Functional requirements for fukutin-related protein in the Golgi apparatus

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Two forms of congenital muscular dystrophy (CMD), Fukuyama CMD and CMD type 1C (MDC1C) are caused by mutations in the genes encoding two putative glycosyltransferases, fukutin and fukutin-related protein (FKRP). Additionally, mutations in the FKRP gene also cause limb-girdle muscular dystrophy type 2I (LGMD2I), a considerably milder allelic variant than MDC1C. All of these diseases are associated with secondary changes in muscle α-dystroglycan expression. To elucidate the function of FKRP and fukutin and examine the effects of MDC1C patient mutations, we have determined the mechanism for the subcellular location of each protein. FKRP and fukutin are targeted to the medial-Golgi apparatus through their N-termini and transmembrane domains. Overexpression of FKRP in CHO cells alters the post-translational processing of α- and β-dystroglycan inhibiting maturation of the two isoforms. Mutations in the DxD motif in the putative active site of the protein or in the Golgi-targeting sequence, which cause FKRP to be inefficiently trafficked to the Golgi apparatus, did not alter dystroglycan processing in vitro. The P448L mutation in FKRP that causes congenital muscular dystrophy changes a conserved amino acid resulting in the mislocalization of the mutant protein in the cell that is unable to alter dystroglycan processing. Our data show that FKRP and fukutin are Golgi-resident proteins and that FKRP is required for the post-translational modification of dystroglycan. Aberrant processing of dystroglycan caused by a mislocalized FKRP mutant could be a novel mechanism that causes congenital muscular dystrophy.

INTRODUCTION

The congenital muscular dystrophies (CMDs) are a heterogeneous group of disorders that are characterized by early onset muscular dystrophy (reviewed in 1, 2). Several of these disorders are also associated with brain defects, mental retardation and abnormalities of the visual system (1–3). Recently, the genes for some of these diseases have been identified (4–6). One of the most severe of these disorders is Fukuyama congenital muscular dystrophy (FCMD), which is caused by mutations in the gene encoding fukutin. FCMD is the second most common muscular dystrophy in the Japanese population and predominates in populations originating from North East Asia (7). FCMD patients are more severely affected than those with Duchenne muscular dystrophy (DMD) and often never walk. These patients are mentally retarded, affected by epilepsy and have severe morphological brain abnormalities indicative of a defect in cortical neuronal migration (8). The majority of FCMD patients have inherited the same mutation, namely the insertion of a retrotransposon in the 3’ untranslated region of the FCMD gene (4). Compound heterozygotes have also been described that have point mutations in one allele of the FCMD gene and the retrotransposon on the second allele (9). However, no patients have been described that are homozygous for the point mutation suggesting that the complete lack of fukutin could result in embryonic lethality (9).

The FCMD gene was predicted to encode a secreted protein called fukutin. However, due to the lack of suitable antibodies, the subcellular location of fukutin has not been determined (4). Interestingly, muscle biopsies from FCMD patients show a dramatic reduction in glycosylated α-dystroglycan, a component of the dystrophin protein complex (see 10 for a

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The authors wish it to be known that, in their opinion, these two authors should be considered as joint First Authors.
Figure 1. Sequences and expression of murine FKRP and fukutin. The sequences of mouse FKRP (A) and fukutin (B). The underlined sequence corresponds to the hydrophobic N-terminal sequence that forms the Golgi signal anchor of FKRP and fukutin. The residues highlighted with bold lettering are the proposed active site DxD of many glycosyltransferases (25). The tissue distribution of the FKRP and fukutin transcripts was determined on northern blots of mRNAs isolated from different mouse tissues (C). The 2.8 kb FKRP transcript is widely expressed with highest levels in brain, lung, heart, kidney and liver (upper panel). The 6.8 kb fukutin transcript is detected predominantly in brain, liver and kidney (middle panel). The same blot was re-hybridized with a β-actin cDNA to show that similar levels of mRNA were loaded (lower panel).
Figure 1 continued.
recent review), suggesting a link between FCMD, DMD and dystroglycan processing (11,12).

A second disorder, MDC1C is caused by mutations in the gene encoding a homologue of fukutin called fukutin-related protein (FKRP) (13). MDC1C patients have severe muscular dystrophy, however only a subset of these patients have brain involvement (14). A milder allelic disorder LGMD2I is also caused by mutations in the FKRP gene (15,16). A wide range of mutations have been described in patients with MDC1C and LGMD2I however, in common with FCMD, patients with two putative null alleles have not been described. Patients with MDC1C and LGMD2I also have abnormally processed α-dystroglycan that has a lower relative molecular mass and abundance suggesting that in common with fukutin, FKRP may be involved in protein modifications such as glycosylation (13,17). However, MDC1C and LGMD2I patients have detectable levels of glycosylated α-dystroglycan as determined by immunoblotting showing that mutations in the FKRP gene affect dystroglycan processing differently when compared to the apparent absence of glycosylated dystroglycan in patients with FCMD (13,16).

