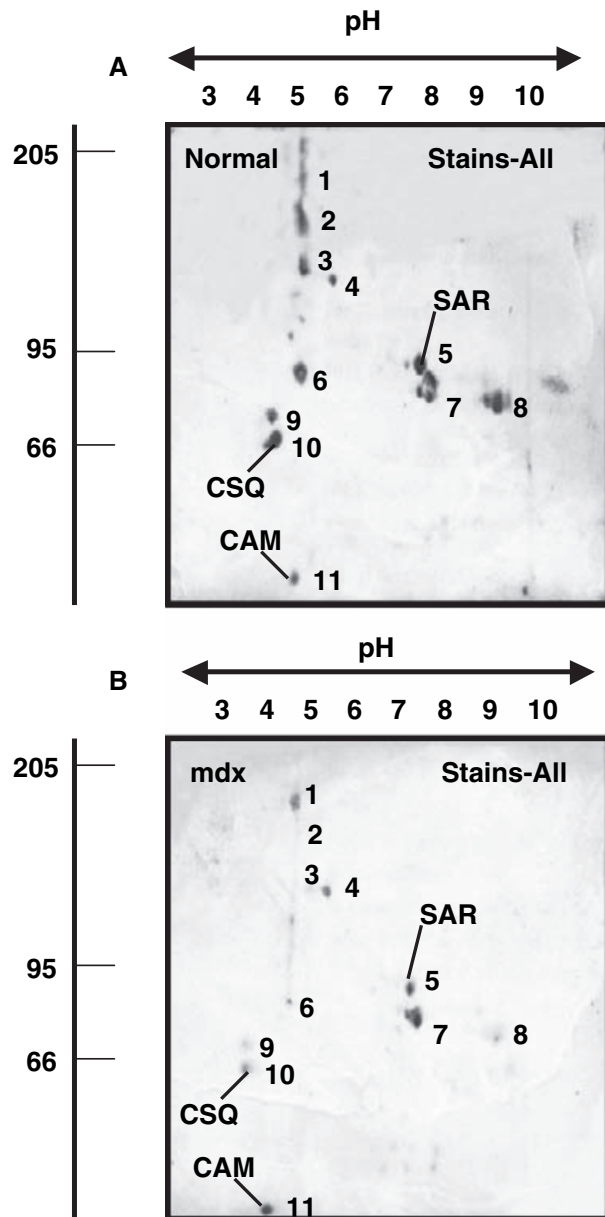


Fig. 3. 2D 'Stains-All' labelling of normal and mdx muscle extracts. Shown are 2D gels of total extracts from normal (A) and dystrophic mdx (B) skeletal muscle labelled with the cationic carbocyanine dye 'Stains-All'. A comparison between the selective dye labelling of protein spots in panel (A) and panel (B) showed that 11 main protein spots are recognized in normal fibres and that eight of these species are greatly reduced in mdx preparations. Taking into account the relatively unique combination of the pI value, molecular mass of individual 2D spots and results from immunoblotting (see Fig. 5), spots 5, 10 and 11 were identified as sarcocalumenin (SAR), calsequestrin (CSQ), and calmodulin (CAM), respectively. The pH values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated at the top and on the left of the panels, respectively.

different fractions by the stepped increase of the applied centrifugal field. The repeated centrifugation at progressively higher speeds and longer centrifugation periods can

