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Fukutin-related protein mutations that cause congenital muscular dystrophy result in ER-retention of the mutant protein in cultured cells

Christopher T. Esapa[1], R.A. Jeffrey McIlhinney[2] and Derek J. Blake[1,*]

[1]Department of Pharmacology and [2]Medical Research Council Anatomical Neuropharmacology Unit, University of Oxford, Oxford, UK

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Mutations in the gene encoding fukutin-related protein (FKRP) cause a spectrum of diseases including congenital muscular dystrophy type 1C (MDC1C), limb girdle muscular dystrophy 2I (LGMD2I) and congenital muscular dystrophies (CMDs) with brain malformations and mental retardation. Although these diseases are associated with abnormal dystroglycan processing, the cellular consequences of the idiosyncratic FKRP mutations have not been determined. Here we show, in cultured cells, that FKRP mutants associated with the more severe disease phenotypes (S221R, A455D, P448L) are retained in the endoplasmic reticulum (ER), whereas the wild-type protein and the mutant L276I that causes LGMD2I are found predominantly in the Golgi apparatus. The ER-retained proteins have a shorter half-life than the wild-type FKRP and are preferentially degraded by the proteasome. Furthermore, calnexin binds preferentially to the ER-retained mutants suggesting that it may participate in the quality control pathway for FKRP. These data provide the first evidence that the ER-retention of mutant FKRP may play a role in the pathogenesis of CMD and potentially explain why the allelic disorder LGMD2I is milder, because the mutated protein is able to reach the Golgi apparatus.

INTRODUCTION

Glycosylation is an important post-translational modification of many proteins in the secretory pathway and is often intrinsically linked to protein folding and quality control (1). The correct processing of N-linked glycans in the endoplasmic reticulum (ER) ensures that misfolded proteins are removed from the cell by the action of the proteasome, a process known as endoplasmic reticulum-associated degradation (ERAD) (2). In addition, mature protein-linked glycans participate in many cellular processes including ligand receptor interactions and juxtacellular communication (3). Mutations in several genes involved in glycan metabolism are known to cause different types of congenital disorders of glycosylation, further emphasizing the importance of protein glycosylation (4). Moreover, at least five different forms of congenital muscular dystrophy (CMD) are caused by genes that encode actual or putative glycosyltransferases (5,6).

Mutations in the gene encoding fukutin-related protein (FKRP) cause congenital muscular dystrophy type 1C (MDC1C) and limb girdle muscular dystrophy type 2I (LGMD2I) (7–9). MDC1C is a rare autosomal recessive disorder characterized by severe muscular dystrophy presenting at birth or in the first few weeks of life (10). On the other hand, LGMD2I is a more common disease and is generally mild with a variable age of onset ranging from childhood to adulthood (7,11). Recently, FKRP mutations have also been described in several patients with CMD, mental retardation and brain abnormalities including cerebellar cysts (10,12). In addition, two patients diagnosed with muscle–eye–brain disease and Walker–Warburg syndrome, both presenting with severe structural eye changes, cobblestone lissencephaly and mental retardation, have been found to have FKRP mutations (13).

FKRP is a type II transmembrane protein that is targeted to the Golgi apparatus through an N-terminal signal anchor (14). Although the function of FKRP has not been determined, it is

*To whom correspondence should be addressed at: Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK. Tel: +44 1865271860; Fax: +44 1865271853; Email: derek.blake@pharm.ox.ac.uk

thought to be a glycosyltransferase or phosphoryl-ligand transferase (8,14,15). This hypothesis is supported by the finding that MDC1C and LGMD2I are associated with a secondary deficiency in the glycosylation of α-dystroglycan, a component of the dystrophin glycoprotein complex (8,16) that functions as a receptor for extracellular matrix proteins including laminin (17), agrin (18,19) and neurexins (20).

At least 36 different missense mutations in the *FKRP* gene have been described to date. No patients have been found with two *FKRP* null alleles suggesting that the complete lack of FKRP might result in embryonic lethality. Furthermore, there are no obvious phenotypic correlations between the nature of the missense mutation and the severity of the disease. For example, patients homozygous for L276I or R54W have LGMD2I (7,21). In addition to patients homozygous for the L276I mutation, several compound heterozygotes have been identified that have the L276I mutation and a different mutation (sometimes even a potential null allele) on the other chromosome (7,22). These patients have LGMD2I suggesting that one copy of FKRP-L276I is sufficient to protect the individual from the severe forms of the disease.

Although the genetic association between *FKRP* mutations and CMD or LGMD2I is well established, the molecular consequences of the allelic variation are unknown. Previously, we have shown that the Golgi localization of FKRP is likely to be an important prerequisite for its function (14). In this study, we show that the mutations associated with the severest phenotypes result in the ER-retention of mutant FKRP, whereas the common LGMD2I mutation, L276I, is found in the Golgi apparatus. We also show that the ER-retained mutants are preferentially degraded by the proteasome and have a shorter apparent half-life than the wild-type protein. These data could explain, in part, why some mutations in the gene encoding FKRP are associated with different disease severities.

RESULTS

Subcellular localization of wild-type and mutant FKRP

We have shown previously that FKRP is a Golgi-resident protein that is targeted to the cis- and medial-Golgi apparatus through an N-terminal signal anchor (14). To determine the subcellular localization of various disease-causing FKRP mutants, transfected COS-7 cells were immunostained with the anti-FKRP antibody 5643 (Supplementary Material, Fig. S1). Importantly, the use of a specific anti-FKRP antibody precludes the need for epitope-tagged constructs that can misfold in heterologous cells (Esapa and Blake, unpublished data). For these experiments, we selected a number of mutations that cause different severities of disease (Table 1). All of the mutations analyzed in this study alter amino acids that are conserved between the human and murine orthologues of FKRP. Western blot analysis of lysates from transfected COS-7 cells showed that all of the mutants produced similar levels of protein identical in molecular weight to wild-type FKRP (data not shown). Wild-type FKRP co-localized with the Golgi matrix protein, GM 130, in the Golgi apparatus (Fig. 1A). In contrast, the mutants S221R, A455D and P448L gave a reticular staining pattern reminiscent of the ER (Fig. 1B). The ER-localization of each of the mutants was confirmed by co-localization with the ER chaperone GRP78 (Fig. 1B). It is therefore possible that each of the mutations associated with the severe disease phenotypes cause FKRP to misfold in the ER where it is retained.

