Naturally Protected Muscle Phenotypes: Development of Novel Treatment Strategies for Duchenne Muscular Dystrophy

Paul Dowling, Philip Doran, James Lohan, Kevin Culligan and Kay Ohlendieck

Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland

Abstract

Primary abnormalities in the dystrophin gene underlie x-linked muscular dystrophy. However, the absence of the dystrophin isoform Dp427 does not necessarily result in a severe dystrophic phenotype in all muscle groups. Distal mdx muscles, namely extraocular and toe fibres, appear to represent a protected phenotype in muscular dystrophy. Thus, a comparative analysis of affected versus naturally protected muscle cells should lead to a greater knowledge of the molecular pathogenesis of inherited neuromuscular disorders. Furthermore, rationalising the protective cellular mechanisms might help in developing new treatment strategies for muscular dystrophy. The rescuing of extraocular and toe muscle fibres has previously been attributed to the special protective properties of fast-twitching small-diameter fibres. More recent biochemical studies have shown that the up-regulation of the autosomal dystrophin homologue named utrophin and the concomitant rescue of dystrophin-associated glycoproteins also plays an important role in the mechanical stabilisation of Dp427-deficient fibres. This result is mirrored in the dystrophic mdx brain where the dystrophin isoform Dp71 seems to be responsible for the preservation of the dystroglycan complex. It is envisaged that future proteomics-based comparisons of naturally protective extraocular, toe and brain tissues with severely affected skeletal muscle fibres will greatly add to our general understanding of the pathophysiology of muscular dystrophy.

Key words: Duchenne muscular dystrophy, Dystrophin, dystrophin-glycoprotein complex, dystroglycan, Dp427, Dp71, mdx, utrophin.

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Duchenne muscular dystrophy (DMD) represents an x-linked muscle disease that belongs to a large group of neuromuscular disorders characterised by the progressive degeneration of voluntary muscles. DMD is the most prevalent type of muscular dystrophy and affects an estimated 1 in 3,500 boys world-wide [28]. The gene responsible for DMD, localized to the Xp21 region, encodes a very large protein named dystrophin. Dystrophin is required inside muscle cells for structural support [36] and is thought to strengthen the muscle periphery by anchoring elements of the membrane cytoskeleton to the surface membrane [13, 17, 46]. Dystrophin-deficient cell membranes become more susceptible to exercise-induced rupturing, so that surface signal transduction mechanisms and sarcolemmal ion fluxes may be disturbed [1, 19, 21]. The subsequent immune response can add to the membrane damage.

The DMD gene is functionally one of the largest known, spanning ~ 2.5 Mb of genomic sequence at the Xp21 region. The full length 14-kb mRNA transcribed from the DMD locus was found to be predominately expressed in skeletal and cardiac muscle with smaller amounts in brain tissue [17, 45]. The resulting protein is made up of 3,685 amino acids and has a relative molecular mass of 427 kDa. Expression of the full-length dystrophin transcript is controlled by three independently regulated promoters termed the brain (B), muscle (M), and purkinje (P) promotors, resulting in the splicing to a common set of 78 exons [9, 29, 45]. The names of these promoters reflect the major site of dystrophin expression. The B promoter drives expression mainly in the brain, cortical neurons and hippocampus regions, while the P and M promoters are primarily concerned with expression in the purkinje cells and muscle fibres, respectively. In addition to these three main promoters, there exist at least four internal promoters that give rise to shorter dystrophin transcripts, specifically a 240 kDa retinal isoform Dp240 [26], a Schwann cell-specific Dp116 [12] and

the brain isoforms Dp71 and Dp140 [39]. The dystrophin isoform Dp71 is detected in most non-muscle tissues including brain, kidney, liver and lungs [31]. Alternative splicing at the 3'-end of the dystrophin gene generates an even greater number of isoforms [8].