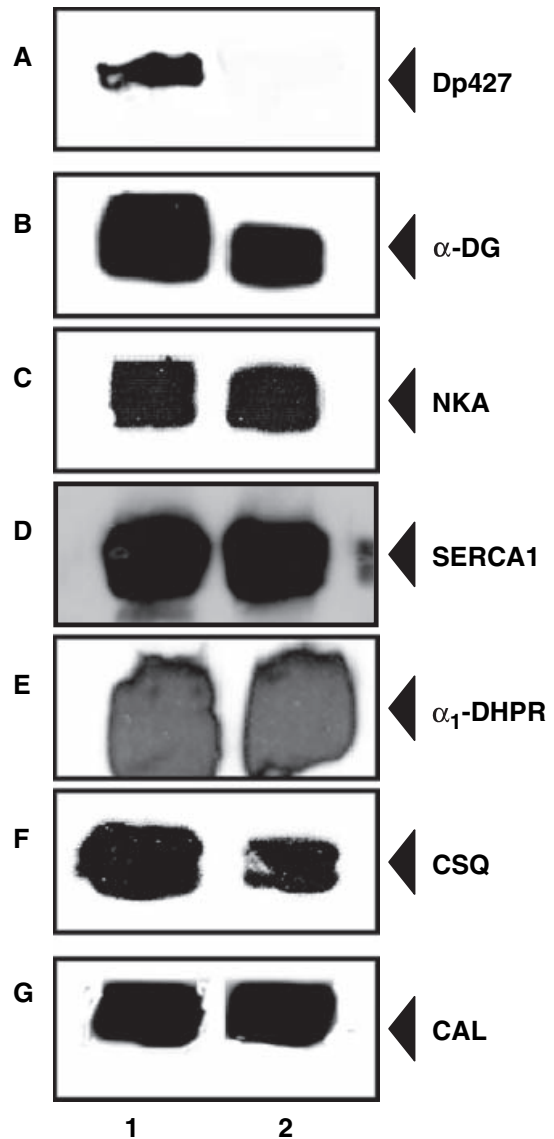


Fig. 4. 1D immunoblot analysis of calsequestrin expression in crude muscle extracts. Shown are identical 1D immunoblots labelled with antibodies to the Dp427 isoform of dystrophin (A), the α -subunit of the dystroglycan complex (α -DG; B), the Na⁺/K⁺ ATPase (NKA, C), the fast SERCA1 isoform of the sarcoplasmic reticulum Ca²⁺ ATPase (D), the α_1 -subunit of the dihydropyridine receptor (E), calsequestrin (F), and calreticulin (CAL). Lanes 1 and 2 represent total protein extracts from normal and dystrophic skeletal muscle fibres, respectively.

fractionate the muscle homogenate into relatively distinct fractions. However, both cross-contamination of vesicular membrane populations and the unintentional enrichment of subspecies of membranes can represent a serious technical problem during comparative subcellular fractionation procedures [44]. Membrane domains originally derived from a similar subcellular location, such as the terminal cisternae region, the junctional sarcoplasmic reticulum or the longitudinal tubules, might form a variety of structures, including right-side-out vesicles, inside-out vesicles and/or membrane sheets. The presence of both leaky and sealed vesicle