Table 1. Constructs used in this study

Construct	Disease association	References
FKRP (wild-type)	Normal	(8,14)
FKRP-S221R	CMD with cerebellar cysts and mental retardation	(10)
FKRP-A455D	CMD with cerebellar cysts and mental retardation	(12)
FKRP-P448L	MDC1C	(8,14)
FKRP-L276I	LGMD2I	(7)
FKRP-EYFP	Normal	This study
L276I-EYFP	LGMD2I	This study
P448L-EYFP	MDC1C	This study
FKRP-N172A/N209A	Not applicable	This study

We also investigated the subcellular location of the FKRP-L276I mutant that is commonly associated with the relatively mild LGMD2I phenotype (Table 1). FKRP-L276I was found predominantly in the Golgi apparatus co-localizing with GM130, although some protein was detected in the ER (Fig. 1A and B). This was also true of wild-type FKRP and is likely to represent the pool of nascent protein before it is trafficked to the Golgi apparatus. These results indicate that the FKRP alleles associated with severest forms of disease are localized in the ER and are unable to reach the Golgi apparatus, whereas the allele associated with the milder phenotype can reach the Golgi apparatus.

Although COS-7 and CHO cells are routinely used in cell biology for studies on protein trafficking and post-translational processing, it was important to determine the subcellular localization of the different proteins in muscle cells which are a more relevant cell type for the study of CMD. To this end, each construct was transfected into C2C12, stained with the anti-FKRP antibody and antibodies against the ER and Golgi components, and examined by confocal microscopy (Fig. 2). As seen in COS-7 cells, the wild-type FKRP and FKRP-L276I co-localized with GM130 in the Golgi apparatus, whereas the FKRP-S221R, FKRP-P448L and FKRP-A455D mutants were found in the ER failing to co-localize with GM130 (Fig. 2). Control experiments on untransfected cells showed that the anti-FKRP 5643 antibody did not detect endogenous protein.

To negate the possibility that the anti-FKRP antibody was detecting conformation-dependent epitopes (for example, denatured or misfolded protein), we produced a series of enhanced yellow fluorescent protein-(EYFP) tagged constructs that were transfected into C2C12 cells (Table 1). For these experiments, EYFP was fused to the C-terminal region of FKRP rather than the N-terminal region because there are several important trafficking determinants at the N-terminal region of the protein (14). FKRP-EYFP and L276I-EYFP co-localized with GM130 in the Golgi apparatus, but did not co-localize with the ER-resident protein calnexin (Fig. 3). In contrast, P448L-EYFP was not detected in the Golgi apparatus, but co-localized with calnexin in the ER (Fig. 3).

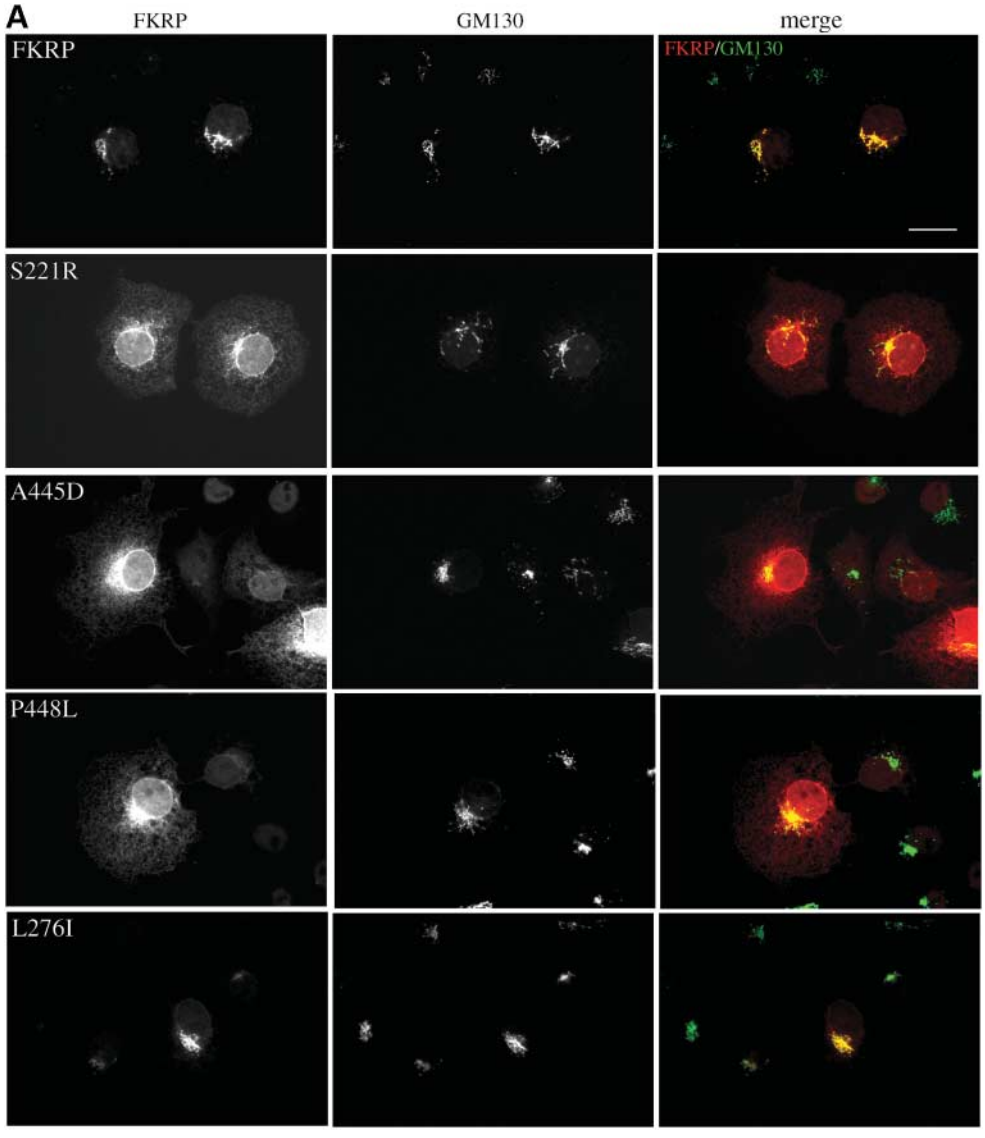

Figure 1. Subcellular localization of FKRP and different FKRP mutants in COS-7 cells. Immunofluorescence images of COS-7 cells transfected with wild-type and mutant FKRP constructs. Cells were labeled with the anti-FKRP antibody and rhodamine-red X anti-rabbit (red) and either anti-GM130 (**A**) and Alexa 488 anti-mouse (Golgi, green) or anti-GRP78 (**B**) and Alexa 488 anti-goat (ER, green) as indicated. The identity of each construct, FKRP (wild-type, S221R, A455D, P448L, L276I) is indicated on the left-hand panel. The merged images are shown in colour. Scale bar is 50 μm.

