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Journal of the Neurological Sciences 250 (2006) 71 – 78

Journal of the Neurological Sciences

www.elsevier.com/locate/jns

Mutation impact on dysferlin inferred from database analysis and computer-based structural predictions

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Received 5 May 2006; received in revised form 6 July 2006; accepted 9 July 2006
Available online 22 September 2006

Abstract

Dysferlin is a large sarcolemmal protein implicated in the repair of surface membrane tears in muscle cells. Mutations in dysferlin result in limb girdle muscular dystrophy type 2B and Miyoshi myopathy. Using a cDNA based approach we identified eight new pathogenic dysferlin alleles. To better understand how missense mutations could lead to reduced or absent dysferlin expression levels, we mapped missense mutations from our own and from published databases ($n=55$) to the secondary protein structure of dysferlin, deduced by computerized structural prediction tools. We found the protein to be very sensitive to the alteration of residues that were predicted to be buried inside the protein structure. We identified seven putative C2 domains, one more than commonly reported, of both type I and type II topology in dysferlin. Missense mutations often affected those structures as well as residues that were highly conserved between members of the ferlin family. Thus, alteration of structurally important residues in dysferlin could lead to improper folding and degradation of the mutant protein.

Keywords: Dysferlin; Limb-girdle muscular dystrophy; Miyoshi myopathy; Mutation analysis; Computer based structural predictions

1. Introduction

Dysferlin is the protein product of the gene that is defective in patients with limb girdle muscular dystrophy type 2 B (LGMD 2B), Miyoshi myopathy (MM) and distal anterior compartment myopathy [1,2]. The dysferlin gene harbors 55 exons, and the cDNA sequence predicts a multi-domain protein of 237 kDa, expressed predominantly in muscle and implicated in sarcolemmal resealing following muscle fiber damage [1–3]. Dysferlin is a type II transmembrane protein with its major part within the cytoplasm anchored to the sarcolemma by its C-terminus. It contains several putative C2 domains thought to be implicated in the membrane resealing processes and two Dysf domains of unknown function. Dysf domains are also found in myoferlin, fer-1, yeast peroxisomal protein [4,5], but are absent in otoferlin. Dysferlin's C2A domain can bind phospholipids *in vitro* [6], however, the lipid binding properties of the remainder C2 domains have not been experimentally characterized to date. Other members of the ferlin family include otoferlin [7,8], and myoferlin [9], that share a high homology with dysferlin and are also predicted to have several C2 domains and a C-terminal transmembrane domain. Otoferlin is expressed in hair cells of the inner ear and mutations in this protein have been linked to a form of recessively inherited deafness [7,8]. Myoferlin is expressed in many tissues and is found predominantly at the plasma membrane of heart and skeletal muscle cells [9], is implicated in myoblast fusion [10] and no human diseases have been reported to be caused by mutations in myoferlin to date [9].

All of the pathogenic dysferlin mutations reported so far affect the protein expression level in skeletal muscle. The mechanism behind the high dysferlin turnover in LGMD or MM patients is easily understood in the case of null mutations or frame shift mutations causing mRNA decay and/or proteosomal degradation of incomplete dysferlin molecules. However, the

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doi:10.1016/j.jns.2006.07.004

fate of dysferlin is not clear in the case of missense mutations which represent the most frequent type of alteration reported in dysferlinopathic patients (40%). Further characterization of dysferlin on a molecular level is needed to better understand the structure and the function of this sarcolemmal protein in normal and pathological conditions. This knowledge may be useful for developing adapted therapeutic strategies for dysferlinopathies, and possibly for other forms of muscular dystrophy, where enhancement of sarcolemmal resealing could be of benefit. One approach to learn more about individual dysferlin domains could come from genotype–phenotype correlation in patients with missense mutations, provided residual amounts of dysferlin protein were produced by the mutant allele to account for the varied phenotypes. However, absence of dysferlin protein with most dysferlin missense mutations reported to date indicates that the protein is very sensitive to amino acid alterations preventing meaningful genotype–phenotype correlation. In that context, bioinformatics prediction tools can assist ongoing fundamental studies in deciphering important structural elements required for proper function of dysferlin in particular and for ferlins in general. In this paper, we first describe structurally important residues found in dysferlin C2 and Dysf domains. We then describe the impact of missense mutations on dysferlin expression levels and on the function of its individual domains as predicted by bioinformatics tools and structure-based sequence alignments.