Localization studies of muscle dystrophin have determined an almost exclusive localization to the cellular periphery on the cytoplasmic facade of the sarcolemma, where it comprises approximately two percent of the isolated plasmalemmal protein complement and at least five percent of the membrane cytoskeleton [17, 46]. The major skeletal muscle dystrophin isoform is a membrane cytoskeletal element of apparent 427 kDa and is a member of the spectrin protein family [37]. The dystrophin molecule can be structurally divided into four separate regions based on sequence homologies and protein-binding capabilities [37]. It is made up of an amino-terminal actin-binding domain followed by central spectrin-like repeats, a cysteine-rich domain, and a unique carboxy-terminal domain. The amino-terminus and a region in the rod domain of dystrophin and utrophin bind directly to cortical actin [57]. The full-length utrophin isoform Up395 represents an autosomal homologue of the dystrophin isoform Dp427 [11, 40]. In humans, its gene is located on chromosome 6. The expressed utrophin protein is about seven percent shorter than x-linked encoded dystrophin, but has a very similar structure and actin-binding function. It is present in many body tissues. In skeletal muscle fibres, it is highly enriched at the neuromuscular junction [47], the region where the motor nerve contacts the muscle surface membrane. Utrophin appears to be able to substitute, at least partially, for missing dystrophin molecules [55]. This was illustrated by a dystrophic mdx mouse model, whose utrophin gene was knocked out experimentally. This double-mutant lacks both dystrophin and utrophin and exhibits an extremely severe dystrophic phenotype [22, 51].

The dystrophin-associated protein complex is composed of a group of surface proteins, which are involved in linking the extracellular matrix to the actin membrane cytoskeleton [13, 16, 46]. The physical coupling between laminin and cortical actin via the dystroglycans and dystrophin is proposed to stabilise the sarcolemma during the rigors of repeated cycles of contraction and relaxation [46]. In addition, the dystrophin-glycoprotein complex transmits the force generated in the muscle sarcomeres to the extracellular matrix. See Figure 1 for a diagrammatic presentation of the dystrophin-associated surface complex from skeletal muscle, including dystrophin, dystroglycans, sarcoglycans, dystrobrevin, sarcospan, and laminin. Also shown is what specific forms of muscular dystrophy can be attributed to changes in the expression levels of particular surface proteins. Depending on the specific type of mutation, genetic abnormalities in the dystrophin gene lead to either severe Duchenne muscular dystrophy or more benign Becker muscular dystrophy [43]. Mutations in the members of the sarcoglycan sub-complex result in the various types of limb girdle muscular dystrophy [13, 27]. Muscle laminin is an amalgamation of various peptide chains [49]. Mutations in the gene expressing this protein cause severe congenital muscular dystrophy [13].

Utrophin and the rescue of mdx extraocular and toe muscle fibres

The fact that small calibre muscle fibres are not severely affected in muscular dystrophy has long been established [35]. Recently, it has been suggested that the protective factor involved in this process is the dystrophin-related protein named utrophin. Full-length utrophin is highly enriched at neuromuscular junctions and co-localises with the nicotinic acetylcholine receptor complex in normal skeletal muscle fibres [47]. The predicted primary structure is similar to that of dystrophin [40, 50]. It has been suggested that levels of utrophin in muscle fibres of patients with DMD could be raised to functionally compensate for the absence of