In control experiments, EYFP produced from the empty vector was found in the nucleus and cytoplasm of transfected C2C12 cells (Fig. 3). Similar results were also obtained in cultured cortical neurons expressing the different EYFP-tagged constructs (McIlhinney *et al.*, unpublished data). Thus, FKRP mutants associated with the severe disease phenotypes are retained in the ER of both COS-7 and C2C12, whereas the wild-type FKRP and FKRP-L276I are found predominantly in the Golgi apparatus.

FKRP mutants are preferentially degraded by the proteasome

Misfolded proteins in the secretory pathway are often removed from the cell by translocation from the ER to the cytoplasm followed by proteasomal degradation. To determine whether wild-type and mutant FKRPs were degraded by the proteasome, transfected CHO cells were incubated in the absence and presence of the proteasome inhibitor lactacystin. Wild-type FKRP and each of the different mutants accumulated in lactacystin treated cells rather than in the untreated cells (Fig. 4A). Lactacystin treatment also increased the abundance of several smaller FKRP immunoreactive proteins that could correspond to differentially glycosylated core protein (discussed subsequently) or degradation products (Fig. 4A). Similar results were obtained in COS-7 cells. To determine the effect of lactacystin treatment on the stability of wild-type and mutant FKRPs, COS-7 cells were transfected with constructs encoding FKRP or FKRP-P448L and treated with cycloheximide (to block protein synthesis) or cycloheximide and

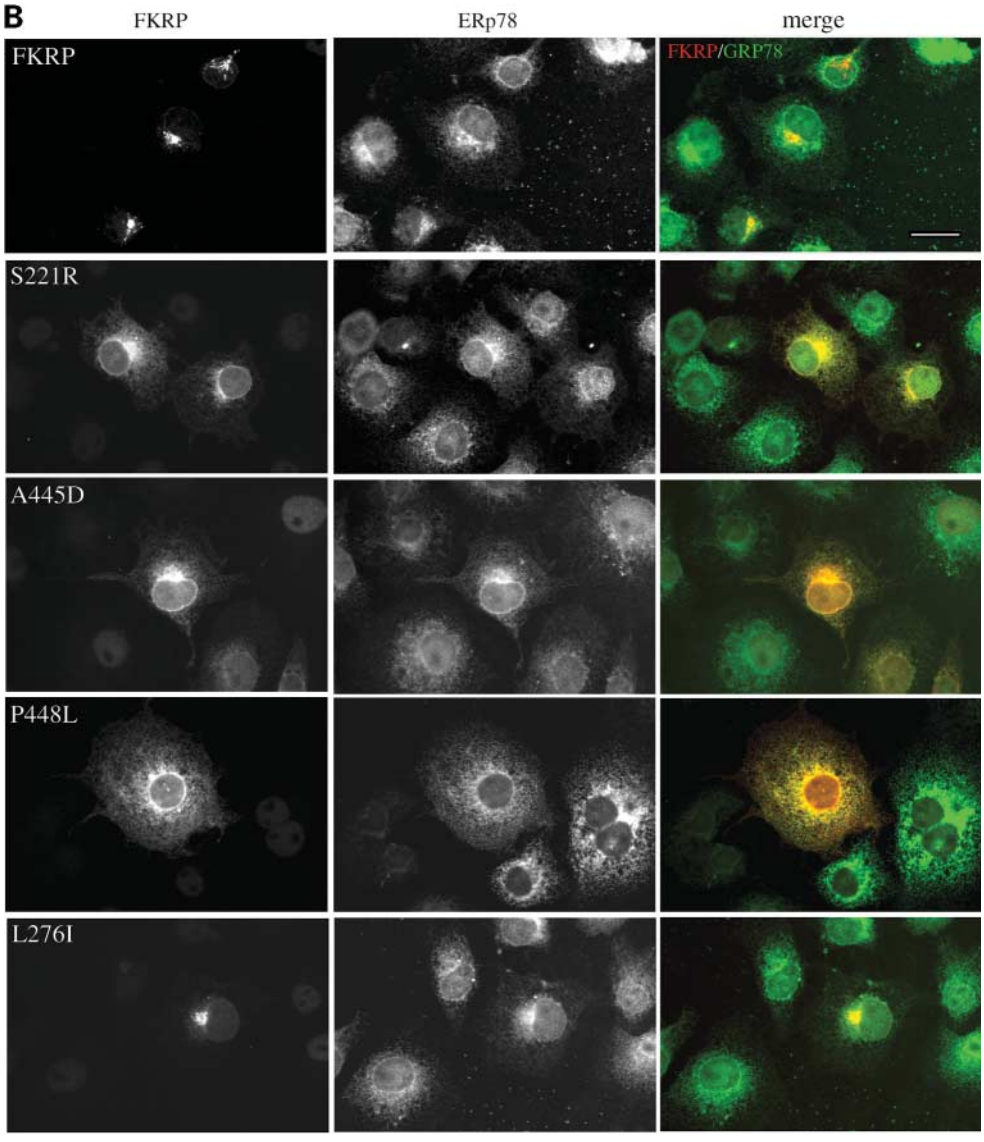

Figure 1. *Continued*

lactacystin. Figure 4B shows the levels of protein remaining after different intervals and representative immunoblots of the time course of each experiment. The levels of wild-type FKRP gradually decline in the presence of cycloheximide with the half-life of the protein being >24 h. In contrast, FKRP-P448L was less stable having an apparent half-life of ~14 h (Fig. 4B). Lactacystin treatment increased the levels of both wild-type and P448L FKRPs over a 24 h period (Fig. 4B) but had a more pronounced effect upon FKRP-P448L mutant. To determine whether lactacystin has an effect upon each of the mutants in this study, the levels of protein produced from each mutant were determined in transfected COS-7 cells treated with lactacystin for 16 h in the absence of cycloheximide. These experiments were performed in triplicate and protein levels were determined by blot densitometry relative to levels of protein in the untreated control (transfected cells cultured for 16 h in the absence of lactacystin), nominally assigned as 100% in each case (Fig. 4C). Lactacystin treatment modestly increased the levels of FKRP and FKRP-L276I but had a dramatic effect upon the steady state levels of the ER-retained mutants, most notably FKRP-P448L whose levels increased almost 3-fold (Fig. 4C). These data suggest that the ER-retained mutants can be preferentially degraded by the proteasome.

