

# Dysferlin is a plasma membrane protein and is expressed early in human development

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Received December 21, 1998; Revised and Accepted February 3, 1999

Recently, a single gene, *DYSF*, has been identified which is mutated in patients with limb-girdle muscular dystrophy type 2B (LGMD2B) and with Miyoshi myopathy (MM). This is of interest because these diseases have been considered as two distinct clinical conditions since different muscle groups are the initial targets. Dysferlin, the protein product of the gene, is a novel molecule without homology to any known mammalian protein. We have now raised a monoclonal antibody to dysferlin and report on the expression of this new protein: immunolabelling with the antibody (designated NCL-hamlet) demonstrated a polypeptide of ~230 kDa on western blots of skeletal muscle, with localization to the muscle fibre membrane by microscopy at both the light and electron microscopic level. A specific loss of dysferlin labelling was observed in patients with mutations in the LGMD2B/MM gene. Furthermore, patients with two different frame-shifting mutations demonstrated very low levels of immunoreactive protein in a manner reminiscent of the dystrophin expressed in many Duchenne patients. Analysis of human fetal tissue showed that dysferlin was expressed at the earliest stages of development examined, at Carnegie stage 15 or 16 (embryonic age 5–6 weeks). Dysferlin is present, therefore, at a time when the limbs start to show regional differentiation. Lack of dysferlin at this critical time may contribute to the pattern of muscle involvement that develops later, with the onset of a muscular dystrophy primarily affecting proximal or distal muscles.

## INTRODUCTION

To date, at least eight forms of autosomal recessive muscular dystrophy have appeared under the general heading of limb-

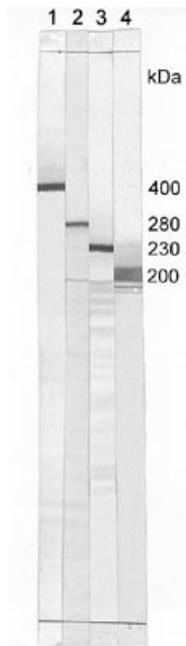
girdle muscular dystrophy (LGMD). These are in two groups: those with abnormal expression of the dystrophin–glycoprotein complex (1) and those where labelling of proteins in this complex is unaffected. Thus, the sarcoglycanopathies (sometimes known as LGMD types 2C, 2D, 2E and 2F) are caused by defects in the genes for  $\gamma$ -,  $\alpha$ -,  $\beta$ - or  $\delta$ -sarcoglycan on chromosomes 13q12, 17q12, 4q12 and 5q33, respectively (2–5). Among the dystrophies where expression of the sarcoglycans is normal, the gene responsible for LGMD2A has been identified as the chromosome 15q15-encoded muscle-specific protease calpain 3 (6), and the gene for LGMD2B was identified recently as the 2p13-located *DYSF* (7,8). Genes for LGMD2G and LGMD2H have been localized to 17q11–q12 (9) and 9q31–q33 (10), and further LGMD genes are inferred (9,11). Although clinically heterogeneous in terms of age of onset and rate of progression, the feature that all these conditions share is weakness that starts with the proximal limb-girdle muscles. Unexpectedly, a disease characterized by the early involvement of distal calf muscles, Miyoshi myopathy (MM), was shown to be caused by mutations in the same gene as LGMD2B (8). Dysferlin, the protein product of the *DYSF* gene, is a novel molecule without homology to any known mammalian protein. However, the gene does have significant homology, throughout its length, to a nematode spermatogenesis factor (*fer-1*), a fact that contributed to the name it was given (7,8). Here we present the first description of dysferlin protein expression.