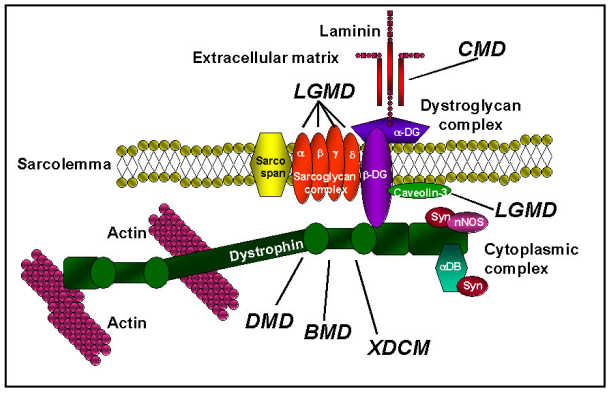

Figure 1. Diagrammatic presentation of the composition of the dystrophin-associated protein complex and its involvement in muscular dystrophies. A trans-sarcolemmal linkage is provided by the interactions between the actin-binding protein dystrophin via the α/β-dystroglycan complex and the extracellular matrix protein laminin. Other dystrophin-associated elements are represented by dystrobrevins, syntrophins, sarcoglycans and sarcospan. Primary genetic abnormalities in members of the dystrophin-glycoprotein complex lead to various forms of muscular disorders: (i) dystrophin – Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), X-linked dilated cardiomyopathy (XLMD, (ii) sarcoglycans: limb-girdle muscular dystrophy, and (iii) laminin – congenital muscular dystrophy (CMD). Another form of limb-girdle muscular dystrophy is triggered by mutations in caveolin.

dystrophin because of the similarities between the two proteins, therefore implicating utrophin as having an important therapeutic value [55]. Comparative immunoblot analysis of gastrocnemius and toe muscle specimens from the naturally occurring mdx mouse, an established model system of DMD pathology [2] revealed that the distal mdx muscle group exhibited an up-regulation of utrophin and a rescuing of the dystrophin-associated protein complex [24]. In dystrophic leg muscles, the absence of dystrophin causes the loss of several sarcolemmal glycoproteins, disrupting the link between the extracellular matrix and the membrane cytoskeleton, thereby rendering the fibres more susceptible to necrosis, stretch induced injury, osmotic shock and exhibit calcium induced net degradation of muscle proteins [1, 6, 17, 19, 46]. Although lacking the dystrophin isoform Dp427, the expression levels of sarcoglycans and dystroglycans are relatively comparable in mdx toe fibres to that of normal muscle fibres. It has been previously shown that utrophin localises extra-junctionally in extraocular and toe fibres from the mdx mouse model [42]. Therefore, utrophin might act as a molecular substitute anchoring the dystrophin-associated protein complex in Dp427-deficient tissue. Hence, utrophin establishes a link between the actin membrane cytoskeleton and the extracellular matrix, helping to prevent down-stream calcium induced protein degradation.

The mdx mouse exhibits a moderate form of muscular dystrophy and a life span greater than one year, whereas the utrophin null mouse shows no muscular dystrophy and has a life span similar to that of wild-type mice. In stark contrast, the dystrophin-utrophin null mouse ($Utrn^{-/-}$/mdx) exhibits a severe muscular dystrophy and a short life span, between four and twenty weeks [22], suggesting an important role for utrophin in sarcolemmal membrane integrity. A comparative histochemical analysis between normal gastrocnemius, mdx gastrocnemius and mdx toe muscle fibres clearly showed a difference in peripherally located nuclei. The observation that mdx toe fibres show mostly peripheral located nuclei indicate strongly that, although lacking Dp427, the fibres do not exhibit the same degree of degeneration as seen in mdx gastrocnemius muscle fibres. This supports the hypothesis that utrophin infers some degree of stability to the dystrophin-associated protein complex [24]. Certainly other cellular factors besides the up-regulation of utrophin are involved in the sparing of the distal mdx muscle fibres. These are a lower mechanical stress per unit surface membrane area and perhaps different metabolic processes. The fact that utrophin is structurally and functionally similar to that of dystrophin opens up an important avenue with respect to gene therapy and pharmacological intervention. Utrophin gene therapy has added advantages over some of the other areas of possible therapy. Since utrophin is naturally produced in low levels by boys suffering from DMD or BMD, their bodies would not recognise utrophin as foreign. This means that if the utrophin gene was inserted as a means of gene therapy to increase utrophin levels the problem of immunological rejection may be overcome. Usually when a foreign substance is seen by the body's immune system, it will recognise it as foreign and will trigger an appropriate immune response. However, since utrophin is not considered foreign to the body, the immune defence system does not react to it unlike dystrophin. Since utrophin is naturally found in various cell types, an increased amount of utrophin in non-muscle tissues does not appear to have any detrimental side effects in the mdx mouse. Consequently a gene therapeutic approach does not necessarily have to target utrophin specifically to skeletal muscle cells.