Preferential association of calnexin with the ER-retained FKRP mutants

The lectin chaperones calnexin and calreticulin are well known for their role in assisting the folding of newly synthesized glycoproteins before they exit the ER. To determine which chaperones may be involved in the quality control of

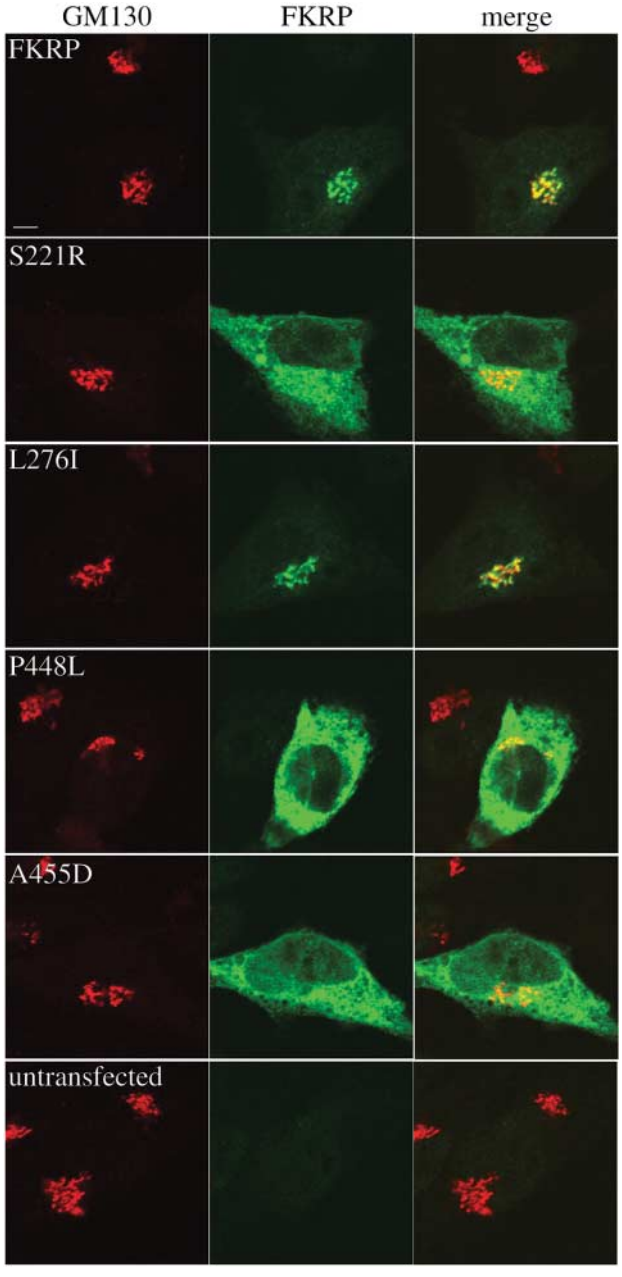

Figure 2. Subcellular localization of FKRP and different FKRP mutants in C2C12 cells. Confocal images from C2C12 cells expressing wild-type and mutant FKRPs stained with the anti-FKRP antibody, Alexa 488 anti-rabbit (green) and anti-GM130 followed by rhodamine-red X anti-mouse (Golgi, red). The identity of each construct, FKRP (wild-type, S221R, L276I, P448L, A455D) is indicated on the left-hand panel. The merged images are shown on the right-hand panel. Untransfected C2C12 have been included as a control to show that the anti-FKRP antibody does not detect the endogenous protein. Scale bar is 5 μm.

FKRP, and to determine whether they contribute to the ER-retention of mutants, we carried out immunoprecipitation of wild-type and mutant FKRP proteins from transfected COS-7 cells using the anti-FKRP antibody. Calnexin is detected in immunoprecipitated material derived from transfection of each construct but is preferentially associated with the ER-retained mutants (Fig. 5A). The highest levels of calnexin are detected in immunoprecipitates from cells transfected with P448L, A445D and S221R (Fig. 5A). Less calnexin was found in immunoprecipitates from cells expressing wild-type FKRP and L276I (Fig. 5A). Although calnexin may participate in the ER-retention of some FKRP mutants, it is possible that the apparent preferential binding of calnexin to the ER-retained mutants is a result of FKRP accumulation in the organelle. In these experiments, immunoprecipitated FKRP co-migrates with the IgG heavy chain, thus obscuring the protein band (Fig. 5A). To avoid these problems, the anti-FKRP antibody was cross-linked to protein G to make immunoaffinity beads that were used for immunoaffinity purification. Using this technique, calnexin was found to co-purify with FKRP, P448L and unglycosylated FKRP mutant where the two sites for N-linked glycosylation on FKRP, N172 and N209 have been mutagenized to alanine (N172A/N209A), in the absence or presence of castanospermine (Fig. 5B). Castanospermine inhibits glucosidase I, an enzyme that trims the terminal glucose from the N-linked glycan added in the ER. Calnexin binds to the monoglucosylated N-linked glycan to aid glycoprotein folding. Thus, addition of castanospermine should prevent calnexin-binding to N-linked glycans on FKRP if calnexin binds in an exclusively glycan-dependent manner (discussed subsequently). These data suggest that calnexin is able to bind to FKRP in a glycan-independent manner as has been shown for proteolipid protein and CD3-epsilon (23,24). Although calnexin is able to bind to unglycosylated FKRP, addition of castonspermine to cells appears to reduce the amount of calnexin associated with FKRP indicated that calnexin might recognize glycans linked to FKRP in addition to the polypeptide backbone. Calnexin was not immunoprecipitated with FKRP from untransfected cells or in immunoprecipitations where the primary antibody was omitted (data not shown). Furthermore, there was no detectable association of wild-type or mutant FKRPs with the ER chaperone GRP78 (glucose-regulated protein 78 or BiP) under the conditions used for the calnexin immunoprecipitation indicating that calnexin association is a specific interaction not simply due to protein aggregation on the beads (discussed subsequently).

To look for additional common chaperones that may play a role in FKRP quality control, we immunoaffinity purified FKRP and FKRP-P448L-containing protein complexes from transfected cells that had been treated with the cross-linker dithiobis[succinimidylpropionate] (DSP). DSP was used so that any weakly bound proteins, such as ERp57 (25), could be detected in the immunoaffinity-purified material after extensive washing with RIPA buffer. Although calnexin is found in the immunoaffinity-purified material, protein disulphide isomerase (PDI) was undetectable (Fig. 5C). In addition to PDI, the chaperones GRP78 (BiP), ERp72, ERp57 and calreticulin were not found in cross-linked protein complexes nor do they co-immunoprecipitate with FKRP.