## RESULTS

### Dysferlin antibody generation

A monoclonal antibody was developed that recognizes an epitope near the C-terminus of dysferlin, within amino acids 1999–2016, just before the predicted transmembrane domain (7,8). Adsorption with the immunizing peptide removed all immunoreactivity. Since the intention was to raise a diagnostically useful antibody that would enable patients with LGMD2B to be identified, the

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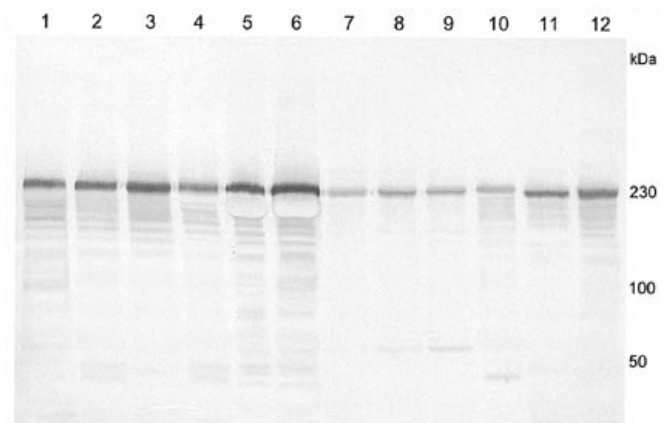
**Figure 1.** Estimation of molecular mass. Four strips from a blot of normal control muscle labelled with antibodies to dystrophin (lane 1, band at ~400 kDa), filamin (lane 2, band at ~280 kDa), dysferlin (lane 3, band estimated to be at ~230 kDa by comparison with the others) and myosin heavy chain (lane 4, band at ~200 kDa).

antibody was given the designation NCL-hamlet (as in '2B or not 2B?—that is the question').

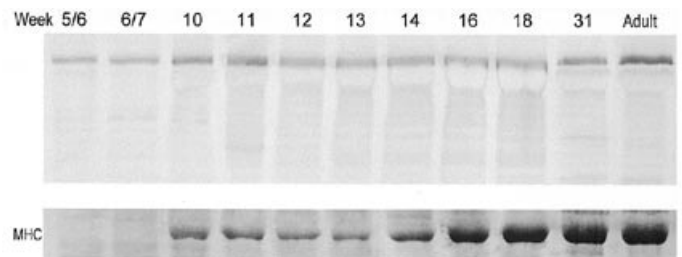
The size of dysferlin is expected to be ~237 kDa, based on the predicted amino acid sequence (8), but it is notoriously difficult to estimate the molecular masses of large proteins on blots. Molecular mass markers are not manufactured in this size range, so the migration distance of the immunolabelled dysferlin band was compared with those for dystrophin (~400 kDa), filamin (250–280 kDa) and myosin heavy chain (~200 kDa). From this, the size of dysferlin was estimated to be ~230 kDa, in keeping with the predicted size (Fig. 1). Bands at 230 kDa were also observed in skeletal muscle from mouse, rat, rabbit, hamster, pig and dog, but not chicken (data not shown), suggesting that the amino acid sequence of this region is conserved between mammals, but differences exist in birds.

### Dysferlin expression in normal tissues

Protein expression in different tissues was examined in rat (Fig. 2). On blots, dysferlin appears to have a ubiquitous distribution, and bands at ~230 kDa were observed in all the tissues tested: skeletal muscle, heart and kidney showed the strongest expression; stomach, lung, uterus, liver and spleen also showed clear labelling, with nervous tissue (cerebellum, brain stem, spinal cord and sciatic nerve) showing less. Blood vessels are present in all the tissues, but vascular smooth muscle represents a minor component of most tissue samples (as judged previously from the smooth muscle myosin content; data not shown). It is possible that the band in the peripheral nerve sample represents a slightly larger protein. In the parts of rat brain available for examination, no clear evidence was found of a smaller protein that might



**Figure 2.** Distribution of dysferlin in different rat tissues. Lane 1, stomach; lane 2, lung; lane 3, kidney; lane 4, uterus; lane 5, skeletal muscle; lane 6, heart; lane 7, cerebellum; lane 8, brain stem; lane 9, spinal cord; lane 10, sciatic nerve; lane 11, liver; lane 12, spleen. The blot was labelled with 1/300 anti-dysferlin antibody, NCL-hamlet.



**Figure 3.** Dysferlin expression during development. Western blot of fetal limb tissues at different stages of development. The times indicated are weeks of fetal age. The blot was labelled with 1/300 anti-dysferlin antibody, NCL-hamlet.

correspond to the reported 4 kb transcript (7,8), but faint bands were seen at ~60 kDa in brain stem and spinal cord (Fig. 2, lanes 8 and 9), and at 40 kDa in sciatic nerve (lane 10). Few fresh human tissues were available for examination: dysferlin was detected on blots of human heart and term placenta, and labelling of skeletal muscle sections showed weak labelling of peripheral nerve and the smooth muscle in blood vessels, but no significant labelling of fibroblasts in the extracellular matrix, or the lymphocytes and macrophages in inflammatory infiltrates and foci of degeneration.