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

The first component of the dystrophin-associated glycoprotein complex to be cloned was dystroglycan, which produces a precursor protein that is processed to generate α- and β-dystroglycan [32]. In skeletal muscle, β-dystroglycan was found to exhibit an apparent molecular mass of 43 kDa. It consists of a single transmembrane domain and is inserted into the muscle plasma membrane with the carboxy-terminus on the cytoplasmic side. The last 15 amino acids of β-dystroglycan, rich in proline residues and containing sites for tyrosine phosphorylation, appear to bind directly to the cysteine-rich region of dystrophin [33, 34, 52], thus establishing the link between the actin cytoskeleton and the sarcolemma. An established feature of dystrophin-deficient skeletal muscle fibres is the drastic reduction in various surface glycoproteins [48]. As illustrated in Figure 2, immunoblotting confirmed the previously shown dramatic reduction of β-dystroglycan in Dp427-deficient mdx gastrocnemius membrane preparations [13, 46]. In contrast, in mdx extraocular and toe fibres the expression level of this key member of the membrane-spanning complex was comparable to that in normal gastrocnemius (Figure 2C). Control blots show that the relative density of the α_2-subunit of the dihydropyridine receptor and laminin is not altered in the three tissues studied (Figure 2A, D).

Comparative immuno fluorescence microscopy confirmed the sarcolemmal localization of β-dystroglycan in normal gastrocnemius muscle and showed a drastically reduced expression in Dp427-deficient mdx fibres [25]. Figure 3A shows the relatively weak surface labelling of β-dystroglycan in mdx gastrocnemius and the predominant central nucleation in dystrophic cells. In contrast to dystrophic gastrocnemius, the degree of central nucleation is considerably less in extraocular mdx fibres (Figure 3B). The active regeneration of muscle to repair or replace lost or damaged fibres is signified by centralized nuclei, small fibre size and basophilic RNA-

rich cytoplasm [6, 10]. Fibres undergoing necrosis and regeneration seem to do so in groups for a reason not fully comprehended, possibly due to incomplete lateral fusion of the myofibres or mast cell mediated myocytotoxicity [30]. The intense labelling of β-dystroglycan in the extraocular mdx fibre periphery demonstrates the sarcolemmal rescue of this dystrophin-associated glycoprotein (Figure 3B) suggesting that extraocular mdx muscles represent a naturally protected phenotype.

Up-regulation of Ca^{2+}-ATPase in extraocular mdx muscle fibres

Abnormal Ca^{2+}-handling has been postulated to play a major role in the end-stage pathology of muscular dystrophy [1, 21]. The calcium hypothesis of muscular

