Identical mutation in patients with limb girdle muscular dystrophy type 2B or Miyoshi myopathy suggests a role for modifier gene(s)

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ABSTRACT

Limb girdle muscular dystrophy type 2B (LGMD2B) and Miyoshi myopathy (MM), a distal muscular dystrophy, are both caused by mutations in the recently cloned gene dysferlin, gene symbol DYSF. Two large pedigrees have been described which have both types of patient in the same families. Moreover, in both pedigrees LGMD2B and MM patients are homozygous for haplotypes of the critical region. This suggested that the same mutation in the same gene would lead to both LGMD2B or MM in these families and that additional factors were needed to explain the development of the different clinical phenotypes. In the present paper we show that in one of these families Pro791 of dysferlin is changed to an Arg residue. Both the LGMD2B and MM patients in this kindred are homozygous for this mutation, as are four additional patients from two previously unpublished families. Haplotype analyses suggest a common origin of the mutation in all the patients. On western blots of muscle, LGMD2B and MM patients show a similar abundance in dysferlin staining of 15 and 11%, respectively. Normal tissue sections show that dysferlin localizes to the sarcolemma while tissue sections from MM and LGMD patients show minimal staining which is indistinguishable between the two types. These findings emphasize the role for the dysferlin gene as being responsible for both LGMD2B and MM, but that the distinction between these two clinical phenotypes requires the identification of additional factor(s), such as modifier gene(s).

INTRODUCTION

The limb girdle muscular dystrophies (LGMDs) are a heterogeneous group of muscle disorders characterized by predominant weakness and wasting of muscles of the pelvic and shoulder girdle. There is broad clinical heterogeneity and this is paralleled by genetic heterogeneity. Both autosomal dominant and recessive forms occur and to date there is evidence for at least 12 different loci (1,2). The second form of autosomal recessive LGMD identified was mapped to chromosome 2p13 and named limb girdle muscular dystrophy type 2B (LGMD2B) (3). Another myopathy affecting initially distal muscles, especially the gastrocnemius, was also mapped to 2p13 (4) and is known as Miyoshi myopathy (MM) (5). The similar map positions of these two myopathies raised the possibility that they might be allelic variants of the same gene (4). Support for the idea that both diseases were caused by the same gene came from the description of two large families in which both clinical phenotypes occurred in each kindred (6,7). Moreover, both types of patient were homozygous for one haplotype in each family, and it was postulated that the diseases were not only caused by the same gene but also the same mutation (6). If correct, the different phenotypes had to be the result of additional factors. Recently, a single gene responsible for both LGMD2B and MM was cloned. It codes for a 237 kDa protein with homology to the Caenorhabditis elegans spermatogenesis factor fer-1, a protein believed to have a role in membrane fusion (8). The gene responsible for these dystrophies was therefore named dysferlin (DYSF) (9,10).

Identification of the gene for LGMD2B/MM allowed us to test the hypothesis that the same mutation in DYSF can cause both LGMD2B and MM. With the development of an antibody to dysferlin (11), it was of particular interest to see whether mutations leading to LGMD2B and/or MM could be distinguished on the basis of dysferlin protein expression in skeletal muscle. Here we report that, in the large Canadian aboriginal
kindred with both LGMD2B and MM patients, all affected individuals are homozygous for one missense mutation, which results in similar reductions of dysferlin expression in both types of patient. As a result, the development of the different phenotypes, at least in the kindred described here, cannot be explained on the basis of allelic diversity, but require additional factor(s), such as modifier gene(s). Observation of the same mutation in patients of two different aboriginal families surrounded by the same core haplotype suggests that this mutation had a common origin.

RESULTS

Mutation detection in Canadian aboriginal pedigrees

The aboriginal kindreds under study have 14 affected individuals including 13 symptomatic patients and one preclinical patient (Fig. 1). Eleven patients are following a course of a fairly rapidly progressive LGMD with onset in the teens and wheelchair requirement by the third decade of life. Two other patients show a milder distal myopathy compatible with MM. All patients have grossly elevated serum creatine kinase (CK) levels (6). The preclinical patient (12 years of age) has asymmetric hypertrophy of the left calf and grossly elevated CK levels, but only very mild proximal muscle weakness.