Direct evidence supporting a role for defects in protein glycosylation causing CMD-like disorders comes from cloning of the genes mutated in Muscle–eye–brain (MEB) disease and the myodystrophy (myd) mouse (6,18). MEB is similar to FCMD and is caused by mutations in the gene encoding O-mannosyl beta-1,2-N-acetylglucosaminyltransferase (POMGnT1) (6). This enzyme is involved in the synthesis of O-mannosyl glycans and is expressed at its highest levels in muscle and heart. MEB patients have a secondary deficiency in α-dystroglycan suggesting that dystroglycan may be a substrate for POMGnT1 (19). The myd mouse is the only naturally occurring model of CMD and is caused by a deletion in the gene encoding the putative glycosyltransferase LARGE (18). In addition to muscular dystrophy, these mice also have sensor-inerial hearing loss suggesting some degree of central nervous system involvement (18). Muscle from this mouse also has incorrectly processed α-dystroglycan further implicating a role for protein glycosylation in the congenital muscular dystrophies.

Recently, it was shown that α-dystroglycan purified from muscle samples from FCMD and MEB patients and the myd mouse has a molecular weight of 90 kDa and is only detected by antibodies raised against the core α-dystroglycan (20). Furthermore, α-dystroglycan derived from these sources had a significantly reduced ability to bind to laminin, neurexin and agrin (20). These studies show that post-translation disruption of the dystroglycan:ligand interface could cause CMD-like changes in muscle. In a parallel study, it was shown that depletion of brain dystroglycan causes a neuronal migration abnormality that is similar to that seen in the myd mouse and in patients with FCMD and MEB (21). Together, these interesting findings show that post-translation modification of α-dystroglycan is required for normal brain development. However, the precise role of fukutin, POMGnT1 and LARGE remains to be formally established.

To determine the function of FKRP we investigated the mechanism for the subcellular localisation of the protein and examined its role in dystroglycan processing. For comparative purposes we also determined the subcellular location of fukutin. We have shown that both proteins are targeted to the Golgi apparatus by their N-termini and transmembrane domains. We also show that FKRP has a direct effect on dystroglycan processing in vitro. Modelling of the mutation P448L found in a consanguineous MDC1C family (13) revealed that the mutant protein is mislocalized in the cell and consequently is unable to alter the processing of dystroglycan. Our data support the idea that FKRP and fukutin are involved in protein modification and suggest that abnormal dystroglycan processing is involved in the pathogenesis of MDC1C and FCMD.

RESULTS

Cloning and expression analysis of FKRP and fukutin
cDNA clones encoding the mouse orthologues of FKRP and fukutin were isolated from a mouse brain library using gene specific PCR probes. The 2.8 kb murine FKRP cDNA encodes a protein of 494 amino acids and has 94% sequence identity to its human orthologue (Fig. 1A). The mouse fukutin cDNA encodes a protein of 461 amino acids and shares 90% sequence identity with its human orthologue (Fig. 1B). These cDNA clones were used to produce the expression constructs described below (Table 1). The high degree of primary sequence similarity between the human and murine orthologues of FKRP and fukutin facilitates the use of these mouse genes for generating in vitro (see later) and in vivo models for MDC1C and FCMD.

For direct comparison, the tissue distribution of the mouse FKRP and fukutin transcripts were determined by hybridising the same northern blot with part of the cognate cDNA. The FKRP transcript is 2.8 kb and is found at highest levels in the brain, lung, heart, kidney and liver (Fig. 1C, upper panel). The fukutin transcript is 6.8 kb and is detected predominantly in brain, liver and kidney (Fig. 1C, middle panel). Both of the transcripts are expressed in the same tissues at similar levels including the heart and skeletal muscle. The blot was stripped and re-hybridized with β-actin cDNA to demonstrate equal loading of mRNA (Fig. 1C, lower panel).

