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Research Article

Mutated fukutin-related protein (FKRP) localises as wild type in differentiated muscle cells

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Abstract

The mechanism of disease in forms of congenital and limb girdle muscular dystrophy linked to mutations in the gene encoding for Fukutin-related protein (FKRP) has previously been associated with the mis-localisation of FKRP from the Golgi apparatus [C.T. Esapa, R.A. McIlhinney, D.J. Blake, Fukutin-related protein mutations that cause congenital muscular dystrophy result in ER-retention of the mutant protein in cultured cells. Hum. Mol. Genet. 14, (2005) 295–305]. In the present report, we have transfected V5-tagged Fukutin-related protein expression constructs into differentiated C2C12 myotubes and the tibialis anterior of normal mice. The transfection of either wild type (WT) or several mutant constructs (P448L, C318Y, L276I) into myotubes consistently showed clear co-localisation with GM130, a Golgi marker. In contrast, whilst WT and the L276I localised to the Golgi of Cos-7 cells, the P448L and C318Y was mis-localised in the majority of these undifferentiated cells. The injection of the same constructs into the tibialis anterior of mice resulted in similar localisation of both the WT and all the mutants. Immunolabelling of FKRP in the muscle of MDC1C and LGMD2I patients was found to be indistinguishable from normal controls. Overall, these data suggest that retention in the endoplasmic reticulum of FKRP is not the main mechanism of disease but that this may instead relate to a disruption of the functional activity of this putative enzyme with its substrate(s) in the Golgi.
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Keywords: Muscular dystrophy; Fukutin-related protein; Myotubes; C2C12 cells; Golgi complex; Dystroglycan

Introduction

A number of forms of congenital muscular dystrophy (CMD) are now known to be associated with mutations in genes encoding for proteins that are either putative or determined glycosyltransferases lending support to the idea that aberrant post-translational modification of proteins may represent a new mechanism of pathogenesis in the muscular dystrophies [2,3]. Four of these are severe forms with associated structural brain defects and variable eye involvement, namely, Fukuyama congenital muscular dystrophy (FCMD) [4], Muscle–Eye–Brain disease (MEB) [5], Walker–Warburg syndrome (WWS) [6] and MDC1D, while in another variant, MDC1C [7], brain and eye involvement are only found in children at the severe end of the spectrum [8]. Interestingly, a milder allelic variant of MDC1C results in a common form of LGMD (limb girdle muscular dystrophy), LGMD2I [7]. All these muscular dystrophies share the hypoglycosylation of α-dystroglycan in skeletal muscle, suggesting that this external membrane protein is either the direct or indirect substrate for the deficient proteins mutated in these conditions.

α- and β-dystroglycan are encoded by a single gene *DAG1*, the transcript from which is post-translationally cleaved to give rise to the two glycoproteins [9]. α-Dystroglycan interacts with several components of the

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extracellular matrix including laminin, neurexin, perlecan and agrin [10–13] whereas β-dystroglycan interacts with the C terminal region of α-dystroglycan and dystrophin, utrophin, caveolin, actin and Grb2 at its N terminus [14–17]. The hypoglycosylated form of α-dystroglycan from the muscle/brain of patients with FCMD, MEB and WWS fails to properly bind laminin, agrin and neurexin on overlay assays thereby severely compromising the linkage which dystroglycan maintains between the extracellular matrix and the muscle fibre/neuronal cell cytoskeleton [13]. Whilst the enzymatic activity of fukutin, the protein deficient in FCMD has yet to be determined, both POMGnT1 (mutations associated with MEB) and POMT1 (mutations associated with a proportion of cases of WWS) encode for enzymes involved in the biosynthesis of O-linked mannosyl structures that have previously been shown to be involved in laminin binding [6,18,19].