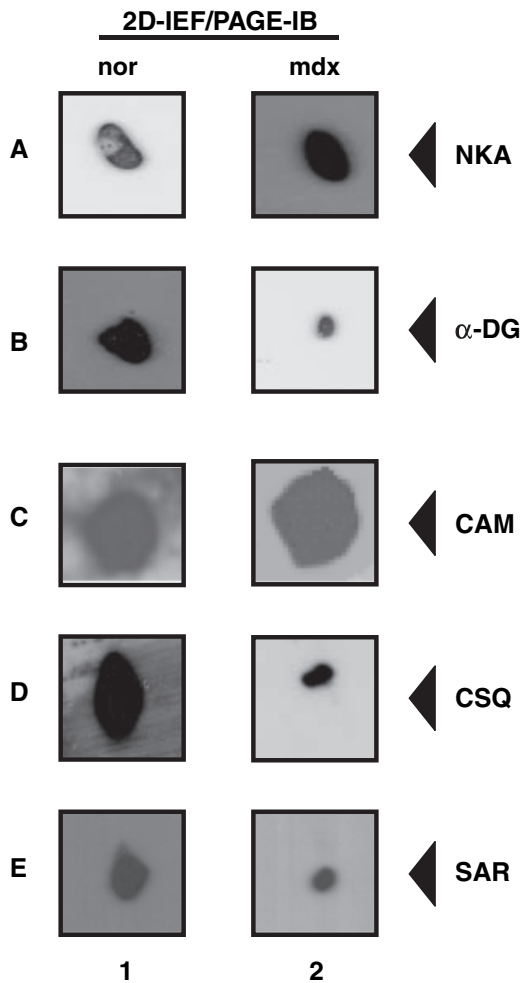


Fig. 5. 2D immunoblot analysis of calsequestrin expression in crude muscle extracts. Shown are identical 2D immunoblots (IB) which correspond to the silver-stained or Coomassie-stained gels in Figs 1 and 3. Blots were labelled with antibodies to the Na^+/K^+ ATPase (NKA; A), the α -subunit of the dystroglycan complex (α -DG; B), calmodulin (CAM; C) calsequestrin (D), and sarcalumenin (SAR). Proteins were separated in the first dimension by IEF and in the second dimension by SDS/PAGE. Lanes 1 and 2 represent total protein extracts from normal and dystrophic skeletal muscle fibres, respectively.

populations further complicates a separation based on density owing to the varying degree of infiltration of different vesicles by the separation medium. In addition, smaller vesicles might become entrapped in larger vesicles. Different membrane systems might aggregate nonspecifically, or bind to or entrap abundant solubilized proteins. Hence, to avoid these problems and to unequivocally show abundance differences between normal and dystrophic muscle fibres, it is advantageous to analyse total tissue extracts instead of microsomal membranes.

As MS and 2D dye labelling, as well as the ECL method in combination with highly specific antibodies, are extremely sensitive detection methods, it was possible to identify specific protein species in such crude muscle preparations. The gel spot pattern presented in this report agrees with previously published studies on skeletal muscle proteomics

[34,35]. The relative 2D position of proteins belonging to the contractile apparatus, such as myosin, actin, troponin and tropomyosin, matched the standardized spot pattern of the Swiss-Prot 2D skeletal muscle data bank [35]. In addition, major protein species, including creatine kinase, aldolase, carbonic anhydrase and albumin, were identified by MS following 2D gel electrophoretic separation. Although the abundance of these proteins was not affected in mdx fibres, our mass spectrometric analysis demonstrated the reproducibility of the 2D technique and thereby set the scene for a proper comparative approach to analyse the fate of Ca^{2+} -binding elements in normal vs. dystrophic fibres.

The major finding of the subproteomics approach presented here, that calsequestrin is reduced in dystrophin-deficient fibres, agrees with a previous dye-binding analysis of Duchenne patient specimens [45] and fully supports the calcium hypothesis of muscular dystrophy [20–22]. Calsequestrin represents a high-capacity, medium-affinity Ca^{2+} -binding protein [46], that exists in a supramolecular membrane assembly in the terminal cisternae region of muscle fibres [47,48]. As the major luminal Ca^{2+} buffer, calsequestrin clusters act as physiological mediators of the excitation–contraction–relaxation cycle [49]. During the initiation phase of excitation–contraction coupling, the transient opening of the ryanodine receptor Ca^{2+} -release channel is triggered by physical coupling to the transverse-tubular $\alpha_1\text{S}$ -dihydropyridine receptor [50]. Ca^{2+} ions bound to junctional calsequestrin are then directly provided for a fast efflux mechanism along a step concentration gradient. Calsequestrin can thus be considered as both a luminal ion trap and an endogenous regulator of the ryanodine receptor complex [51]. It is therefore not surprising that the reduced expression of this important regulatory component plays a central role in the pathophysiological pathway leading to fibre degeneration. Although it is not fully understood whether calsequestrin complexes operate at their full ion-binding capacity under normal conditions, it can be expected that even small changes in individual steps involved in ion-binding and ion-fluxes may render skeletal muscles more susceptible to necrosis. Owing to the enormous complexity of the triadic signal transduction mechanism [52], skeletal muscle proteomics has not yet identified the full complement of excitation–contraction coupling elements expressed in various fibre types. It is not clear how many auxiliary proteins are involved in the fine regulation of Ca^{2+} storage, Ca^{2+} uptake and Ca^{2+} release. However, based on the results presented here, it appears to be that an abnormal luminal protein expression pattern is involved in X-linked muscular dystrophy.