Sub-cellular localisation of FKRP and fukutin

Hydrophobicity plots and secondary structure analysis predict that FKRP and fukutin are type II membrane proteins (N-terminus outside, C-terminus inside). To determine the subcellular location of FKRP and fukutin, several different cell lines were transfected with expression constructs encoding each protein (Table 1). Myc-tagged FKRP co-localized precisely with α-mannosidase II identifying the medial-Golgi apparatus in NRK cells (Fig. 2A). Similar results were obtained with fukutin indicating that both proteins are Golgi-residents (Fig. 2A). Comparison of myc-tagged FKRP with untagged FKRP showed that the addition of an epitope tag at the C-terminus of the protein had no apparent effect on the location of FKRP (data not shown). Treatment of the cells with brefeldin A (BFA) or nocodazole (NC) resulted in disassembly of the Golgi apparatus and the redistribution of each protein into the endoplasmic reticulum (BFA) or into discrete clusters (NC, data not shown) indicative of Golgi-resident proteins.
FKRP and fukutin are predicted to be phospholigand transferases and are possibly glycosyltransferases (13,17). Many glycosyltransferases are Golgi-residents and are maintained in the organelle using a variety of different strategies. To determine the mechanism that targets FKRP and fukutin to the Golgi apparatus, several constructs were produced that contained part of each protein fused to green fluorescent protein (GFP). The N-terminal 33 amino acids of FKRP are sufficient to recruit the GFP chimera to the Golgi apparatus (Fig. 2B). This region contains the transmembrane domain with the N-terminal amino acids in the cytoplasm. Similar results were obtained with the N-terminus of fukutin fused to GFP (Fig. 2B). These data suggest that both proteins are targeted to the Golgi apparatus by a non-cleavable signal anchor sequence. The remaining lumenal domain of FKRP fused to the C-terminus of GFP was not targeted to the Golgi apparatus and remained diffuse within the cytoplasm of the cell and excluded from the nucleus (Fig. 2B). The corresponding region of fukutin again behaves identically to the lumenal region of FKRP in that it remains in the cytoplasm of the cell (Fig. 2B). In control experiments the two empty GFP vectors were not targeted to the Golgi apparatus but were found in the cytoplasm and nucleus of transfected NRK cells (data not shown).

Several Golgi-resident proteins are recruited to the Golgi apparatus by a process known as kin recognition that involves the stem region of the protein (see 22 for a review). The stem lies between the transmembrane domain and catalytic domain of Golgi-resident glycosyltransferases. To determine whether FKRP and fukutin could be targeted to the Golgi apparatus by kin-recognition, FK-pClNeo was co-transfected with GFP-FKRP-CT (Fig. 2C). GFP-FKRP-CT encodes the entire C-terminus of FKRP and will only be targeted to the Golgi apparatus through a potential association with fukutin. COS-7 cells expressing both proteins were examined for co-localisation. FK-pClNeo is efficiently targeted to the Golgi apparatus but was unable to recruit the C-terminal FKRP-GFP construct to the same site (Fig. 2C). Similarly, FK-CT was not recruited to the Golgi apparatus following co-transfection with full length FKRP (data not shown). These data show that FKRP and fukutin are targeted to the Golgi apparatus through their N-termini and transmembrane domains and are unable to recruit each other to the organelle by kin association.

Expression of FKRP alters dystroglycan processing

in vitro

One of the salient features of MDC1C and FCMD are the secondary changes in α-dystroglycan (12,13). It has been postulated that these qualitative differences in α-dystroglycan are caused by an abnormality in dystroglycan modification. To determine the effect of FKRP on the processing of α-dystroglycan CHO cells were co-transfected with dystroglycan and FKRP expression constructs and mutants thereof (Table 1). CHO cells were chosen because they have previously been shown to produce highly glycosylated and correctly processed α- and β-dystroglycan (23). In these experiments we used a chick dystroglycan expression construct (ChDG) and antibodies specific for the chick core protein. This experimental paradigm allowed us to examine the effects of FKRP on dystroglycan processing without using antibodies against glycosylated epitopes. We also used FKRP without the epitope tags because this could potentially interfere with the function of FKRP. Furthermore, using antibodies specific to the α-dystroglycan core protein and 9E10 that detects a myc epitope tag placed at the C-terminus of β-dystroglycan, we were able to identify the different dystroglycan isoforms.