DISCUSSION

In this study, we show that mutations in the *FKRP* gene associated with the most severe phenotypes (CMD with and

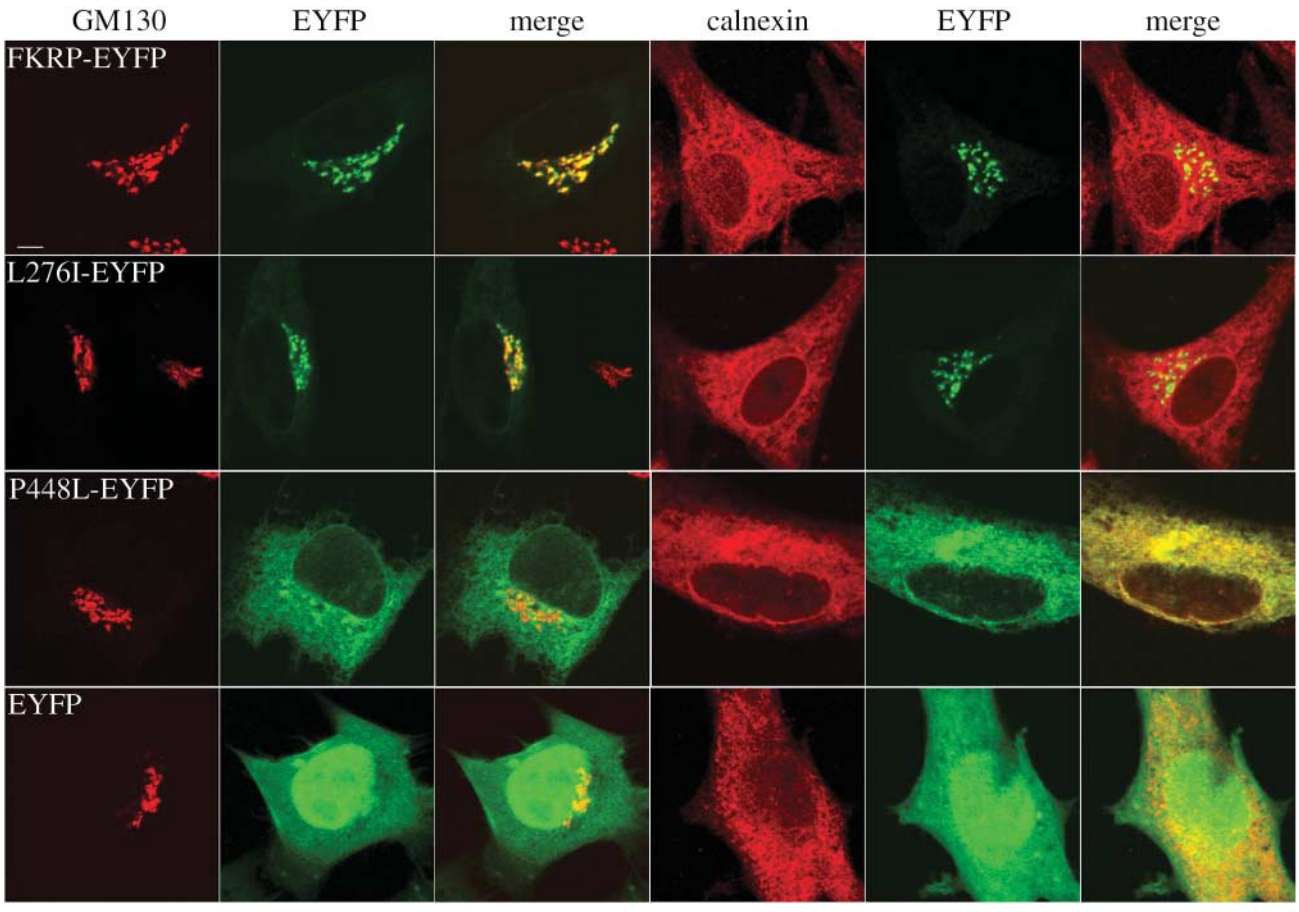

Figure 3. Subcellular localization of EYFP-tagged FKRPs in C2C12 cells. Confocal images from C2C12 cells expressing FKRP-EYFP, L276I-EYFP, P448L-EYFP and EYFP co-stained with anti-GM130 followed by rhodamine-red X anti-mouse (Golgi, red) or anti-calnexin followed by rhodamine-red X anti-rabbit (ER, red). Merged images are shown as indicated. Scale bar is 5 μm.

without brain involvement) cause the FKRP protein to accumulate in the ER where it can be removed from the cell by the proteasome. In contrast, wild-type FKRP and FKRP-L276I, the most common cause of the milder allelic disorder LGMD2I, are trafficked to the Golgi apparatus and are less susceptible to proteasomal degradation.

Our experiments on cultured COS-7 and C2C12 cells have revealed important differences in the trafficking of some FKRP mutants relative to wild-type FKRP and the common LGMD2I mutation, such as L276I. ER-retention of the mutants S221R, P448L and A455D is seen both in COS-7 cells that are commonly used in cell biology to study protein trafficking and folding (26) and in physiologically relevant C2C12 muscle cells. Furthermore, using an antibody-independent technique involving the transfection of EYFP-tagged constructs, we have shown that the wild-type FKRP and the L276I mutant are localized in the Golgi apparatus in C2C12 cells, whereas the P448L mutation causes the protein to be retained in the ER (Fig. 3). These data support our initial findings that FKRP and fukutin are Golgi-resident proteins (14). A recent paper has suggested that FKRP is localized in the rough ER where it is likely to be involved in the initial step of O-mannosylation on α-dystroglycan (27). However, protein *O*-mannosyltransferases-1 and −2 (POMT1 and POMT2) have been shown to initiate the first step of protein-linked O-mannosylation on α-dystroglycan (28). Furthermore, mammalian POMT2 and the POMT1 and POMT2 homologues in yeast are ER-resident proteins (29,30). Thus, as originally suggested, FKRP (and fukutin) is unlikely to function in the ER (14). It is therefore possible that the protein detected by Matsumoto *et al.* (27) in the ER represents a folding intermediate of FKRP. Interestingly the authors of this report also show partial co-localization of FKRP with GM130 in muscle cells and state that FKRP co-localizes with fukutin in transfected CHO cells.