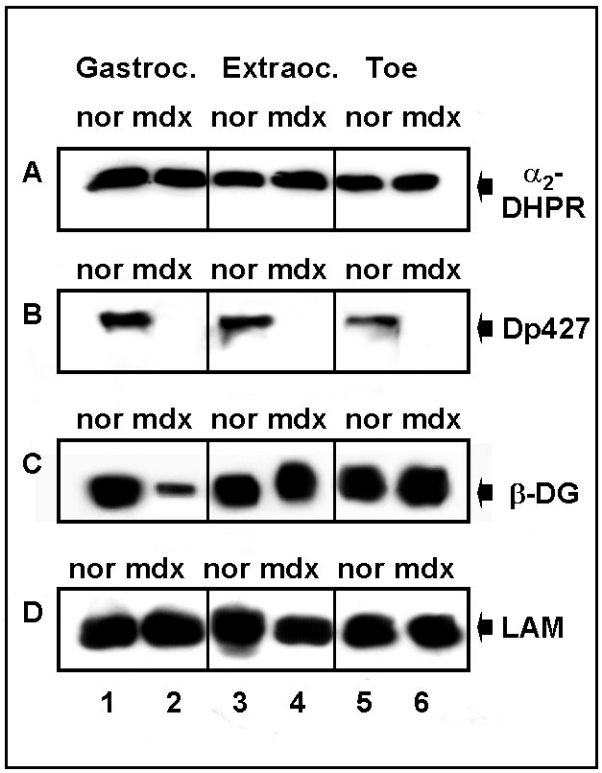

Figure 2. Comparative immunoblot analysis of microsomes isolated from normal and dystrophic mdx gastrocnemius, extraocular and toe muscle fibres. Shown are immunoblots labelled with monoclonal antibodies to the α_2-subunit of the dihydropyridine receptor of the transverse tubules (α_2-DHPR) (A), the full-length Dp427 isoform of the membrane cytoskeletal protein dystrophin (B), the 43 kDa sarcolemmal glycoprotein β-dystroglycan (β-DG) (C), and the extracellular matrix protein laminin (LAM) (D). Lanes 1 to 6 contain membranes from normal (nor) and dystrophic (mdx) gastrocnemius (Gastroc.), extraocular (Extraoc.) and toe muscle fibres, respectively. Membranes were prepared and analysed by standard methodology [21]. Although deficient in dystrophin, both extraocular and toe mdx microsomes contain β-dystroglycan levels comparable to normal muscle fibres. This strongly indicates that these mdx fibres represent a naturally protected phenotype.

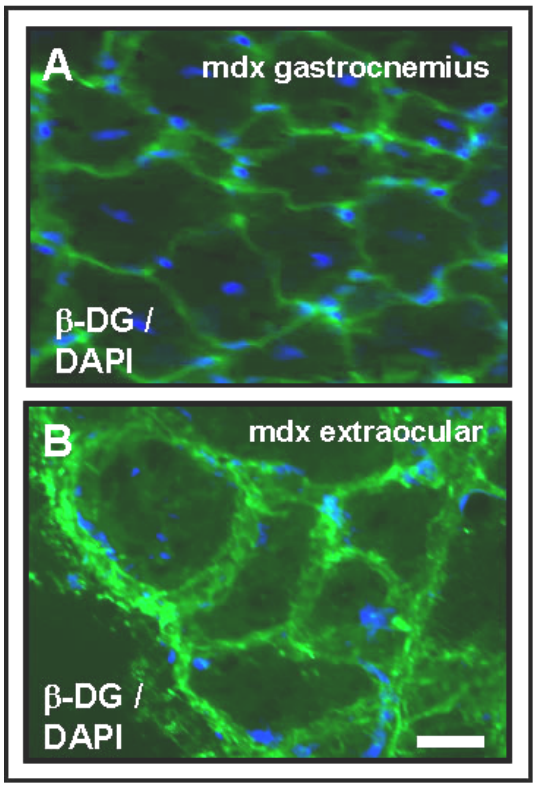

Figure 3. Comparative immunofluorescence analysis of dystrophic gastrocnemius and extraocular fibres. Shown is the immuno-decoration of mdx gastrocnemius (A) and mdx extraocular muscle fibres (B) using 10 μm cyrosections stained with primary antibodies to β-dystroglycan (β-DG) and secondary fluorescein-conjugated antibodies [25]. Incubation with the DNA-binding dye diamidino-phenyindole (DAPI) was used to label nuclei. The direct comparison of the fluorescein labelling intensity of β-dystroglycan demonstrated a higher expression levels of β-dystroglycan in mdx extraocular fibres as compared to mdx gastrocnemius muscle. DAPI staining revealed a high degree of central nucleation in mdx gastrocnemius sections and more peripheral nucleation in mdx extraocular fibres indicating that degeneration/regeneration-cycles are not as active in the extraocular fibres as compared to gastrocnemius cells.