<table>
<thead>
<tr>
<th>Name</th>
<th>Vector</th>
<th>Description</th>
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<tr>
<td>FKRP</td>
<td>pCIneo</td>
<td>Full length mouse FKRP cds</td>
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<tr>
<td>FKRP-myc</td>
<td>pCIneo</td>
<td>Full length FKRP with a C-terminal myc tag.</td>
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<tr>
<td>FK</td>
<td>pCIneo</td>
<td>Full length mouse fukutin cds</td>
</tr>
<tr>
<td>NT-FKRP-GFP</td>
<td>pEGFP-N3</td>
<td>aa 1–33 of FKRP fused to the N-terminus of GFP</td>
</tr>
<tr>
<td>GFP-FKRP-CT</td>
<td>pEGFP-C2</td>
<td>aa 29-end fused to the C-terminus of GFP</td>
</tr>
<tr>
<td>NT-FK-GFP</td>
<td>pEGFP-N3</td>
<td>aa 1–36 of fukutin fused to the N-terminus of GFP</td>
</tr>
<tr>
<td>GFP-FK-CT</td>
<td>pEGFP-C2</td>
<td>aa 36-end of fukutin fused to the C-terminus of GFP</td>
</tr>
<tr>
<td>ChDG</td>
<td>pCMV-Tag1</td>
<td>Full length chick dystroglycan</td>
</tr>
<tr>
<td>FKRP-NNN</td>
<td>pCIneo</td>
<td>Full length FKRP with aa 362–364 mutated from DVD to NNN</td>
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<tr>
<td>FKRP-R2E, R5E</td>
<td>pCIneo</td>
<td>Full length FKRP with aa 2 and 5 mutated from R to E</td>
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<td>pCIneo</td>
<td>FKRP with aa 448 mutated from P to L</td>
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<td>pCIneo</td>
<td>FKRP-myc with aa 448 mutated from P to L</td>
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<td>FP</td>
<td>pCIneo</td>
<td>Full length mouse FKRP cds</td>
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<td>FKRP</td>
<td>pCIneo</td>
<td>Full length mouse fukutin cds</td>
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**cds:** Coding sequence; **aa:** amino acids.
Figure 2. Golgi-targeting of FKRP and fukutin. Golgi localization of FKRP and fukutin (A). Transfected NRK cells were stained for FKRP-myc (upper panel, green), fukutin (lower panel, green) and α-mannosidase II (manII, middle panels, red). The merged images show precise co-localization of FKRP and fukutin with α-mannosidase II. Golgi targeting sequences in FKRP and fukutin (B). NRK cells transfected with the GFP constructs shown on the left of the figure were stained with an anti α-mannosidase II antibody to identify the medial part of the Golgi apparatus. Two channel confocal images were captured to show the location of the Golgi apparatus (red channel, α-mannosidase II) and the different GFP constructs as indicated (green channel). The merge images are shown in the right hand panels. Only the N-terminal constructs, NT-FKRP-GFP and NT-FK-GFP are targeted to the Golgi apparatus. No targeting is seen with the constructs containing the luminal domains of FKRP and fukutin (GFP-FKRP-CT and GFP-FK-CT) or with the two empty GFP vectors (pEGFP-C2 and pEGFP-N3) used in this experiment (data not shown). Testing kin recognition between FKRP and fukutin (C). COS-7 cells transfected with GFP-FKRP-CT and full length fukutin were examined for co-localization of the two antigens. GFP-FKRP-CT stains punctae in the cytoplasm whereas fukutin is restricted to the Golgi apparatus. There is no apparent co-localization of the two antigens suggesting that fukutin cannot recruit FKRP to the Golgi apparatus. Scale bar = 20 μm.
originating from cleavage and processing of the precursor protein.

We also compared the processing of dystroglycan with two FKRP mutants. FKRP-NNN has a mutation of residues 362 to 364 such that the sequence DVD is mutated to NNN. The DxD motif in FKRP is indicative of enzymatic function and is highly reminiscent of motifs found in many other inverting and non-inverting transferases that add sugars to other sugars, phosphates and proteins (24–26). Mutations of the DxD motif to NNN have been used to abolish the function of many putative glycosyltransferases (see 27 for example). The other mutant assayed in our expression system was FKRP-R2E, R5E that is poorly targeted to, or retained in, the Golgi apparatus (Fig. 3).

Expression of ChDG alone in CHO cells produces dystroglycan that is processed into three major proteins, \( \beta \)-dystroglycan (43 kDa), \( \alpha \)-dystroglycan (160 kDa) and an \( \alpha \)-dystroglycan isoform of 90 kDa (Fig. 4A and B). The 90 kDa and 160 kDa proteins are not detected with 9E10 showing that they are differentially processed forms of \( \alpha \)-dystroglycan (data not shown). Co-expression of ChDG and FKRP alters the pattern of dystroglycan immunoreactivity such that there is a marked decrease in the levels of 160 kDa dystroglycan and an increase in the 90 kDa isoform and the appearance of a \( \beta \)-dystroglycan cross-reactive protein of 60 kDa (Fig. 4A and B). Interestingly, FKRP also alters the processing of \( \beta \)-dystroglycan increasing the abundance of a smaller \( \beta \)-dystroglycan isoform of 41 kDa (Fig. 4A and C). We then examined the effect of two FKRP mutants on dystroglycan processing. Both mutants behave in a similar manner by reducing the levels of the 90 kDa dystroglycan (compared to the expression of wild-type FKRP) but leaving the levels of 160 kDa dystroglycan apparently unaltered (Fig. 4A and B). Thus it can be concluded that each mutant is unable to affect the processing of dystroglycan \textit{in vitro}. In control experiments the presence of the FKRP transcript in each of the appropriate transfections was demonstrated by RT–PCR (Fig. 4D). Each of these experiments was performed at least three times with a similar outcome. Two antibodies against each dystroglycan isoform were also used to ensure that each band was correctly assigned. Antibodies VIA41 and IIH6 that recognise glycan-dependent epitopes on human dystroglycan did not cross-react with chick dystroglycan.