The primary objective of this study was to determine the cellular fate of disease-associated FKRP mutants *in vitro*, and to determine why different FKRP mutations predispose to different disease severities. We selected two mutations (S221R, A455D) described in CMD patients with cerebellar cysts and mental retardation (10), one MDC1C mutation (P448L) (8,14) and the most common mutation (L276I) reported in LGMD2I patients (7). Using well-established techniques in cell biology, we have shown that the severe CMD

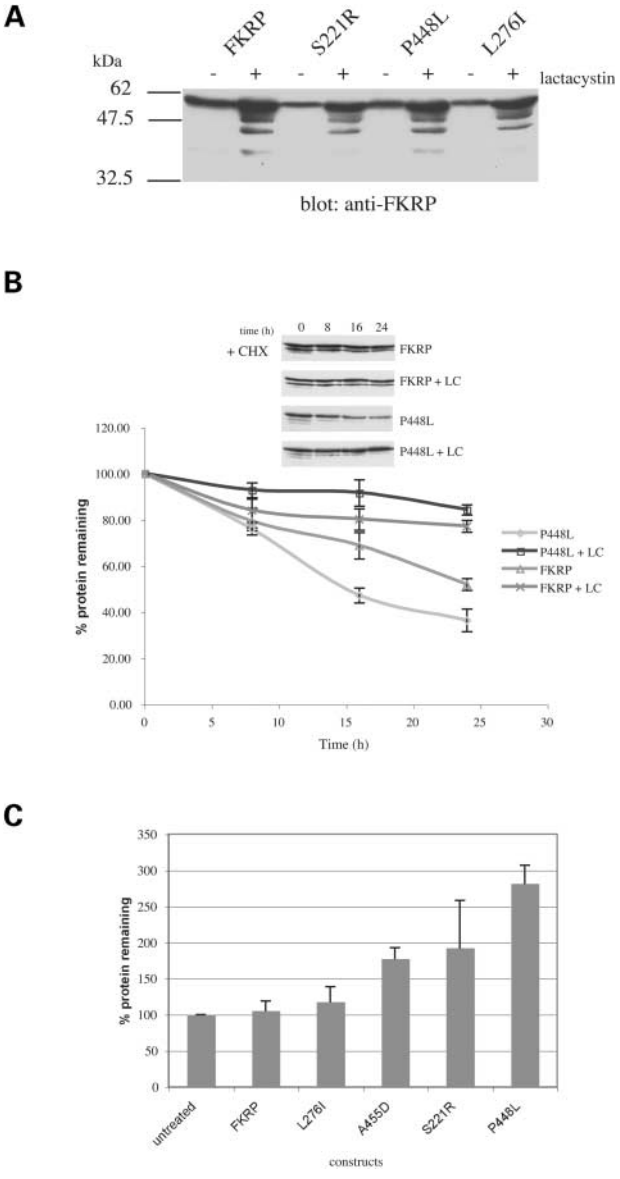

Figure 4. FKRP is an ERAD substrate. Western blot showing the steady state levels of wild-type and mutant FKRPs in CHO cells chronically treated with the proteasome inhibitor lactacystin (LC) for 16 h (**A**). A single 60 kDa protein is detected in untreated cells (−), whereas lactacystin treatment (+) produces a ladder of FKRP-reactive proteins and increases the abundance of the 60 kDa mature protein. Graphical representation and representative immunoblots of protein levels in COS-7 cells expressing FKRP or FKRP-P448L treated with cycloheximide (CHX) and lactacystin (**B**). FKRP-448L has a shorter half-life (14 h) compared with >24h for the wild-type protein. Addition of lactacystin reduces the degradation of both proteins but has a more pronounced effect upon the levels of the ER-retained P448L mutant. Error bars show the standard error of the mean. Lactacystin treatment (16 h) preferentially increases the steady state levels of the ER-retained FKRP mutants in COS-7 cells (**C**). Experiments were performed in triplicate and the error bars show the standard error of the mean.