Patients 8 and 9 (Fig. 1A) were selected for mutation analysis because patient 8 is one of three patients who were reported to be heterozygous for the haplotype of the candidate region and therefore expected to be heterozygous for the disease-causing mutation, and patient 9 was reported to be homozygous for the common haplotype spanning a >4 cM region between D2S291 and D2S286 (6). A search for mutations in DYSF was performed on amplified exons using single strand conformation polymorphism (SSCP) and heteroduplex analyses. Despite the haplotype findings in these two patients, both were found to be homozygous for a C→G transversion found in both patients at position 2745 of DYSF (Fig. 2). This mutation changes the proline at position 791 to arginine and abolishes an HpaII site, thus allowing for easy mutation detection (Fig. 2B) in the entire pedigree. Pro791 is a conserved residue between dysferlin and fer-1, the C.elegans protein mentioned above (8) (alignment not shown).

The mutation segregates in an autosomal recessive fashion in the entire pedigree (Fig. 1A). It was not seen in 100 unrelated control chromosomes nor in other dysferlin-associated muscular dystrophies studied to date. The same mutation was found in four other LGMD patients from two apparently unrelated families from another remote inbred aboriginal community (Fig. 1B). The diagnosis of dysferlin-associated muscular dystrophy has therefore been confirmed in these families as well.

Haplotype analyses

Haplotype analyses were performed on the patients and their family members with four microsatellites flanking the dysferlin gene, two DYSF intragenic markers and the DYSF C2745G mutation (Fig. 1A). As expected, patients 1–5, 9 and 10 are homozygous for the same D2S292–D2S443–Cy172-H32–DYSF C2745G–104-sat–D2S291–D2S2110 haplotype [184–247–199–(–)–156–184–139]. Patients 6–8 are heterozygous for D2S292, D2S443 and D2S2110. However, these patients are homozygous for the Cy172-H32–DYSF C2745G–104-sat–D2S291 core-haplotype [199–(–)–156–184]. The four patients (nos 11–14) from the other Canadian aboriginal kindreds are also homozygous for D2S292–D2S443–Cy172-H32–DYSF C2745G–104-sat–D2S291–D2S2110 haplotype.
The recent cloning of the LGMD2B/MM gene, DYSF, and the current work have proven that both LGMD2B and MM in this large Canadian aboriginal pedigree are caused by the same gene (9,10) and by the same mutation in this gene.

There is strong supporting evidence that the Pro791Arg mutation is disease-causing in the patients studied here. This includes evidence that: (i) the mutation segregates correctly for an autosomal recessive disease in this large kindred and the two other small families; (ii) the mutation has not been seen on 100 control chromosomes; (iii) MM and LGMD2B patients homozygous for the mutation show an identical reduction of dysferlin protein; (iv) the Pro→Arg change should have dramatic effects on the conformation of the protein; and (v) this Pro residue is conserved between the C.elegans protein fer-1 and dysferlin.

Surprisingly, patients 6–8 (Fig. 1A), who had been reported to be heterozygous for the critical haplotype and, therefore, were expected to be compound heterozygotes (6), are also homozygous for the Pro791Arg mutation. This points to a common ancestry in all the patients of the pedigree and to ancient recombination events closely flanking the chromosomal segment around the disease gene in the mother of patients 6–8. This interpretation is supported further by the haplotype analysis using microsatellites within and very closely flanking the dysferlin gene, which indicate that patients 6–8 are homozygous for the same core haplotype as all other patients in this family (Fig. 1A).

The presence of four patients (nos 11–14, Fig. 1B) in an apparently unrelated aboriginal community homozygous for the same Cy172–H32–DYSF C2745G–104-sat–D2S2110 haplotype suggests that there may be common ancestry between these two communities as well. The 158 bp allele of the intragenic marker 104-sat in these patients differs from the disease-associated allele of the large family at that locus by 2 bp. As the alleles flanking this marker are identical to those on the disease-associated haplotype, it is most likely that a mutation of the 104-sat microsatellite had occurred.

There appears to be a slight discrepancy between the very minimal staining of dysferlin at the membrane in tissue sections of patients and the clearly visible 230 kDa band on western blots that represents between 11 and 15% of the staining intensity of control muscle. While this may in part just reflect the aggregate staining of all dysferlin molecules in one band, it may also be an indication of a possible misfolding of the protein that may interfere with correct integration into the membrane. Whatever the reason, it is clear from this study and the accompanying paper that the antibody to dysferlin will offer a marked enrichment of the tools for the very difficult field of LGMD diagnostics (12,13). The results presented here also point out that it appears unlikely that one will be able to distinguish LGMD2B and MM on the basis of dysferlin protein expression. This does not rule out the possibility that a large series of biopsies from different muscles of LGMD2B and MM patients might still show differences between MM and LGMD2B. However, such a study is clearly not readily feasible.