In conclusion, based on the original formulation of the calcium hypothesis of muscular dystrophy [53] that preceded the discovery of dystrophin and its associated glycoproteins [2,3], the subproteomics analysis presented here has further elucidated the molecular basis of abnormal Ca^{2+} cycling through the dystrophic sarcoplasmic reticulum. Pharmacological agents, which modulate Ca^{2+} homeostasis and Ca^{2+} -dependent mechanisms, can counteract dystrophic symptoms [6,7]. Ca^{2+} pumps, Ca^{2+} -binding proteins, Ca^{2+} -release channels and/or Ca^{2+} exchangers appear to represent excellent therapeutic targets for preventing muscle fibre degeneration. Thus, drug-based alterations in Ca^{2+} cycling may be useful in avoiding

Ca²⁺-related proteolytic processes, and future trials will show whether a long-term improvement of muscle mass and strength can be achieved in dystrophic patients.

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References

- Emery, A.E. (2002) The muscular dystrophies. *Lancet* **359**, 687–695.
- Koenig, M., Hoffman, E.P., Bertelson, C.J., Monaco, A.P., Feener, C. & Kunkel, L.M. (1987) 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.
- Ervasti, J.M., Ohlendieck, K., Kahl, S.D., Gaver, M.G. & Campbell, K.P. (1990) Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* **345**, 315–319.
- Kapsa, R., Kornberg, A.J. & Byrne, E. (2003) Novel therapies for Duchenne muscular dystrophy. *Lancet Neurol.* **2**, 299–310.
- Khurana, T.S. & Davies, K.E. (2003) Pharmacological strategies for muscular dystrophy. *Nat. Rev. Drug Discov.* **2**, 379–390.
- Badalamente, W.A. & Stracher, A. (2000) Delay of muscle degeneration and necrosis in mdx mice by calpain inhibition. *Muscle Nerve* **23**, 106–111.
- Tidball, J.G. & Spencer, M.J. (2000) Calpains and muscular dystrophy. *Int. J. Biochem. Cell Biol.* **32**, 1–5.
- Bogdanovich, S., Krag, T.O.B., Barton, E.R., Morris, L.D., Whittemore, L.A., Ahima, R.S. & Khurana, T.S. (2002) Functional improvement of dystrophic muscle by myostatin blockade. *Nature* **420**, 418–421.
- Partridge, T.A. (2002) Myoblast transplantation. *Neuromuscul. Disord.* **12**, S3–S6.
- Partridge, T.A. (2003) Stem cell route to neuromuscular therapies. *Muscle Nerve* **27**, 133–141.
- Bachrach, E., Li, S., Perez, A.L., Schienda, J., Liadaki, K., Volinski, J., Flint, A., Chamberlain, J. & Kunkel, L.M. (2004) Systemic delivery of human microdystrophin to regenerating mouse dystrophic muscle by muscle progenitor cells. *Proc. Natl Acad. Sci. USA* **101**, 3581–3586.
- Ahmad, A., Brinson, M., Hodges, B.L., Chamberlain, J.S. & Amalfitano, A. (2000) Mdx mice inducibly expressing dystrophin provide insights into the potential of gene therapy for Duchenne muscular dystrophy. *Hum. Mol. Genet.* **9**, 2507–2515.
- Wang, B., Li, J. & Xiao, X. (2000) Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy. *Proc. Natl Acad. Sci. USA* **97**, 13714–13719.
- Chamberlain, J.S. (2002) Gene therapy of muscular dystrophy. *Hum. Mol. Genet.* **11**, 2355–2362.
- Van Deutekom, J.C. & van Ommen, G.J. (2003) Advances in Duchenne muscular dystrophy gene therapy. *Nat. Rev. Genet.* **4**, 774–783.
- Tinsley, J.M. & Davies, K.E. (1993) Utrophin: a potential replacement for dystrophin? *Neuromuscul. Disord.* **3**, 537–539.
- Emery, A.E. (2002) Muscular dystrophy into the new millennium. *Neuromuscul. Disord.* **12**, 343–349.