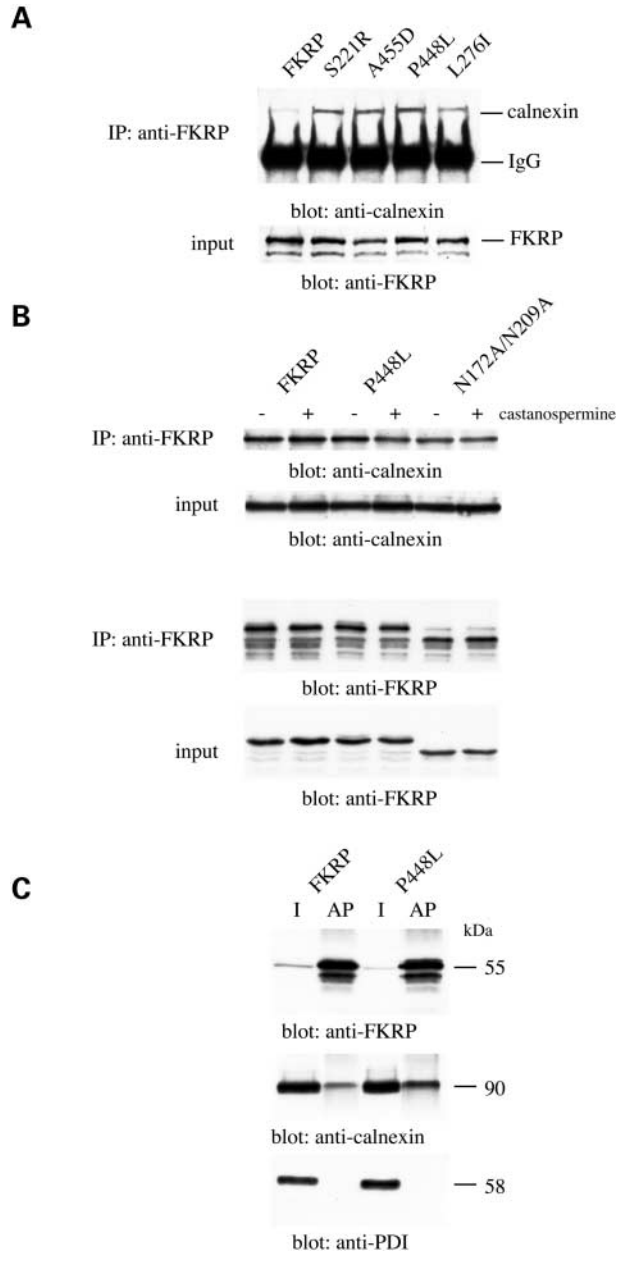

Figure 5. Association of calnexin with FKRP and mutants. Immunoprecipitation of calnexin with FKRP and mutants thereof using the anti-FKRP antibody (**A**). The anti-calnexin blot shows that higher levels of calnexin are associated with the ER-retained mutants when compared with FKRP and LGMD2I. The levels of FKRP and the mutants in the cell lysates (input) are shown below the immunoprecipitation. Calnexin was not detected when the precipitating antibody was omitted (data no shown). Immunoaffinity purification of FKRP and calnexin (**B**). FKRP, P448L and FKRP-N172A/N209A were purified from cell lysates that were treated (+) with castanospermine or untreated (−). Calnexin is detected in the immunoaffinity-purified material from each construct irrespective of presence of castanospermine. The presence of immunoaffinity purified FKRP cross-reactive proteins is shown along with the lysates for each protein. In this experiment, FKRP is not obscured by rabbit IgG as in (A). Calnexin does not bind non-specifically to the immunoaffinity matrix used in this experiment (data not shown). Immunoaffinity purification of FKRP and calnexin after cross-linking (**C**). DSP-cross-linked proteins were immunoaffinity purified with anti-FKRP conjugated beads. Calnexin was co-purified with FKRP and P448L, whereas PDI was undetectable in the AP material but was present in the input (I) cell lysates. Note that more calnexin co-purifies with P448L than with FKRP.

mutants are retained in the ER, whereas the LGMD2I-specific mutant (L276I) is found predominantly in the Golgi apparatus (Figs 1–3). Although the activity of each protein is unknown, the differences in disease severity could be explained by differential trafficking of the mutant proteins in the cell. This

raises the possibility that each of the mutants may retain some function but are unable to act on substrates, such as dystroglycan, because they cannot reach, or are poorly trafficked to the Golgi apparatus (14). The most common mutation in cystic fibrosis, cystic fibrosis transmembrane conductance regulator (CFTR) ΔF508, is retained in the ER due to a temperature-sensitive defect in protein folding (31,32). However, when this mutant is expressed in oocytes, it functions as a chloride channel showing that the protein retains some activity (33). Once the function of FKRP has been elucidated, it will be important to determine whether the severe alleles could retain partial function when they are forcibly localized in the Golgi apparatus. Clearly, the L276I mutant has some activity because LGMD2I patients can often have almost normal α-dystroglycan immunoreactivity associated with a relatively mild myopathy (7). Furthermore, some individuals that are homozygous for another LGMD2I mutation are asymptomatic suggesting that a modifier locus might influence the expression of the phenotype (34). It is also important to note that every mutation in the *FKRP* gene is unlikely to cause the ER-retention of the mutant protein. Many mutations in the gene encoding CFTR are associated with plasma membrane delivery of partially functional protein, thereby evading the quality control mechanism that retains CFTRΔF508 in the ER (35).

Although the ER-retention of mutant plasma membrane [for example, CFTR, pendrin and podocin (36,37)] and secreted proteins [for example, retinoschisin and fibrillin-1 (38,39)] is well documented, there is a paucity of data on the quality control pathway of the Golgi-resident proteins and their association with disease. The chaperones calnexin and its soluble counterpart calreticulin are particularly important for the folding of glycoproteins in the ER (40). They bind to protein-linked monoglucosylated N-linked glycans to promote correct folding after which the terminal glucose residue is cleaved by glucosidase II abrogating the interaction with calnexin (1). Failure to adopt the correct conformation results in repeated re-glucosylation of the N-glycans and re-association with calnexin (41). In this study, we used co-immunoprecipitation, affinity purification and cross-linking (Fig. 5) to show a steady state interaction between calnexin and FKRP indicating its involvement in assisting with protein folding. We also observed a more persistent interaction of calnexin with the ER-retained FKRP mutants suggesting that calnexin plays a role in the actual ER-retention mechanism. Other common chaperones, such as GRP78 and PDI, did not associate with wild-type or mutant FKRP under any of the conditions used for calnexin immunoprecipitation. Prolonged association of proteins, such as CFTRΔF508 (42) and mutated tyrosinase (in oculocutaneous albinism type 1) (43), with calnexin cause intracellular retention of each protein in the different diseases. Thus, calnexin may participate in the quality control of FKRP folding in the ER.

In summary, we have shown that several missense mutations in the gene encoding FKRP that are associated with severe disease states cause the protein to be retained in the ER. In contrast, the milder L276I mutation is found predominantly in the Golgi apparatus where it can participate, albeit in an impaired capacity, in the post-translational processing of proteins such as α–dystroglycan. To the best of our knowledge, this is the first example of a disease being caused by the ER-retention, presumably through protein misfolding, of a Golgi-resident protein.

MATERIALS AND METHODS

Antibodies

The stem region of FKRP (encoding amino acid residues 29–130) was amplified by PCR, cloned into pQE-30 UA (Qiagen) and transformed into *Escherichia coli* XL1-blue competent cells (Stratagene) for protein production. The fusion protein was purified using Talon resin (Clontech) according to the manufacturer's instructions and used to immunize New Zealand White rabbits (Sigma-Genosys). Antisera (anti-FKRP 5643) were affinity purified (AP) on Sulfolink coupling gel (Pierce) and used as described subsequently. The Golgi marker anti-GM130 was purchased from BD Biosciences, and the ER marker anti-GRP 78 (also known as BiP) was purchased from Santa Cruz Biotechnology. Antibodies raised against calnexin, ERp57, ERp72 and PDI were purchased from Bioquote Limited (UK).