### Dysferlin expression during fetal development

Dysferlin expression was also examined in samples of human fetal limb tissue of different ages. Western blot analysis demonstrated a clear 230 kDa band from the earliest time point examined, at Carnegie stage 15 or 16, embryonic age 5–6 weeks (Fig. 3). No fetal material was available for sectioning at this time.

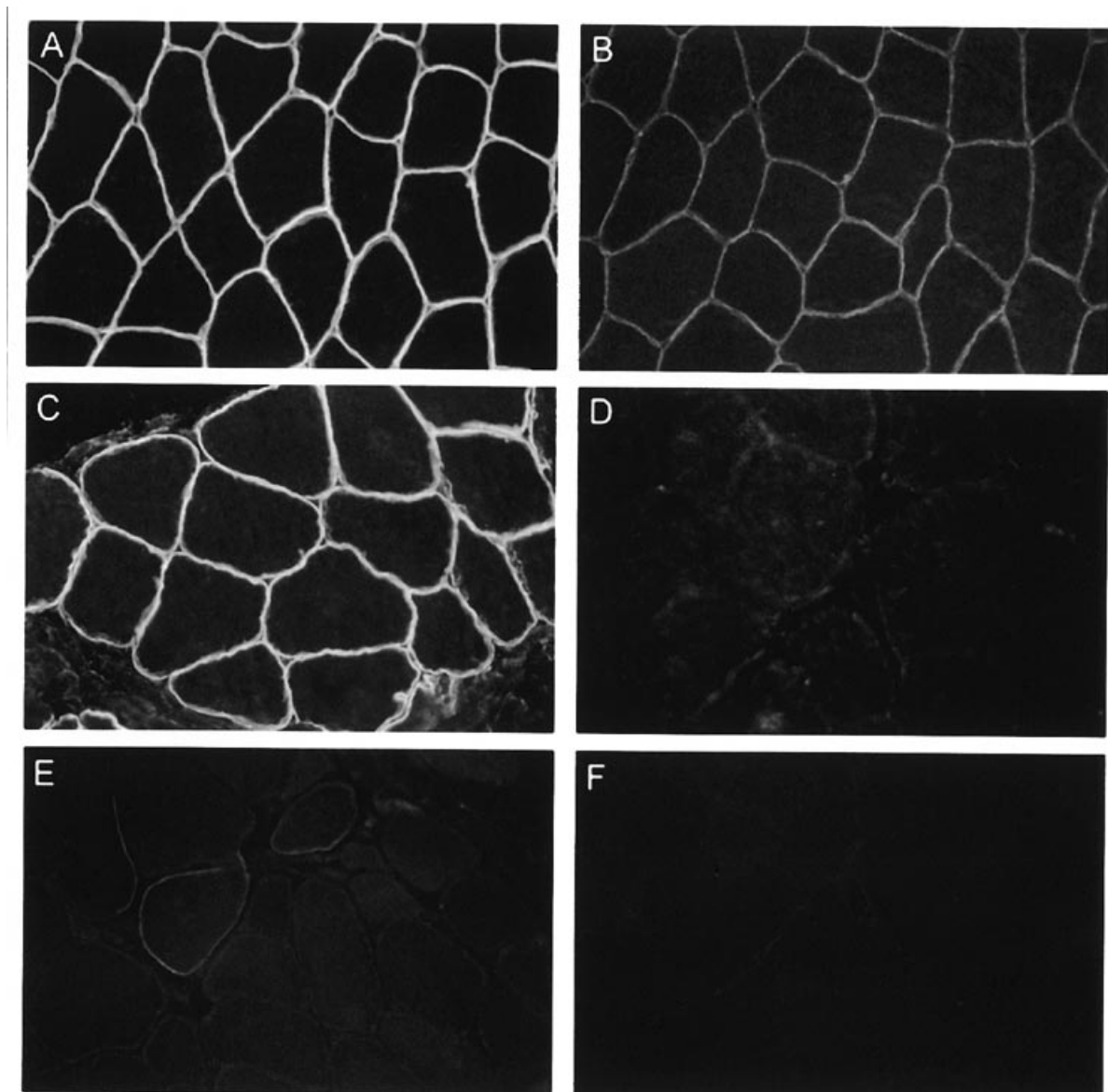
### Localization of dysferlin in skeletal muscle