Comparative analysis of dystroglycan in the presence and absence of FKRP by two dimensional gel electrophoresis showed an increase in the abundance of low molecular weight \( \alpha \)-dystroglycan isoforms (Fig. 4E). The pattern of dystroglycan immunoreactivity on 2D gels detected with an antibody that recognises the core protein shows a major broad band of 160 kDa, indicative of heterogeneous glycosylation, with a low pI (Fig. 4E). Other weaker bands can also be seen that correspond to the 90 kDa \( \alpha \)-dystroglycan isoform. In cells transfected with FKRP and ChDG, three major proteins of 160 kDa, 90 kDa and 60 kDa are detected (Fig. 4E). Expression of FKRP results in a dramatic reduction in the levels of 160 kDa \( \alpha \)-dystroglycan when compared to cells only expressing Figure 3. Mutational analysis of the Golgi-targeting sequence in FKRP (A) Sequence alignment of the cytoplasmic N-terminus and transmembrane domains of FKRP, fukutin, LARGE, POMGnT1, \( \alpha \)-mannosidase II and beta4GalT I identifies a pair of conserved basic amino acids (asterisks) immediately adjacent to the transmembrane. Both arginine codons were mutated to glutamic acid in FKRP (mut-FKRP). COS-7 cells transfected with the mutant (R2E, R5E) and wild-type FKRP (red) show that the mutant is not precisely targeted or retained by the Golgi apparatus (green) but labels diffusely around the Golgi stack and the nuclear membrane. By contrast wild-type FKRP precisely co-localizes with marker at the marker apparatus. Scale bar = 20 \textmu m.
ChDG (Fig. 4E). In the cells expressing FKRP and ChDG the lower molecular weight α-dystroglycan cross-reactive proteins predominate (asterisks in Fig. 4E). The 90 kDa and 60 kDa dystroglycan immunoreactive proteins do not appear to be heterogeneous since they have a discrete isoelectric point (cf 160 kDa α-dystroglycan Fig. 4E). An RT–PCR assay for FKRP expression was performed on the transfected CHO cells (D). FKRP is detected in lanes 2–4 but not in lane 1 or the lane with no template (nt). 2D–PAGE analysis of dystroglycan produced in CHO cells (E). CHO cells were transfected as indicated. Proteins extracted from the cells were analysed by 2D–PAGE. In the first dimension proteins were separated by isoelectric point whilst the second dimension separates proteins by relative molecular mass. α-dystroglycan, detected with the Sheep 1 antibody, appears as a broad major band in cells expressing ChDG. In cells expressing FKRP the 160 kDa α-dystroglycan band is reduced whilst two partially processed intermediates of 90 kDa and 60 kDa (asterisks) are found.

Functional analysis of the P448L mutation in the FKRP gene

Given that our experimental system has allowed us to analyse the localization and presumed function of FKRP, we used this-paradigm to model some of the mutations seen in MDC1C patients. For our initial studies we chose mutations identified in severely affected patients. The mutation P448L alters a highly conserved amino acid in the C-terminus of FKRP. Proline appears to be invariant at this position in vertebrate FKRP, insect FKRP, a distant bacterial homologue Rickettsia prowazekii, RP-689 and fukutin (Fig. 5A). FKRP-P448L localised to the nuclear membrane with a discrete but diffuse perinuclear extension (Fig. 5B). When this mutant is co-transfected with ChDG in CHO cells it has no apparent effect on the processing of dystroglycan (Fig. 5C). The pattern of dystroglycan immunoreactive proteins differs considerably from the control transfections with
ChDG and FKRP and is indistinguishable from the dystroglycan profile of cells only transfected with ChDG (Fig. 4C). Interestingly the P448L-FKRP mutant is reminiscent of the R2E, R5E-FKRP mutant (see Fig. 3 for comparison) in that it is not properly targeted to the Golgi apparatus and has no apparent effect upon the processing of dystroglycan. These data suggest that the FKRP-P448L is mistargeted in the cell and therefore cannot alter the processing of α-dystroglycan.

**Figure 5.** The P448L mutation is inefficiently trafficked to the Golgi apparatus. Sequence alignment of the region of FKRP at proline 448. P448 (asterisk) is conserved in a variety of different FKRP orthologues and also in fukutin. Dr, *Danio rerio*; Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*; Rp, *Rickettsia prowazekii*; Mm, *Mus musculus*; Ci, *Ciona intestinalis*. COS-7 cells transfected with FKRP-P448L show that the mutant protein does not precisely co-localize with the Golgi marker (B). FKRP-P448L immunoreactivity is spread diffusely around the region around the Golgi apparatus and is also seen at the nuclear membrane. Scale bar = 20 μm. Extracts were prepared from CHO cells transfected with ChDG (lane 1), ChDG and FKRP (lane 2), ChDG and FKRP-P448L (lane 3) and western blotted with an antibody against α-β-dystroglycan and α-dystroglycan (C). The mutant protein does not produce any discernible difference in dystroglycan when compared to cells only expressing ChDG. By contrast, expression of wild-type FKRP produces a markedly different pattern of immunoreactive proteins. The asterisks mark the positions of the different isoforms of α- and β-dystroglycan.