Expression constructs and site-directed mutagenesis

The construct encoding full-length mouse FKRP cDNA has been described previously (14). Site-directed mutagenesis was used to introduce point mutations in the FKRP cDNA (14). All constructs were verified by sequencing. The primers used are as follows with mutated nucleotides underlined:

FKRP-S221Rf: 5′-GCTGGCCACCAGACTCTTCCTACAGACCGCC-3′
FKRP-S221Rr: 5′-GGCGGTCTGTAGGAAGAGTCTGGTGGCCAGC-3′
FKRP-L276If: 5′-CGTTGGGAATCCGTATCGTGAGCTGGGAAG-3′
FKRP-L276Ir: 5′-CTTCCCAGCTCACGATACGGATTCCCAACG-3′
FKRP-P448Lf: 5′-GCACTTCCTGCAGCTACTTGTCCCCCTGC-3′
FKRP-P448Lr: 5′-GCAGGGGGACAAGTAGCTGCAGGAAGTGC-3′
FKRP-A455Df: 5′-GTCCCCCTGCCCTTTGACGGTTTCATGGCACAGGC-3′
FKRP-A455Dr: 5′-GCCTGTGCCATGAAACCGTCAAAGGGCAGGGGGAC-3′
FKRP-N172Af: 5′-GGTGCCTAGCTCTGGCCGTCAGCCTGCGGG-3′
FKRP-N172Ar: 5′-CCCGCAGGCTGACGGCCAGAGCTAGGCACC-3′
FKRP-N209Af: 5′-TCCCGCGACCTCTTCGCCCTCTCGGTGCCC-3′
FKRP-N209Ar: 5′-GGGCACCGAGAGGGCGAAGAGGTCGCGGGA-3′

EYFP-tagged constructs were produced by PCR amplification of the full-length sequence and were subcloned into the *Eco*RI site of pEYFP-N1 (Clontech).

Cell culture, transfection and drug treatment

COS-7 and C2C12 cells were grown in Dulbecco's modified Eagle's medium supplemented with Glutamax, 10% fetal calf serum and penicillin/streptomycin. CHO cells were grown in Ham's F-12 medium supplemented with Glutamax, 10% fetal calf serum and penicillin/streptomycin. Cells grown in six-well plates with and without cover slips or in 10 cm tissue culture dishes were transfected with 1 or 6 μg of each expression construct using Fugene 6 reagent (Roche), according to the manufacturer's instructions. Lactacystin (10 μM, Sigma) and cycloheximide (50 μg/ml, Sigma) were applied to the cells 24 h after transfection as described previously (44). Cells were incubated with the drugs for 16–24 h and were then processed as described subsequently. The glucosidase I inhibitor castanospermine (50 μg/ml) was used as described previously (44).

Immunocytochemistry

Twenty-four hours after transfection, cells grown on glass cover slips were fixed with 4% (w/v) paraformaldehyde in PBS at 4°C for 15 min. Cells were permeabilized with 0.1% Triton X-100 in PBS at 4°C for 15 min and washed with PBS. Slides were incubated with the purified anti-FKRP-stem antibody, together with either the Golgi marker anti-GM130 (1 : 200) or the ER marker anti-GRP78 (1 : 100). Slides were washed and then incubated with rhodamine red X-conjugated anti-rabbit IgG (Jackson Immunoresearch) and Alexa 488-conjugated anti-mouse IgG (Molecular Probes) or Alexa 488-conjugated anti-goat IgG (Molecular Probes). Slides were examined by fluorescent microscopy using a Leica DMRA2 microscope. Confocal images were captured on a Leica LSM 510 microscope in MultiTrack mode using an HeNe laser at 543 nm and the Ar laser at 488 nm with the pinhole adjusted to two Airy units for both excitation wavelengths.

Western blot analysis

Forty-eight hours after transfection, cells were lysed with SDS–PAGE sample buffer containing 5% 2-mercaptoethanol. Cell lysates were separated by SDS–PAGE, blotted onto nitrocellulose membrane (Schleicher and Schuell) and probed with the purified anti-FKRP antibody (1:250), followed by HRP-conjugated anti-rabbit IgG. Protein bands were visualized using enhanced chemiluminescence (Pierce) and autoradiography. Band intensity was quantified using a Bio-Rad Gel-Doc System using Quantity One software (Bio-Rad).

Cross-linking

Transfected COS-7 cells were lysed in PBS buffer containing 1% Triton X-100, 0.5% deoxycholate, 1 mM EGTA and the homobifunctional cross-linker DSP (Pierce) at a final concentration of 2 mM. The reaction was incubated for 30 min at room temperature. Residual DSP was quenched by incubating the reaction mixture for 10 min in the presence of 15 mM Tris, pH 7.4 before centrifugation to remove cell debris. Cleared lysates were used for immunoprecipitation as described subsequently.

Immunoaffinity purification and immunoprecipitation

Two milligrams of AP anti-FKRP antibody was bound to 0.5 ml of packed protein G-Sepharose beads (Amersham Pharmacia) by rotating at 4°C overnight in borate buffer (0.2 M di-sodium tetraborate, 0.2 M boric acid, pH 9) as described by Greaves co-workers (45). The beads were spun down at 2000 rpm for 5 min and washed twice with 5 ml of borate buffer. The antibody was covalently cross-linked to the bound protein G using 20 mM dimethyl pimelimidate (Pierce) for 30 min at room temperature. The beads were spun down and washed with borate buffer containing 0.1 M ethanolamine (pH 8.0) before quenching unreacted dimethyl pimelimidate by rotating beads in 5 ml of ethanolamine for 2 h at room temperature. The beads were washed twice with PBS followed by 100 mM glycine pH 3.0, to remove any uncoupled antibody, and were finally washed twice with PBS before re-suspending in PBS/0.05% sodium azide as a 50% slurry.

COS-7 cells transfected with wild-type and mutant FKRP constructs were washed twice with PBS and solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton X-100, 0.5% deoxycholate, 1 mM EGTA) at 4°C for 30 min. Cell debris was removed by centrifugation. Lysates were precleared by rotating with protein G at 4°C for 1 h and spun down to remove the beads. For immunoprecipitation, the lysates were then mixed with anti-FKRP-protein G beads and rotated at 4°C for 3 h, after which the beads were washed three times with RIPA buffer. Immunoprecipitated proteins were eluted with 50 mM diethylamine pH 11, freeze-dried and reconstituted in SDS–PAGE sample buffer and used for SDS–PAGE and western blotting. FKRP was detected with the anti-FKRP antibody (1 : 250) and calnexin was detected using a rabbit polyclonal anti-calnexin antibody (1 : 2000).

For standard immunoprecipitations, COS-7 cell extracts were prepared as described earlier. Proteins were immunoprecipitated with the rabbit polyclonal anti-FKRP antibody (1:200) at 4°C for at least 4 h. Immune complexes were captured by incubating with MagnaBind goat anti-rabbit IgG beads (Pierce) at 4°C overnight. The beads were washed three times with RIPA buffer and dissolved in treatment buffer (75 mM Tris, pH 6.8, 3.8% SDS, 4 M urea, 20% glycerol, 5% 2-mercaptoethanol). Immunoprecipitated proteins were released from the beads by boiling for 5 min followed by SDS–PAGE and western blotting.

SUPPLEMENTARY MATERIAL

Supplementary material is available at HMG Online.

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