The localization of dysferlin in skeletal muscle was first determined by light microscopy. Like dystrophin (Fig. 4A and C), labelling for dysferlin was observed at the periphery of the muscle

fibre (Fig. 4B). The resolution of light microscopy makes it impossible to determine whether the labelling was at the plasma membrane or in the basal lamina. Electron microscopic immunogold cytochemistry revealed labelling sites for dysferlin that were concentrated at the periphery of the myofibres, many being closely adjacent to the plasma membrane (Fig. 5). The basal lamina, external to the plasma membrane, was not labelled. No nuclear membrane labelling was observed, or labelling of the endoplasmic or sarcoplasmic reticulum. It is not yet possible to confirm that the N-terminus and bulk of the molecule lie on the inside of the plasma membrane, but examination of the available photographs shows labelling of the NCL-hamlet epitope with 78% of the gold particles on the inside (Fig. 5).

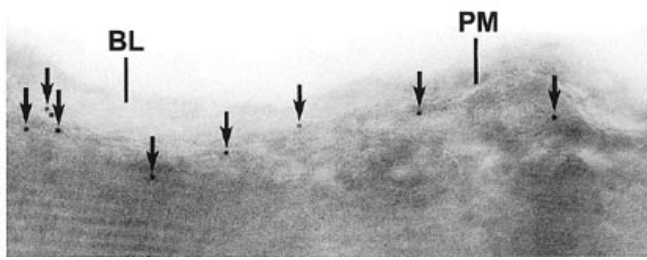
#### Dysferlin expression in patients

Immunocytochemical analysis of dysferlin expression was undertaken in LGMD2B patients with homozygous frameshifting mutations that would cause premature termination of translation before the amino acids of the antibody-binding site. Some slight labelling was seen around some fibres (e.g. Fig. 4D), but a single fibre and parts of adjacent fibres were more strongly labelled in one patient (Fig. 4E). This pattern of labelling is strikingly similar to that seen for dystrophin in patients with Duchenne muscular dystrophy (DMD) where faint labelling may occur and 'revertant' dystrophin-positive fibres are a common feature (12–14). Immunolabelling of serial sections showed that these fibres were not



**Figure 4.** Immunocytochemical labelling of skeletal muscle sections from: (A) a normal control labelled with 1/10 Dy8/6C5 to dystrophin (C-terminus); (B) a normal section labelled with NCL-hamlet to dysferlin; (C) the Libyan Jew with LGMD2B (homozygous frameshifting mutation), labelled with Dy4/6D3 to dystrophin (rod); (D) the same patient, labelled with NCL-hamlet to dysferlin; (E) the Palestinian Arab with LGMD2B (homozygous frameshifting mutation) labelled with NCL-hamlet; (F) the normal control and is labelled with the tissue culture medium containing 20% fetal calf serum that was used to grow the NCL-hamlet hybridoma cells (negative control).





**Figure 5.** Ultrastructural immunocytochemistry. Dysferlin is located at the periphery of the myofibre. Gold particles (10 nm; arrowed) are seen close to the plasma membrane (PM). BL, basal lamina. Magnification,  $\times 75\,000$ .