The higher level of dysferlin detected in the preclinical patient (33%; Fig. 4, lane 2) appears to agree with the lack of symptoms at this stage of the disease. Given the limited number of biopsies studied, it is unknown whether these levels will continue to decline as the disease develops or whether such different dysferlin levels could represent variations one may encounter even in
patients with the same mutation. Given the large size of the dysferlin gene (>150 kb with 55 exons), we cannot rule out that additional mutations in DYSF may cause the variation in expression, nor can we rule out, for that matter, that such second mutations could determine the LGMD2B or MM phenotype.

LGMDs characteristically show strong clinical heterogeneity (1,14). Contributing to this heterogeneity are 12 different genes and their many different mutations (1,2). Furthermore, even in families with identical mutations of the same gene, clinical heterogeneity has been observed. This is generally typified by varying degrees of severity (15,16). In the case of LGMD2B and MM, the different clinical phenotypes led originally to the designation of different disease entities, which are characterized by the primary involvement of proximal or distal muscle groups, respectively. Mapping of these two diseases to the same chromosomal region suggested that they may be allelic (4,7), similar to Duchenne and Becker muscular dystrophies (17). The difference, however, between LGMD2B and MM is not just one of severity as is generally seen in the other examples described for allelic diversity, but in the primary muscle group involvement. The fact that an identical mutation has been described in patients who vary with respect to severity and initial muscle involvement clearly points out that additional factors, such as modifier gene(s), must be involved in modifying the clinical phenotype. Modifier genes or multiple genes have been postulated previously for LGMDs (6,9,15,16,18,19). Given that the principal difference in the clinical phenotype between MM and LGMD2B patients is in the type of muscles involved initially, finding a modifier gene might reveal the mechanism for the muscle involvements, characteristic for specific types of muscular dystrophy. The clear-cut differences in clinical phenotype of the LGMD2B and MM patients in the pedigree described here and in a second, similarly large, family reported from Russia (7) may hold the clue for identifying such modifier gene(s).
Materials and Methods

Subjects

The pedigrees showing 13 affected individuals, one preclinical patient and five consanguinity loops are presented in Figure 1A and B. The pedigrees as illustrated have been slightly modified for reasons of confidentiality. Onset of weakness was noted in a 20-year-old patient (no. 1, Fig. 1A) with MM, and a deltoid left calf (no. 10, Fig. 1A), a gastrocnemius biopsy from a 26-year-old LGMD2B patient (no. 12, Fig. 1B). Muscle biopsies were obtained from three patients. A gastrocnemius biopsy of a 12-year-old preclinical patient homozygous for the Pro791Arg mutation, intronic primers 62.2F (5′-GGGCTTATGTTGGGAAATACGA-3′) and 62.2R (5′-AGCTAGAGTCACTACGGTTCG-3′) were used to amplify a 292 bp product using the following conditions: (i) 94°C for 4 mins; (ii) 30 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 1.5 mins; and (iii) 72°C for 10 mins. This sequence has two HpaII sites. The first site is located 10 bp downstream of the intron–exon boundary at nt 2745 surrounding the mutation (C2745G). The second site is located 90 bp downstream of the intron/exon boundary at nt 2824 serving as a convenient internal control for complete digestion. The 292 bp PCR product was restricted with HpaII and fragments were separated on an 8% acrylamide gel and stained with ethidium bromide. After digestion, three fragments of 80, 100 and 112 bp were detected in DNA from normal controls; four fragments of 80, 100, 112 and 192 bp were detected in DNA from carriers; and two fragments of 100 and 192 bp were detected in DNA from patients homozygous for the C2745G mutation. One hundred control chromosomes of individuals from Northern England were tested for this mutation. Numbering of base pair positions is as described by Liu et al. (9), GenBank accession no. AF075575 for dysferlin cDNA.

Haplotype analysis

DNA from patients and their immediate family was genotyped for two dysferlin intragenic microsatellites, Cy172-H32 (9) and 104-sat (10) as well as four microsatellites surrounding DYSF. Haplotypes were constructed using the known map order: tel-D2S292–D2S443–Cy172-H32–DYSF–C2745G–104-sat–D2S291–D2S1100-cen (9,10) assuming a minimal number of recombination events.