**DISCUSSION**

In this paper we have shown that FKRP and fukutin are Golgi-resident proteins and that the N-terminus and transmembrane domain of each protein is sufficient to define their subcellular location. Using an *in vitro* expression system we also show that FKRP overexpression alters the processing of dystroglycan but that several mutants, including the mutation P448L that results in a severe form of muscular dystrophy are unable to modify
α-dystroglycan. These data strongly support our initial proposal that FKRP and fukutin are proteins that are required for the post-translational modification of dystroglycan (5,16).

It is now clear that several forms of congenital muscular dystrophy are associated with secondary alterations in α-dystroglycan processing. These abnormalities are seen in patients with MDC1C, FCMD, MEB and in the myd mouse. Bio-informatic clues about the function of the genes that cause these disorders and the secondary changes in α-dystroglycan in the muscle suggest that each of these proteins is potentially a glycosyltransferase. However, thus far only POMGnT1 has been shown to directly catalyse a glycosylation reaction and in no case has a direct effect on dystroglycan been demonstrated.

Transfection of genes encoding glycosyltransferases into cells and subsequent analysis of their glycome is a powerful new approach to defining substrates and activities for glycosyltransferases with unknown activities (28). We have used a variation of this strategy to examine the processing of dystroglycan in CHO cells in the presence of FKRP. Overexpression of FKRP clearly has an effect on dystroglycan maturation such that there is a reduction in the 160 kDa isoform of α-dystroglycan and a concomitant increase in 90 kDa and 60 kDa α-dystroglycan cross-reactive proteins (Fig. 4). Whilst the molecular identities of the 90 kDa and 60 kDa dystroglycan isoforms are unknown, these proteins could represent immature processing intermediates or proteolytic fragments of α-dystroglycan (see below). These changes in dystroglycan processing appear to be a direct consequence of functional FKRP since the three mutants that were constructed had little or no effect on dystroglycan processing in vitro (Fig. 4 and Fig. 5). Our studies provided the first demonstration that FKRP has a direct effect on dystroglycan processing in vitro.

Whilst the precise activity of FKRP and fukutin is unknown we have been able to show that FKRP directly affects dystroglycan processing. The downward shift in relative molecular weight of α-dystroglycan is possibly explained by the addition of terminating glycans to the dystroglycan backbone such that no additional modifications can be made or by altering the stability of dystroglycan rendering it susceptible to endogenous proteases. It is well-documented that glycosylation increases the stability of proteins in the extracellular matrix (ECM) and in plasma as well as having a role in receptor:ligand interactions (29). Thus overexpression of FKRP could render dystroglycan sensitive to matrix or secreted proteases. It is also interesting to note that FKRP also affects β-dystroglycan processing again reducing the relative molecular weight of the protein. This effect could again be explained by proteolysis or incomplete glycosylation of β-dystroglycan.

It still remains to be established whether FKRP, fukutin and LARGE have any catalytic activity. However, by comparison to POMGnT1 each protein may be involved in O-linked mannosylation. Several proteins in yeast are required for O- and N-linked mannosylation (30). Of these the proteins, Mnn4p has significant primary sequence similarity to FKRP. S. cerevisiae strains with mutations in the MNN4 gene have reduced mannansphosphate transferase activity and reduction in mannansphosphate content of cell wall mannan (31,32). No catalytic activity has been ascribed to Mnn4p, however it has been proposed that Mnn4p regulates the levels or activity of other enzymes in the mannosylation pathway (30,32). Therefore it has been suggested that MNN4 in yeast has a dominant (positive) regulatory role (30). Given that a yeast homologue of FKRP appears to be a non-catalytic regulator of mannosylation, it is formally possible that FKRP has such a role in humans.

Several studies have shown that α-dystroglycan is a heavily sialylated mucin-like glycoprotein that is found in different variants that are formed by tissue-specific glycosylation (for review see 33,34). Dystroglycan glycosylation is important for binding to laminin and agrin in the basal lamina (35–37). Abnormalities in the basal lamina have also been described in the muscles and brains of FCMD patients. These defects include reduction in the levels of several components of the muscle and neuronal ECM and alterations in immunoreactivity of members of the dystrophin-associated protein complex (11,38). Furthermore, alterations in laminin-α2 immunoreactivity have been described in the muscles of patients with FCMD (38,39). Thus, FCMD may result from defects in the ECM or basal laminae that could involve components of the dystrophin-associated protein complex (40). Alterations in the processing of α-dystroglycan could have several important effects on basal lamina formation and function. By analogy to FCMD, patients with MDC1C could have similar basal lamina defects.