- Tkatchenko, A.V., Le Cam, G., Leger, J.J. & Dechesne, C.A. (2000) Large-scale analysis of differential gene expression in the hindlimb muscles and diaphragm of mdx mouse. *Biochim. Biophys. Acta* **1500**, 17–30.
- Chen, Y.W., Zhao, P., Borup, R. & Hoffman, E.P. (2000) Expression profiling in the muscular dystrophies: identification of novel aspects of molecular pathophysiology. *J. Cell Biol.* **151**, 1321–1336.
- Alderton, J.M. & Steinhardt, R.A. (2000) Calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. *J. Biol. Chem.* **275**, 9452–9460.
- Culligan, K. & Ohlendieck, K. (2002) Abnormal calcium handling in muscular dystrophy. *Bas. Appl. Myol.* **12**, 147–157.
- Alderton, J.M. & Steinhardt, R.A. (2000) 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.
- Ohlendieck, K., Matsumura, K., Ionasescu, V.V., Towbin, J.A., Bosch, E.P., Weinstein, S.L., Sernett, S.W. & Campbell, K.P. (1993) Duchenne muscular dystrophy: deficiency of dystrophin-associated proteins in the sarcolemma. *Neurology* **43**, 795–800.
- Ohlendieck, K. & Campbell, K.P. (1991) Dystrophin-associated proteins are greatly reduced in skeletal muscle from mdx mice. *J. Cell Biol.* **115**, 1685–1694.
- Ohlendieck, K. (1996) Towards an understanding of the dystrophin-glycoprotein complex: linkage between the extracellular matrix and the subsarcolemmal membrane cytoskeleton. *Eur. J. Cell Biol.* **69**, 1–10.
- Clarke, M.S., Khakee, R. & McNeil, P.L. (1993) Loss of cytoplasmic basic fibroblast growth factor from physiologically wounded myofibers of normal and dystrophic muscle. *J. Cell Sci.* **106**, 121–133.
- Mallouk, N., Jacquemond, V. & Allard, B. (2000) Elevated subsarcolemmal Ca²⁺ in mdx mouse skeletal muscle fibres detected with Ca²⁺-activated K⁺ channels. *Proc. Natl Acad. Sci. USA* **97**, 4950–4955.
- Turner, P.R., Westwood, T., Regen, C.M. & Steinhardt, R.A. (1988) Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. *Nature* **335**, 735–738.
- Culligan, K., Banville, N., Dowling, P. & Ohlendieck, K. (2002) Drastic reduction of calsequestrin-like proteins and impaired calcium binding in dystrophic mdx muscle. *J. Appl. Physiol.* **92**, 435–445.
- Dowling, P., Doran, P. & Ohlendieck, K. (2004) Drastic reduction of sarcalumenin in Dp427-deficient fibres indicates that abnormal calcium handling plays a key role in muscular dystrophy. *Biochem. J.* **379**, 479–488.
- Dowling, P., Lohan, J. & Ohlendieck, K. (2003) Comparative analysis of Dp427-deficient mdx tissues shows that the milder dystrophic phenotype of extraocular and toe muscle fibres is associated with a persistent expression of beta-dystroglycan. *Eur. J. Cell Biol.* **82**, 222–230.
- Campbell, K.P., MacLennan, D.H. & Jorgensen, A.O. (1983) Staining of the Ca²⁺-binding proteins, calsequestrin, calmodulin, troponin C, and S-100, with the cationic carbocyanine dye 'Stains-all'. *J. Biol. Chem.* **258**, 11267–11273.
- Murray, B.E. & Ohlendieck, K. (1997) Crosslinking analysis of the ryanodine receptor and α_1 dihydropyridine receptor in rabbit skeletal muscle triads. *Biochem. J.* **324**, 689–696.
- Isfort, R.J. (2002) Proteomic analysis of striated muscle. *J. Chromatogr. B* **771**, 155–165.
- Sanchez, J.C., Chiappe, D., Converset, V., Hoogland, C., Binz, P.A., Paesano, S., Appel, R.D., Wang, S., Sennitt, M., Nolan, A.,