Sequence alignments

Protein sequence alignments were performed with dysferlin (GenBank accession no. 1373333) and fer-1 (GenBank accession no. 3600028) using CLUSTALW 1.7 from the Institute for Biomedical Computing (http://www.ibc.wustl.edu/service/msa/index.html), ALIGN from the Genestream Resource Centre (http://vega.crbm.cnrs-mop.fr/bin/align-guess.cgi) and BLASTP (Blast 2 Sequences) from National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gorf/bl2.html).

Antibody preparation

The monoclonal antibody NCL-hamlet was generated against a peptide near the membrane spanning domain at the C-terminus of dysferlin comprised of amino acids 1999–2016 (Fig. 2A) as described in the accompanying paper by Anderson et al. (11).

Muscle biopsies

Open muscle biopsies were obtained from three patients. A gastrocnemius biopsy of a 12-year-old preclinical patient homozygous for Pro791Arg with asymptomatic hypertrophy of her left calf (no. 10, Fig. 1A), a gastrocnemius biopsy from a 20-year-old patient (no. 1, Fig. 1A) with MM, and a deltoid biopsy from a 26-year-old LGMD2B patient (no. 12 in Fig. 1B).
Each biopsy was divided into three portions. The third sample was snap frozen in isopentane which had been cooled in liquid nitrogen. This portion was used for immunocytochemistry and western blot analysis.

**Western blot analysis**

Polyacrylamide gel electrophoresis and western blotting were performed as described previously (20). All tissue samples were weighed frozen, and homogenized in 19 vol of electrophoresis treatment buffer, giving a loading concentration of ~200 mg in 30 ml. For the densitometric analysis, dried gels and blots were scanned at 400 d.p.i. on an Epson GT8000 flatbed scanner using white light for gels, and blue for blots. Each image was stored as a bit-map where 8 bits = 1 pixel, and each pixel was graded from 0 (pure black) to 255 (pure white) on a 256 greyscale. Customized software written for the Optimas v5.2 image analysis package was used for the densitometric analysis. In this analysis, greyscale values were converted to OD units using a Kodak SR-37 step-wedge so that the scanner was calibrated as a true densiometer. The outline of each band was defined by a software algorithm involving background measurements and the greyscale value for each pixel within the band was automatically converted to an OD value, producing a volume OD measurement for the band.

Pathological muscle samples contain a variable amount of fat and fibrous connective tissue; therefore, myosin heavy chain staining on the post-blotted gel was used as an indication of how much true muscle protein was loaded in each sample. Thus, the volume OD value for each dysferlin band was divided by the corresponding value for myosin heavy chain in that sample to produce a density value that was ‘normalized volume OD’ (20).

The normalized volume OD for each patient was then expressed as a percentage of the average value for three control samples run on the same blot, resulting in an estimate of protein abundance expressed as ‘percentage of normal’ for each patient sample. Very little tissue was available for most patient samples, so it was not possible to undertake the analysis multiple times for statistical analysis. The method outlined here, while not perfect, represents the best attempt we were able to make at obtaining meaningful results from a single shot experiment.

**Immunocytochemistry**

Immunocytochemistry at the light level was performed as described previously (21) and in the accompanying paper by Anderson et al. (11), with 6 µm unfixed frozen sections, monoclonal primary antibodies and 1/100 Dako R260 rhodamine conjugated secondary antibody. The primary antibodies were used undiluted, or at the dilutions specified in each figure legend.

**ABBREVIATIONS**

CK, creatine kinase; DYSF, gene symbol for dysferlin; LGMD, limb girdle muscular dystrophy; LGMD2B, limb girdle muscular dystrophy type 2B; MHC, myosin heavy chain; MM, Miyoshi myopathy; SSCP, single strand conformation polymorphism.

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**REFERENCES**


[Other references are listed here...]

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This is a sample page from a journal article with a focus on muscular dystrophies. The text describes methods used for immunocytochemistry and western blot analysis, and provides details on tissue handling, protein measurement, and statistical analysis. The article also includes acknowledgments and references for further reading. The abbreviations section lists the symbols and acronyms used in the study. The acknowledgments thank the families and physicians involved, and mention the support from various organizations and funding bodies.