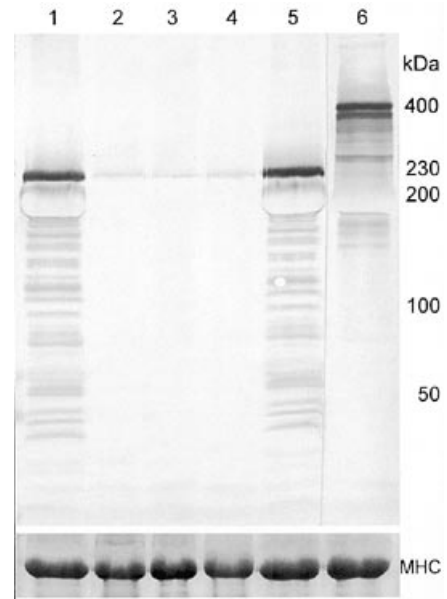
labelled with an antibody to developmental myosin heavy chain, indicating that they were not in the process of regenerating. Western blot analysis of the patient biopsies demonstrated very faint bands at 230 kDa, representing an abundance of  $<5\%$  of normal (Fig. 6, lanes 2–4). Patients with non-2p13-linked forms of muscle disease (e.g. dystrophinopathy, sarcoglycanopathy, calpainopathy, inflammatory myopathy, metabolic myopathy) showed dysferlin labelling on sections and blots that was indistinguishable from normal (e.g. Fig. 6, lane 5). Dysferlin immunoanalysis was also undertaken in patients with a common *DYSF* mutation but different clinical phenotypes (LGMD2B versus MM) (15).

#### Immunoreactivity to fragments on blots and sections

In control skeletal muscle (normal or non-2p13-linked patient), multiple bands of smaller size were observed in addition to the intensely labelled 230 kDa band (Fig. 6, lanes 1 and 5). These lower bands were missing in patients with mutations in the *DYSF* gene (lanes 3 and 4), indicating that these represent fragments of dysferlin protein, as opposed to protein products of other genes. A comparable range of lower molecular mass bands below the full-size protein band is also seen with antibodies to dystrophin (e.g. Fig. 6, lane 6). Large muscle proteins take a long time to be synthesized (16) and to be broken down (17), and it is likely that these bands represent metabolic fragments of the full-size protein. In this context, it is interesting that the Dy8/6C5 antibody, to an epitope at the C-terminus of dystrophin, recognizes a single band on blots and provides very clean labelling on sections (Fig. 4A). In contrast, the rod antibody Dy4/6D3, which recognizes multiple fragments of dystrophin on blots (shown in Fig. 6), demonstrates a slight cytoplasmic labelling (Fig. 4C), suggesting that the smaller fragments are not able to locate correctly. Some faint dysferlin labelling was also observed within the muscle cytoplasm (Fig. 4B), and this may also be attributable to unattached fragments of the full-size protein.

#### DISCUSSION

This report describes the production of a monoclonal antibody to dysferlin and its use in the examination of protein expression in normal and pathological situations. Dysferlin is a ubiquitously expressed 230 kDa molecule that is localized to the periphery of muscle fibres. It therefore appears that the putative transmem-



**Figure 6.** Western blot of human skeletal muscle biopsies. Lanes 1 and 6, normal control subjects (amputated leg muscles); lane 2, deltoid sample from a Palestinian LGMD2B patient linked to chromosome 2p13 (unknown mutation); lane 3, quadriceps sample from a Palestinian Arab LGMD2B patient (homozygous frameshifting mutation); lane 4, deltoid sample from a Libyan Jewish patient with LGMD2B (homozygous frameshifting mutation); lane 5, quadriceps sample from a patient with genetically confirmed LGMD2A. Lanes 1–5 were labelled with 1/300 anti-dysferlin antibody, NCL-hamlet. Lane 6 was labelled with 1/50 Dy4/6D3 to an epitope in the rod domain of dystrophin. The lower panel shows the corresponding myosin heavy chain (MHC) bands from the Coomassie blue-stained gel, and the outline of these bands can be seen at 200 kDa in lanes 1, 5 and 6. The MHC bands on the post-blotted gel are used to indicate how much muscle protein, as opposed to fat and fibrous connective tissue, is loaded in each lane. Densitometric estimates of dysferlin/myosin abundance were expressed as a percentage of the normal sample in lane 1: lane 2, 1.5%; lane 3, 1.8%; lane 4, 4.7%; lane 5, 100.8%.

