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Subcellular Localization of Fukutin and Fukutin-Related Protein in Muscle Cells

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Fukuyama-type congenital muscular dystrophy and congenital muscular dystrophy 1C are congenital muscular dystrophies that commonly display reduced levels of glycosylation of α-dystroglycan in skeletal muscle. The genes responsible for these disorders are *fukutin* and *fukutin-related protein* (*FKRP*), respectively. Both gene products are thought to be glycosyltransferases, but their functions have not been established. In this study, we determined their subcellular localizations in cultured skeletal myocytes. FKRP localizes in rough endoplasmic reticulum, while fukutin localizes in the *cis*-Golgi compartment. FKRP was also localized in rough endoplasmic reticulum in skeletal muscle biopsy sample. Our data suggest that fukutin and FKRP may be involved at different steps in *O*-mannosylglycan synthesis of α-dystroglycan, and FKRP is most likely involved in the initial step in this synthesis.

Key words: *cis*-Golgi,, fukutin, fukutin-related protein, rough endoplasmic reticulum, skeletal muscle.

Abbreviations: DG, dystroglycan; ER, endoplasmic reticulum; FCMD, Fukuyama-type congenital muscular dystrophy; FKRP, fukutin-related protein; MDC1C, congenital muscular dystrophy 1C; POMT1, protein *O*-mannosyltransferase 1.

Congenital muscular dystrophy comprises a genetically heterogeneous group of disorders. Fukuyama-type congenital muscular dystrophy (FCMD), the most common congenital muscular dystrophy in Japan, is characterized by muscle weakness and hypotonia from early infancy, and is associated with mental retardation and a brain anomaly called type II lissencephaly. The causative gene was cloned in 1998 and named *fukutin*, and a 3-kb retrotransposal insertion in the 3'-untranslated region of *fukutin* is found in most patients with FCMD (*1*). Fukutin-related protein (FKRP) was characterized as a homolog of fukutin in 2001 (*2*), and mutations in *FKRP* were also shown to cause muscular dystrophies (congenital muscular dystrophy 1C (MDC1C) and limb-girdle muscular dystrophy 2I) (*2*, *3*).

Skeletal muscle biopsy samples from FCMD and MDC1C patients show a marked reduction in α-dystroglycan (α-DG) by immunostaining with an antibody against the glycosylated epitope (*2*, *5*), whereas α-DG could still be visualized even in FCMD skeletal muscle with an antibody that recognizes the peptide epitope (*6*). The reduction in α-DG glycosylation in skeletal muscle leads to a loss in its ability to bind to extracellular ligands such as laminin, agrin and neurexin (*6*). Thus, the abnormality in posttranslational glycosylation of α-DG plays a crucial role in the pathogenesis of FCMD and MDC1C. The functions of fukutin and FKRP have not been clarified. However, they are supposed to act as glycosyltransferases, because they both are type-II transmembranous proteins and possess a DXD motif in the C-terminal side that is often found in glycosyltransferases (*4*). These facts strongly indicate that these two proteins may participate in the glycosylation of α-DG.

Protein glycosylation is a highly organized orderly process. Newly synthesized naked proteins are sequentially modified *en route* by glycosyltransferases during transport from the rough ER to the *trans*-Golgi network (*7*). Therefore, the expression and localization of a glycosyltransferase must be precisely regulated to synthesize the specific glycostructure in each tissue and cell. Determining the localization of putative glycosyltransferases will help to predict their functions. In this study, we determined the precise localization of fukutin and FKRP in muscle cells.

MATERIALS AND METHODS

Antibodies—Anti-FKRP and anti-fukutin antibodies were raised in rabbits against the recombinant fragments comprising the carboxyl-terminal 112 and 121 amino acids, respectively. Expression vectors for the FKRP and fukutin fragments with a glutathione-*S*-transferase tag at the N-terminus were constructed by inserting the cDNA fragments into pGEX-4T (Amarsham). Both antibodies were affinity-purified on antigen-immobilized activated thiol-Sepharose 4B (Amersham Pharmacia Biotec). Other antibodies used in this study are anti-emerin (Novocastra Laboratories), anti-BiP, anti-calnexin, anti-

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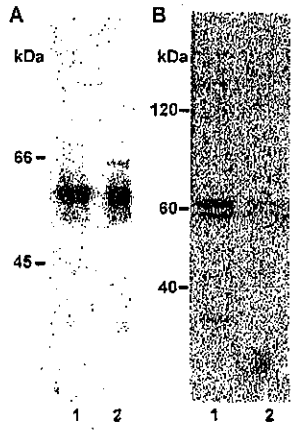

Fig. 1. Characterization of newly raised polyclonal antibodies by Western blotting. A: The anti-FKRP antibody detected a band at approximately 60 kDa in COS cell lysates with transiently expressed myc-FKRP (lane 1) and in human skeletal muscle cells (lane 2). B: Western blotting of a C2C12 cell lysate transfected with myc-fukutin. Both anti-fukutin (lane 1) and anti-myc (lane 2) antibodies detected doublet bands at approximately 60 kDa.