- Cawthorne, M.A. & Hochstrasser, D.F. (2001) The mouse SWISS-2DPAGE database: a tool for proteomics study of diabetes and obesity. *Proteomics* **1**, 136–163.
36. Westermeier, R. & Naven, T. (2002) *Practical Proteomics*. Wiley-VCH-Verlag, Weinheim, Germany.
37. Froemming, G.R. & Ohlendieck, K. (2001) The native dihydropyridine receptor exists as a supramolecular complex in skeletal muscle. *Cell. Mol. Life Sci.* **58**, 312–320.
38. Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA* **76**, 4350–4354.
39. Bradd, S.J. & Dunn, M.J. (1993) Analysis of membrane proteins by Western blotting and enhanced chemiluminescence. *Methods Mol. Biol.* **19**, 211–218.
40. Maeda, T., Chapman, D.L. & Stewart, A.F. (2002) Mammalian vestigial-like 2, a cofactor of TEF-1 and MEF2 transcription factors that promotes skeletal muscle differentiation. *J. Biol. Chem.* **277**, 48889–48898.
41. Watchko, J.F., O'Day, T.L. & Hoffman, E.P. (2002) Functional characteristics of dystrophic skeletal muscle: insights from animal models. *J. Appl. Physiol.* **93**, 407–417.
42. Durbeej, M. & Campbell, K.P. (2002) Muscular dystrophies involving the dystrophin-glycoprotein complex: an overview of current mouse models. *Curr. Opin. Genet. Dev.* **12**, 349–361.
43. Sicinski, P., Geng, Y., Ryder-Cook, A.S., Barnard, E.A., Darlison, M.G. & Barnard, P.J. (1989) The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* **244**, 1578–1580.
44. Ohlendieck, K., Ervasti, J.M., Snook, J.B. & Campbell, K.P. (1991) Dystrophin-glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. *J. Cell Biol.* **112**, 135–148.
45. Niebroj-Dobosz, I., Kornguth, S., Schutta, H., Siegel, F.L. & Hausmanowa-Petrusewicz, I. (1989) 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.
46. MacLennan, D.H. & Reithmeier, R.A. (1998) Ion tamers. *Nat. Struct. Biol.* **5**, 409–411.
47. Glover, L., Quinn, S., Ryan, M., Pette, D. & Ohlendieck, K. (2002) Supramolecular calsequestrin complex. *Eur. J. Biochem.* **269**, 4607–4616.
48. Franzini-Armstrong, C., Kenney, L.J. & Varriano-Marston, E. (1987) The structure of calsequestrin in triads of vertebrate skeletal muscle: a deep-etch study. *J. Cell Biol.* **105**, 49–56.
49. MacLennan, D.H., Abu-Abed, M. & Kang, C. (2002) Structure-function relationships in Ca²⁺-cycling proteins. *J. Mol. Cell. Cardiol.* **34**, 897–918.
50. Leong, P. & MacLennan, D.H. (1998) Complex interactions between skeletal muscle ryanodine receptor and dihydropyridine receptor proteins. *Biochem. Cell Biol.* **76**, 681–694.
51. Ohkura, M., Furukawa, K., Fujimori, H., Kuruma, A., Kawano, S., Hiraoka, M., Kuniyasu, A., Nakayama, H. & Ohizumi, Y. (1998) Dual regulation of the skeletal muscle ryanodine receptor by triadin and calsequestrin. *Biochemistry* **37**, 12987–12993.
52. Murray, B.E., Froemming, G.R., Maguire, P.B. & Ohlendieck, K. (1998) Excitation-contraction-relaxation cycle: Role of Ca²⁺-regulatory membrane proteins in normal, stimulated and pathological skeletal muscle fibres (review). *Int. J. Mol. Med.* **1**, 677–697.
53. Duncan, C.J. (1978) Role of intracellular calcium in promoting muscle damage: a strategy for controlling the dystrophic condition. *Experientia* **34**, 1531–1535.