brane domain identified in the dysferlin sequence (7,8) does exist, with the plasma membrane being the target in skeletal muscle. Thus, dysferlin joins the group of sarcolemmal proteins located at the plasma membrane or within the basal lamina, whose reduced expression cause inherited degenerative myopathies. Proteins in this group include dystrophin (18),  $\alpha$ - (3),  $\beta$ - (19),  $\gamma$ - (2) and  $\delta$ -sarcoglycan (5), the laminin  $\alpha 2$  chain of merosin (20), the integrin  $\alpha 7$  chain (21) and caveolin 3 (22). The function of some or all of these proteins may be structural, whereby the loss of protein leads to muscle fibre degeneration. Dystrophin and the associated proteins (dystroglycans and sarcoglycans) are unaffected in LGMD2B/MM (7), so dysferlin is unlikely to be a close member of that complex. Given the homology of dysferlin to a nematode spermatogenesis factor that is required for successful membrane fusion (7,8), it is also conceivable that the lack of dysferlin may cause faulty myotube fusion and thereby impair muscle regeneration.

Dysferlin molecules of  $\sim 230$  kDa were expressed in all the tissues tested, with less apparent variation in abundance (per mg of sample) than might be expected from the distribution of RNA. Dysferlin RNA appears to be expressed predominantly in skeletal muscle, although it is also present in heart and placenta, and weakly expressed in liver, lung, kidney and pancreas (7,8). There

have been no reports of tissues other than skeletal muscle being affected in either LGMD2B or MM. It should be noted that  $\beta$ -sarcoglycan also has a widespread tissue distribution although the clinical symptoms of  $\beta$ -sarcoglycanopathy (or LGMD2E) are restricted to skeletal muscle (4,23). The distribution (and functions) of dysferlin in non-muscle tissues will also need elucidation.

Patients with 2p13-linked MD were differentiated from those with other conditions on the basis of reduced dysferlin expression on sections and blots. The generation of a diagnostically useful antibody is important since the dysferlin gene has >50 exons, and mutation analysis is extremely laborious. It has been estimated that LGMD2B may be a very common form of milder limb-girdle dystrophy (24). The widespread tissue distribution may mean that it is possible to undertake dysferlin immunoanalysis in a sample other than a muscle biopsy.

In this preliminary study, we found very low levels of immunolabelling in sections and blots of muscle from two patients with homozygous frameshifting mutations in the *DYSF* gene. The amount of labelling, >5% of normal, is similar to that of dystrophin seen in cases of DMD with terminating mutations (12,13). It seems likely that, by analogy, the reading frame has been restored by exon skipping (25–29) in a small proportion of dysferlin transcripts. There are many small exons in the dysferlin gene, which may predispose this gene to this activity. An alternative explanation for the low level of immunoreactive protein detected is the existence of a homologous protein of identical size and location. However, the observation of occasional more brightly labelled 'revertant' fibres argues against this possibility. The lack of correlation with a marker of regeneration (developmental myosin heavy chain) indicates that the immunolabelling in these few fibres is unlikely to represent re-expression of a fetal protein.

Muscle weakness in LGMD2B starts in the proximal muscles of the pelvic girdle and presents as difficulty with running and climbing stairs. In contrast, initial muscle weakness in MM characteristically is restricted to the gastrocnemius muscle, as indicated by an inability to stand on the toes (30–34). The finding that both these conditions are caused by mutations in the same gene is interesting because each type of muscular dystrophy is defined by the muscles involved and, although the clinical phenotype of LGMD2B and MM may overlap to varying degrees (30), the possibility of such different patterns of muscle involvement is unique among the known dystrophies. For example, Duchenne and Becker muscular dystrophy have different clinical profiles yet the actual muscle involvement is identical in both cases (35). This certainly raises the possibility that dysferlin may interact with a modifying protein that is the product of another gene (31). It is also possible that dysferlin levels vary in different muscle groups, or that it plays a role in development of the distal and proximal musculature in the fetus. Dysferlin was clearly detected at Carnegie stage 15 or 16 (embryonic age 5–6 weeks) and is therefore present at a stage of development when the limbs start to show regional differentiation. Lack of dysferlin at this critical time may contribute to the pattern of muscle involvement that develops later, with the onset of a muscular dystrophy primarily affecting proximal or distal muscles.