2. Patients and methods

2.1. Patients

Patients included in this study were selected if the clinical phenotype was consistent with LGMD or distal myopathy, if histological sections of skeletal muscle showed a dystrophic pattern, and if Western-blotting of skeletal muscle homogenates showed loss or a marked reduction of the amount of dysferlin. All clinical material was obtained for routine diagnostic purposes with informed consent. A total of ten patients from nine unrelated families were selected for genetic analysis of dysferlin. Family IX includes a mother (IX-1) and her daughter (IX-2), previously described by us [11].

2.2. Immunoblotting

Western blot analysis for dysferlin was carried out on muscle biopsy homogenates from patients and normal controls. 12 μg of protein from skeletal muscle homogenate was loaded per lane. Proteins were separated on a 5.5% SDS-PAGE gel for 1.5 h at 100 V and transferred to a nitrocellulose membrane at 100 V for 1 h. After transfer, the nitrocellulose membrane was probed with an anti-dysferlin (NCL-Hamlet, Novocastra) antibody diluted 1/400 and incubated overnight at 4 °C. This monoclonal antibody recognizes an epitope encoded by exon 52. Signals were revealed with an anti-mouse IgG-peroxydase diluted 1/2000 (Dako) and the ECL Western blotting detection reagents (Amersham Biosciences).

2.3. Mutation screening

Mutational analysis was performed using mRNA extracted from skeletal muscle biopsies with the RNAeasy kit (Qiagen). Direct sequencing of the coding region of the dysferlin gene was performed using nine overlapping amplicons obtained with the one step RT-PCR system (Qiagen) with primer pairs previously described [12] and the MasterCycler instrument (Eppendorf). The cycling conditions were 30 min at 50 °C, followed by 40 cycles of (94 °C for 1 min; 57 °C for 1 min.; 72 °C for 1 min) and an extension step at 72 °C for 10 min. Mutations were verified by sequencing of PCR products of corresponding genomic DNA fragments. Nucleotides were numbered according to the dysferlin sequence GenBank NM_003494, with the first base of the Met-codon counted as position 1.

2.4. Multiple sequence alignment and secondary structure prediction

We used the program PROF [13] of the PredictProtein server, http://www.predictprotein.org, for secondary structure prediction and solvent accessibility of all 2080 amino acid residues of dysferlin. Multiple sequence alignment of the human ferlins dysferlin (GenBank:NP_003485), myoferlin (Genbank:AAF27176), otoferlin (GenBank:NP_919224) and the nematode *C. elegans* fer-1 (GenBank:AAB02243) was performed with the MULTIALIN program. The C2 domain alignment was performed manually using dysferlin C2 domains, the mouse synaptotagmin III C2A domain (GenBank: O35681) and the C2 domain of PKCδ (GenBank: AAA03176). The alignment of residues important for the structure of beta-strands of the C2 domains was performed according to an amino acid consensus obtained from the sequence alignment of 65 published C2 domains [14]. The sequence information on Dysf domains was found in the conserved domain database from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). DysfN accession number (smart00693) and DysfC (smart00694).

2.5. Database analysis

We used the Leiden muscular dystrophy database (http://www.dmd.nl/) for the selection of dysferlin missense mutations since it is the most complete and standardized dataset available of dysferlin alleles. Fifty five unique missense mutations were selected for the study including one novel missense mutation detected in this study.

3. Results

3.1. Novel dysferlin mutations in LGMD2B and MM patients

The entire cDNA of dysferlin was sequenced in ten of our dysferlinopathy patients (Table 1). Dysferlin was weakly expressed in skeletal muscle of patient VI who harbored an Arg2042Cys mutation on one allele (Fig. 1) and patient IX-1,