dystrophy [19] assumes that the deficit of the linkage between the extracellular matrix component laminin and the actin membrane cytoskeleton, normally provided by the dystrophin-associated protein complex, impairs the structural integrity of the muscle plasmalemma [15]. As a result, dystrophin-deficient skeletal muscle membranes yield to exercise-induced membrane ruptures more frequently than those of normal fibres. Smaller diameter fibres, such as extraocular and toe muscle fibres, do not appear to be as severely affected as bulk muscle fibres such as gastrocnemius tissue [21, 35]. Transient micro-rupturing allows for the introduction of proteolytically activated Ca^{2+}-leak channels into the sarcolemma [1] during the natural processes of cell membrane resealing [7]. This triggers a localized sub-sarcolemmal accumulation of Ca^{2+}-ions [56]. A pathophysiological consequence of abnormal Ca^{2+}-handling [1] and membrane defects [44] is impaired excitation-contraction coupling [23]. The raised cytosolic Ca^{2+}-levels allow for activation of Ca^{2+}-dependent proteases, causing further channel activation and general protein degradation. An example being the calpains, which are muscle specific and potent proteolysis instigators [1, 5, 54]. In addition, the reduced expression of several luminal calsequestrin-like proteins (CLPs) and a general decrease in the ability of the sarcoplasmic reticulum (SR) to properly sequester Ca^{2+}-ions allows for abnormal Ca^{2+}- buffering [21]. This might be a contributing aspect to the raised cytosolic Ca^{2+}-levels. In contrast, mitochondria in close proximity to ryanodine receptor Ca^{2+}-release channel complexes at the membrane face of the sarcoplasmic reticulum buffer a certain degree of elevated Ca^{2+}-levels. Irregularities in mitochondrial function, however, are postulated to lead to the generation of pro-apoptotic factors, resulting eventually in muscle degeneration [38, 41, 53].

Ca^{2+} re-uptake is a vital step during muscle relaxation, it was therefore of interest to study the fate of the major sarcoplasmic reticulum Ca^{2+}-ATPase isoform SERCA1 in muscular dystrophy [25]. The Ca^{2+}-ATPase activity was shown to be drastically increased in extraocular mdx fibres. Comparative immunoblotting also uncovered an increase in the expression level of the SERCA1 ion pump species, indicating a potential adaptive response in these dystrophin-deficient fibres. The increased removal of cytosolic Ca^{2+}-ions from small-diameter fibres, via the up-regulation of this predominant Ca^{2+}-pump, might protect dystrophin-deficient muscle cells from Ca^{2+}-induced myonecrosis [25]. Since calsequestrin-like protein levels are reduced in mdx gastrocnemius muscle, a decreased capacity for Ca^{2+}-ion buffering and storage exists in the dystrophic sarcoplasmic reticulum, with a consequence of increased cytosolic proteolysis via Ca^{2+}-mediated enzymes. Therefore, the increased removal of excess Ca^{2+}-ions by up-regulated SERCA1 units might counter-act these pathophysiological changes. A more detailed study of the differences between an affected muscle group and a protected phenotype might pave the way for vital therapeutic and pharmacological intervention, as is demonstrated by the deployment of protease inhibitors such as carnitine-linked leupeptin in order to inactivate Ca^{2+}-dependent calpain activity [5]. Histologic analysis of treated muscles, in comparison to untreated mdx myofibers, consistently showed increases of myofiber diameter. In addition, there was a clear correlation between increased myofiber size and decreased calpain activities in leupeptin treated muscles [5]. Pharmacological intervention directed towards key calcium handling proteins, abnormally expressed in dystrophic tissue, such as the SERCA1 pump and the CLPs might offer a worthy target in the treatment of the muscular dystrophies.