One possible explanation for the severe muscular dystrophy in these patients is that mutations that abolish the function of FKRP produce α-dystroglycan that has a reduced capacity to bind to extracellular laminin and thus disrupts the basal lamina of the muscle (Fig. 6). Consistent with this hypothesis, MDC1C patients often have secondary changes in laminin immunoreactivity (16). The simplest explanation for the phenotype of patients with MDC1C is that mutations in Golgi-resident FKRP cause aberrant or incomplete glycosylation of α-dystroglycan and possibly other ECM proteins such that dystroglycan has a reduced capacity to bind the ECM components effectively weakening the bridge between the DPC and the ECM (Fig. 6). For diseases such as FCMD and MEB and in the myd mouse it has been shown that low molecular weight α-dystroglycan has a reduced affinity for several ECM proteins supporting the model presented in Figure 6 (20).

In this paper we have determined the mechanism for Golgi-targeting and retention of FKRP. Both FKRP and fukutin are targeted to the Golgi apparatus through their N-termini and transmembrane domains. Both proteins co-localise with the medial Golgi marker α-mannosidase II. We have shown that in addition to the transmembrane domain of FKRP the N-terminal arginine residues play an important role in Golgi-targeting and/or retention. β-1,4-galactosyltransferase is also targeted to Golgi apparatus via its membrane-spanning domain (41). However, single cysteine and histidine residues within the transmembrane domain are required for oligomerization and Golgi-retention (42). The transmembrane domain of FKRP does not have a cysteine or histidine residue suggesting that homo-oligomerization is unlikely to be a retention mechanism for FKRP. Furthermore, we have shown that the N-terminal arginine residues of FKRP are important determinants for Golgi-targeting or retention. This pair of basic residues is found in many medial Golgi-residents, including those implicated in CMD (Fig. 3), and may govern the Golgi-localization of a subset of Golgi-resident proteins. We have also shown that the
FKRP mutant P448L is poorly targeted to or retained in the Golgi apparatus (Fig. 5). Given that the N-terminus of FKRP is critical for Golgi targeting (Fig. 3), the FKRP-P448L mutant protein may be inefficiently retained targeted to, or retained in, the Golgi apparatus because it is misfolded. These data demonstrate the importance for the Golgi localization of FKRP and identify several sequence elements that are required for this process.

Figure 6. A potential mechanism for congenital muscular dystrophy. The schematic shows how mutations in the FKRP gene could cause muscular dystrophy by interfering with the processing of dystroglycan. The most likely affect of FKRP and fukutin mutations is to change the glycosylation of α-dystroglycan. This may interfere with the interactions of dystroglycan with laminin-2 and other ECM proteins or could reduce the stability of dystroglycan in the ECM.

In summary, we have shown that FKRP and fukutin are Golgi-resident proteins that are targeted to the organelle using a non-cleavable signal anchor. FKRP directly affects the processing and/or maturation of dystroglycan in vitro and that this function is perturbed by several different mutations in the gene. We have also demonstrated a functional requirement for FKRP targeting to the Golgi apparatus such that the MDC1C patient mutation, P448L, is mistargeted and unable to affect dystroglycan processing. These data provide the first functional characterization of FKRP and offer a potential molecular mechanism to explain the muscle disease in MDC1C patients. These studies will also shed light on the other CMDs that are associated with secondary changes in α-dystroglycan.

MATERIALS AND METHODS

Cloning, expression constructs and northern blotting

Mouse cDNA clones encoding FKRP and fukutin were isolated from a normalised mouse brain cDNA library cloned in pUC86 (Invitrogen) by colony hybridization and sequenced using standard methods. The EMBL accession numbers for each sequence are AJ511806 (FKRP) and AJ511807 (fukutin). The complete FKRP cDNA was subcloned into the NotI site of pCI neo (Promega). Golgi-targeted GFP constructs were made by PCR amplification of the regions encoding amino acids 36–461 was PCR amplified using the primers, FKRPmycF (5'-TGGAATTCTCAAGCTTACATCAACAAAATC) and FKRPmycR (5'-GCATATGACGTTGATGTAATGTTAGTGTAAGG) and subcloned into pEGFPC2 (Clontech). The remainder of the fukutin cDNA encoding amino acids 36–461 was PCR amplified using the primers FK2gf (5'-TGAATTCCGGACCGGTTCATC) and FK2gr (5'-CGCTCGAGAAGGAGACGTTATG) and subcloned into pEGFPC2 (Clontech). The remainder of the FKRP cDNA lacking the region encoding the N-terminus and transmembrane domain was amplified by PCR using the primers, FKRP3gf (5'-CCGAATTCCACCAGCCGAAATCCTCCC) and FKRP3gr (5'-CGCTCGAGAAGGAGACGTTATG) and subcloned into pEGFPC2 (Clontech). The remaining fukutin cDNA encoding amino acids 36–461 was PCR amplified using the primers FK3gf (5'-TGAATTCCGGACGGAACGTTAATCC) and FK3gr (5'-CTGTTTTGTTCTCAAGCTTACATCAACAAAATC) into the HindIII/KpnI sites at the 3' end of the FKRP coding sequence. All clones were verified by sequencing. Northern blots were purchased from OriGene (OriGene Technologies, Rockville, MD) and hybridized with the mouse FKRP and fukutin cDNAs in Rapid-hyb buffer (Amersham-Pharmacia), according to the manufacturer's instructions. RT-PCR was performed on RNA extracted from CHO cells transfected with FKRP expression constructs and derivatives thereof using standard protocols.