Mutations in Fukutin-related protein (FKRP) give rise to a wide range of clinical phenotypes with and without brain involvement [7,8,20–22]. The function of FKRP is unknown; however, there is indirect evidence for a role for this putative glycosyltransferase in α-dystrolgycan processing [7,23,24] and recent data suggest that FKRP is localised in the Golgi complex [25]. The reason for the difference in severity in different patients is presently unclear although recent work in Cos-7 and C2C12 myoblasts suggests that mutations associated with the more severe MDC1C phenotypes result in the retention of FKRP in the endoplasmic reticulum [1]. We have recently found, however, that the immunolocalisation of endogenous FKRP in the muscle biopsy of MDC1C patients is not different from controls (Brown et al., unpublished observation and present paper). In the present work, we extend these findings and show that the cellular localisation of mutant FKRP tagged at its C terminus with the V5 epitope is not significantly different from wild type either in vivo after intramuscular plasmid injections, or in vitro following the transfection of V5-tagged FKRP constructs into differentiated C2C12 myotubes. However, several of these constructs were mis-localised in undifferentiated Cos-7 cells. These observations, together with those previously published [1], suggest that FKRP protein localisation is dependent on cell type and that protein retention into the endoplasmic reticulum is not the main mechanism of disease in MDC1C.

Materials and methods

Antibodies

Rabbit polyclonal antibodies (UW150 and UW151) was raised against a synthetic peptide which encompassed amino acids 250–264: C-taharwkaeregrar (16mer) of FKRP. A second rabbit polyclonal antibody (N1) was raised against a C terminal fusion protein and was a kind gift of Dr. Nishino [25].

Cell lines and tissue culture

C2C12 (ECCAC), Cos-7 cells and fetal human myoblasts were cultured in 10% FCS in DMEM (high glucose) supplemented with 2 mM L-glutamine. C2C12 cells were plated onto glass coverslips and allowed to fuse for 7 days in 5% horse serum (Gibco, Invitrogen) after which they were fixed in paraformaldehyde (2%) for 10 min. Cells were removed from the dish using trypsin:EDTA (Sigma). Where indicated, cells were incubated with growth medium supplemented with 5 μg/ml of brefeldin A (Sigma) for 60 min at 37°C and fixed in 2% paraformaldehyde as described above. For immunocytochemistry, cells were permeabilised before staining using PBS-X100 Triton 0.5%. To generate stable transfectants pcDNA3.1/V5-His©TOPO®TA coding for the fusion protein FKRP-V5 was linearised with *Bst*ZI and transfected into C2C12 cells. Stably transfected cells were selected with G418 (600 μg/ml) (Geneticin; GIBCO BRL). Myoblasts were differentiated into multinucleated myotubes, using 5% horse serum in DMEM supplemented with 2 mM L-glutamine.

Construction of expression plasmids

The complete coding sequence of human FKRP (NCBI accession number NM_024301) was amplified using (*forward primer* CAGCTAGCCCCAGACTTC and *reverse primer GCCGCTTCCCGTCAGACTC*) and Advantage HF Polymerase (Clontech). The amplified product was then cloned into the pcDNA 3.1/V5-His TOPO® expression vector (Invitrogen) which synthesises a fusion protein with the V5 tag at the C-terminus of FKRP. Site-directed mutagenesis was performed on plasmid DNA using Advantage HF polymerase. Amplified products were treated with *Dpn*I (10 U) to remove template plasmid DNA and were transformed into One Shot® TOP10 chemically competent *E. coli* (Invitrogen). All constructs were verified by sequencing. Primers used for mutation generation are shown below (mismatches underlined):

FKRP-L276I

F: CGC TGG GCA TCC GC**A** TAG TGA GCT GG
R: CCA GCT CAC TA**T** GCG GAT GCC CAG CG

FKRP-P448L

F: CTT CCT GCA GC**T** GCT GGT GCC CC
R: GGG GCA CCA GC**A** GCT GCA GGA AG

FKRP-C318T

F: CGC TGG ACG CCC CCC TGC T**A**C CTG CGC
R: GCG CAG G**T**A GCA GGG GGG CT CCA GCG

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