Persistent Dp71 expression in mdx brain tissue

The major dystrophin protein product in brain, Dp71, is expressed in astrocytes and glial cells [4, 14]. It contains the carboxy-terminal and the cysteine-rich domains of dystrophin, and seven additional amino acids at the amino-terminus. It lacks the entire large central domain of spectrin-like repeats and the actin-binding domain of dystrophin [4]. The Dp71 isoform represents the major DMD gene product in brain and other non-muscle tissues. The trans-membrane link is maintained by an interaction between Dp71 and β-dystroglycan, which in turn interacts with α-dystroglycan, thus establishing the membrane spanning dystroglycan sub-complex [20]. Our analysis of normal and mdx brains showed the expression levels of Dp71 to be similar in the two tissues, with the abundance levels of both α- and β-dystroglycan exhibiting no major difference (Figure 4D, E). While the protein complement of normal and mdx brain microsomes was found to be very similar, the dystrophin isoform Dp427 is completely absent in mdx brain (Figure 4A, B). Although Dp71 co-localizes with β-dystroglycan, the lack of full-length brain dystrophin seems to trigger a disturbed organization of the dystroglycan sub-complex [18]. In dystrophic forebrain, β-dystroglycan expression is not drastically affected, possibly due to the up-regulation of utrophin isoforms which partially compensate for the deficiency in brain dystrophins. Together with Dp71, which exists in contrast to its normally oligomeric form in *mdx* brain as a monomeric protein, brain utrophins confer a degree of stability on the dystrophin-associated protein complex in Dp427-deficient brain [18]. In analogy to the naturally protected extraocular and toe mdx fibres, comparative investigations into the adaptive changes in the dystrophic mdx brain might lead to the discovery of new therapeutic targets in the treatment of muscular dystrophy.

Proteomics analysis of Muscular Dystrophy

Potential problems associated with myoblast and gene therapy, namely immune rejection and cytotoxic side effects, have led to novel approaches in tackling muscular dystrophy [3, 55]. One of them is the usage of high-resolution two-dimensional gel electrophoresis in conjunction with mass spectroscopy in order to identify novel proteins involved in this disorder. Proteomics-based research strategies directed towards the determination of abnormally expressed proteins in control versus dystrophic leg muscle, as compared to that of protected phenotypes (exemplified by mdx extraocular, toe and brain tissues) represent a new scientific angle in the treatment for muscular dystrophy. The general protocol of such an approach is outlined in the flow chart of Figure 5. For example, high-throughput analyses based on mass spectroscopy might detect rescuing pathways by which utrophin abolishes the dystrophic phenotype. Utrophin up-regulation, whether it occurs through gene transfer or pharmacological intervention, is an exciting option. The increased expression of the Up395 protein in