Site-directed mutagenesis

Site-directed mutagenesis was performed on plasmid DNA (10 ng) using Pfu Turbo DNA polymerase (Stratagene) in a 20 μl volume with 12.5 ng of each primer and 0.2 mM dNTPs. PCR cycles were as follows: 95°C for 30 s followed by 18 cycles of 95°C for 30 s, 55°C for 1 min, 68°C for 16 min. The mutated DNA template was digested with DpnI (10 U) to remove the parental plasmid and used to transform XL1-Blue competent cells. Plasmid DNA was extracted from transformed XL1-Blue cultures using the Qiagen EndoFree Maxiprep kit according to the manufacturer's instructions. Mutations were confirmed by DNA sequencing. The primers used to make the different mutants were: FKRP-NNNF, 5'-CCT TGG GAC TAC AAC AAC AAC CTG GGC ATC TAC C
Antibodies and immunocytochemistry

Polyclonal anti-FKRP antibodies (FKRP-FP) were produced in rabbits immunized with a thioredoxin fusion protein spanning the entire luminal domain of the protein (amino acids 36–end). Polyclonal anti-fukutin antibodies (FK-CT) were produced in rabbits against a thioredoxin fusion protein spanning the last 60 amino acids of mouse fukutin. All antibodies were affinity purified and pre-absorbed against thioredoxin by column chromatography using proteins covalently attached to Sulfolink (Pierce and Warriner). The anti-FKRP and fukutin antibodies only worked on recombinant protein produced by heterologous expression in mammalian cells. Other groups have failed to produce anti-fukutin antibodies in spite of using multiple immunogens and different strategies (4). The sheep anti-dystroglycan antibodies have been described previously (43). Mouse anti-mannosidase II was purchased from BabCO (Berkley, CA) and the 9E10 anti-myc monoclonal antibody was purchased from Roche. Expression constructs were transfected into COS-7 (monkey kidney), NRK (normal rat kidney fibroblasts) and CHO (Chinese hamster ovary) cell lines using Fugene-6 (Roche) following the manufacturer’s instructions. For immunolocalization of FKRP and fukutin, cells grown on glass cover slips were fixed in 4% (w/v) paraformaldehyde in PBS at 4°C for 15 min and permeabilized in 0.1% (v/v) Triton X-100 in PBS for 15 min at 4°C. Cells were extensively washed in PBS before applying the antibodies. Antigens were detected by indirect immunofluorescence with the following primary antibodies, FKRP-CT (diluted 1:25), FK-CT (diluted 1:100), anti-myc 9E10 (diluted 1:200) and anti-α-mannosidase II (diluted 1:10 000) followed by the secondary antibodies, rhodamine-Red X anti-rabbit-IgG (Jackson Immunoresearch) and Alexa 488-labelled anti-mouse-IgG (Molecular Probes). Slides were examined by fluorescence microscopy using a Leica DMRE microscope or by laser confocal microscopy using a Leica TCS SP2 microscope. Images were adjusted for brightness and contrast using Adobe Photoshop version 6. For studies on the FKRP mutants, expression constructs were transfected into COS-7 cells as described above. The Golgi apparatus was identified by co-transfection of a Golgi-localized GFP construct (NT-FK-GFP). The antigens were identified by immunofluorescence as described above. Slides were viewed with a Leica microscope and images were captured using a Hamamatsu Orca ER CCD camera and OpenLab 3.0.9 software (Improvision).

2D-gel electrophoresis and immunoblotting

Proteins were prepared from transfected CHO cells that were lysed with 2D rehydration sample buffer (8 M urea, 2% w/v Chaps, 50 mM DTT, 0.2% v/v bio-lyte 3/10 ampholytes) and separated by isoelectric focusing in the first dimension using IPG strips and a Protein IEF Cell (BioRad) and SDS-PAGE in the second dimension (10% Criterion gels, BioRad). Proteins were transferred to a nitrocellulose membrane (BA83, Schleicher and Schuell) and probed with antibodies against dystroglycan (diluted 1:1000) or the myc-epitope tag (diluted 1:100). For western blots, protein samples were prepared for SDS-PAGE in SDS/urea buffer (4 M urea, 3.8% SDS, 20% glycerol, 75 mM Tris pH 6.8, 5% 2-mercaptoethanol) and immunoblotted as described previously (44).

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