## MATERIALS AND METHODS

### Antibodies

To generate the new antibodies to dysferlin, the 2080 amino acid sequence (GenBank accession no. AF075575) was assessed with Omega v1.1 analytical software from Oxford Molecular, and a peptide (C-ERPAGQGRDEPNMNPKE) corresponding to amino acids 1999–2016 was chosen for synthesis and conjugation to keyhole limpet haemocyanin. Following immunizations and test tail bleeds, a CD1 mouse that had responded well was chosen, given a final boost of intravenous immunogen in saline, and killed. The harvested splenocytes were fused with X63.Ag8.653 myeloma cells using polyethylene glycol solutions (36). The resultant hybridoma wells were screened for specific antibody activity on sections and blots, before being cloned four times at 0.5 cells/well to ensure monoclonality. The antibody was used undiluted on tissue sections and at 1/300 dilution on blots. Antibodies to two epitopes on dystrophin were also used: Dy4/6D3 (rod domain) and Dy8/6C5 (C-terminus) (13). The antibody to filamin is a commercially available one (Novocastra, Newcastle upon Tyne, UK), and antibodies to myosin heavy chain were generated by immunization with myosin purified from rat muscle.

### Patients

Muscle samples from five normal control subjects were obtained, with permission, from amputated leg tissue. Muscle biopsies from patients with various muscle diseases were taken as part of the routine diagnostic protocol, and stored in a liquid nitrogen archive. Samples from >30 patients with non-2p13-linked diseases were examined. Six patients from five different families with 2p13-linked LGMD2B/MM were studied. One Palestinian Arab patient with classical LGMD2B (30) has a homozygous frameshifting mutation where a 23 bp insertion produced a stop codon at position 1633 (7). A further Palestinian patient with the LGMD2B phenotype was from a family linked to 2p13, in whom the mutation has not yet been identified. A Libyan Jewish patient from a large kindred with LGMD2B (33) was identified as having a homozygous single base deletion producing a stop codon at position 1729 (7). Other patients with defined mutations have been examined (15).

### Histopathology

All the biopsies showed the classical signs of a muscular dystrophy including an increase in fibre size variation and foci of necrosis and regeneration. However, features of note included the presence of large inflammatory infiltrates or other evidence of inflammatory processes in half the patients.

### Immunoelectrophoresis and western blotting

Polyacrylamide gel electrophoresis, western blotting and densitometric analysis were performed as described in detail previously (37). All tissue samples were weighed frozen and homogenized in 19 vol electrophoresis treatment buffer (e.g. 20 mg + 380  $\mu$ l buffer), giving a loading concentration of ~200  $\mu$ g in 30  $\mu$ l (17).

## Labelling of sections

Immunocytochemistry at the light level was performed as described previously (38) with 6  $\mu\text{m}$  unfixed frozen sections, monoclonal primary antibodies and 1/100 Dako R260 rhodamine-conjugated secondary antibody (Dako, Cambridge, UK) diluted in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 0.1 M lysine. The primary antibodies were used undiluted, or at the dilutions specified in each figure legend. Immunogold labelling was also carried out as described previously (39,40). Briefly, the control muscle sample (from a case with no evidence of any form of muscular dystrophy) was fixed, cryoprotected and plunge-frozen in liquid nitrogen. Sections were cut at a thickness of 80 nm at a block temperature of  $-90^{\circ}\text{C}$  and a knife temperature of  $-110^{\circ}\text{C}$  using a Reichert Ultracut E microtome fitted with an FC4D cryomicrotomy attachment. The primary antibody, NCL-hamlet, was applied undiluted. The secondary antibody, British BioCell EM.GAM10 goat anti-mouse immunoglobulins, conjugated with 10 nm gold particles was diluted 1/20 in PBS containing 0.5% BSA. Sections processed without a primary antibody, or with an antibody to dystrophin (Dy8/6C5) were used as negative and positive controls, respectively.

## ABBREVIATIONS

BL, basal lamina; BSA, bovine serum albumin; DMD, Duchenne muscular dystrophy; LGMD, limb-girdle muscular dystrophy; MHC, myosin heavy chain; MM, Miyoshi myopathy; PBS, phosphate buffered saline; PM, plasma membrane.

## ACKNOWLEDGEMENTS

Grants for our work have been awarded by the Muscular Dystrophy Group of Great Britain and Northern Ireland, the Medical Research Council of Great Britain, the Association Française contre les Myopathies and the Italian Telethon.

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