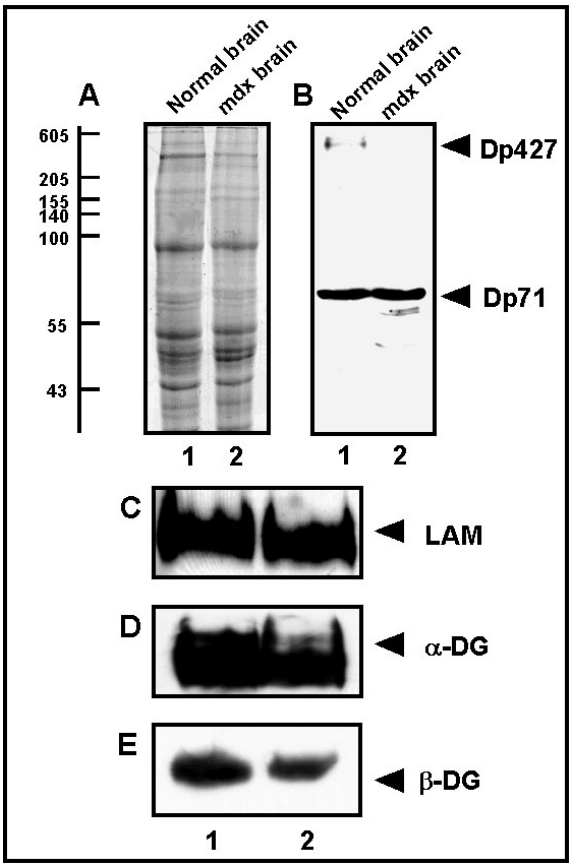

Figure 4. Comparative immunoblot analysis of normal and dystrophic mdx brain microsomes. Shown is a Coomassie-stained gel (A) of normal (lane 1) and dystrophic (lane 2) brain membranes, as well as identical immunoblots lebelled with antibodies to the two brain dystrophin isoforms of 427 kDa (Dp427) and 71 kDa (Dp71) (B), laminin (LAM) (C), α-dystroglycan (α-DG) (D), and β-dystroglycan (β-DG). The major dystrophin isoform in brain, Dp71, appear to be unaffected in the Dp-427-deficient brain tissue and might thus compensate for the lack of full-length dystrophin in stabilising the dystrophin-associated complex. Brain membranes were prepared and analysed by standard methodology [18]. Molecular mass standards (in kDa) are indicated at the left.

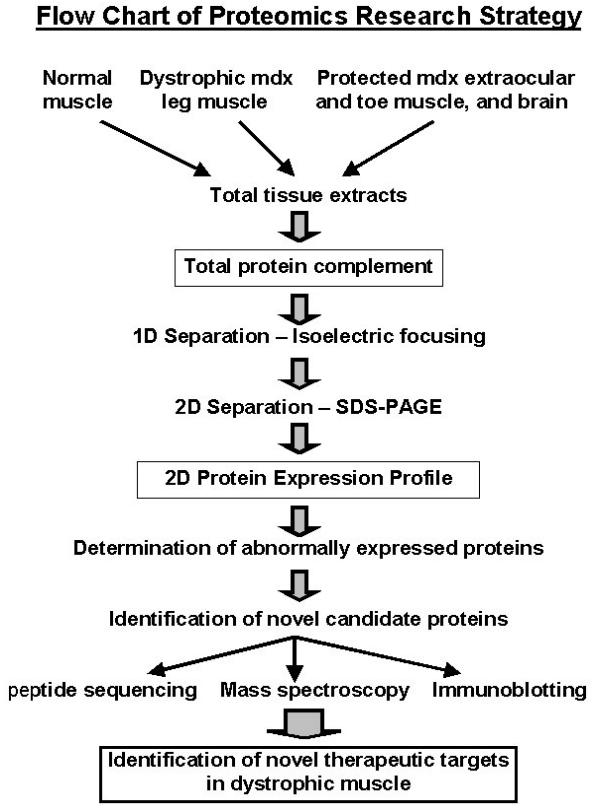

Figure 5. Flow chart of a proteomics-based research strategy to identify novel therapeutic targets in dystrophic muscle. Total skeletal muscle extracts from normal tissues and mdx gastrocnemius, mdx extraocular, and mdx brain will be subject to one-dimensional separation using isoelectric focusing. Subsequently high-resolution sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) will be performed in the second dimension in order to establish a total protein expression profile. Abnormally expressed proteins can then be determined by density scanning and identified by peptide sequencing, mass spectroscopy and/or immunoblotting. This high-throughput approach might be useful in the initial characterization of novel candidate proteins implicated in the protection of the dystrophin-associated protein complex.

mdx toe fibres appears to functionally compensate for the absence of dystrophin in Dp427-deficient tissue. Substituting utrophin seems to establishing a proper linkage between the extracellular matrix and actin membrane cytoskeleton in certain dystrophic fibres. Dystrophin-related proteins, along with the various Ca^{2+}-handling proteins, merit consideration in identifying novel therapeutic targets in dystrophic muscle. Through the processes of protein isolation, electrophoretic separation, peptide digestion and sequencing, mass spectroscopy and verification by immunoblotting, new candidate proteins may be identified.

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Address correspondence to:

Prof. Kay Ohlendieck, Department of Biology, National University of Ireland, Maynooth, Co. Kildare, Ireland; Tel. (353) (1) 708-3842; Fax: (353) (1) 708-3845 E-mail: kay.ohlendieck@may.ie

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