GM130 (BD Biosciences Pharmingen), and anti-myc 9E10 (Oncogene Science). Primary antibodies were used at the following dilutions: anti-FKRP (1:200), anti-fukutin (1:100), anti-myc (1:100), anti-BiP (1:100), anti-calnexin (1:100), anti-GM130 (1:500) and anti-emerin (1:500). Alexa 488-labeled anti-rabbit IgG and Alexa 568-labeled anti-mouse IgG (Molecular probes) were used as secondary antibodies.

cDNA Transfection and Immunocytochemistry—The open reading frames of FKRP and fukutin were amplified by PCR and subcloned into the expression vector CMV-myc (BD Clontech). These expression vectors encoding myc-epitope tagged FKRP and fukutin were transfected into the C2C12 cell line using Lipofectamine as described in the manufacturer's instructions (Invitrogen Carlsbad). The C2C12 cells and human skeletal muscle cells were induced to differentiate as described in a previous report (*8*). The immunostaining of myocytes was performed as described earlier (*9*). Laser confocal fluorescence images were obtained using an Olympus FLUOVIEW confocal microscope (Olympus).

Western Blotting and Immunohistostaining of Human Skeletal Muscles—Western blotting and immunohistostaining of human skeletal muscle were performed as described previously (*5*, *9*). The immunoreactive bands on Western blot were visualized using an ECL kit (Amersham Pharmacia Biotec). The immunostained sections were observed under a Zeiss Axiophot2 microscope (Carl Zeiss)

Muscle Biopsy Sample—Control muscle was obtained from surgical discards from a 24-yr-old male patient. Informed consent was obtained using the form approved by the Ethical Review Board at NCNP.

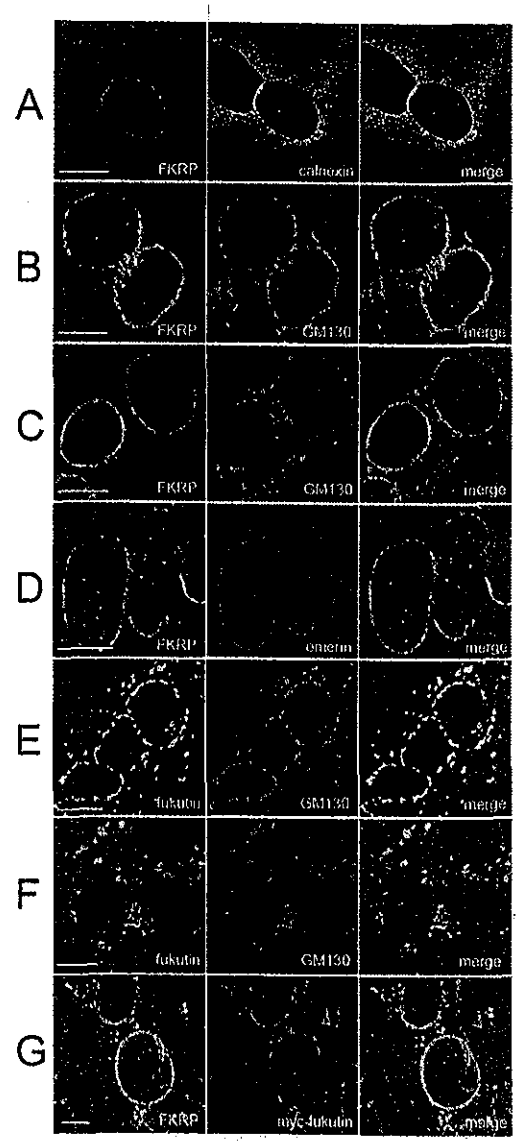

Fig. 2. Subcellular localization of FKRP and fukutin. A: FKRP and calnexin staining of human skeletal myotubes. FKRP (red) was continuously stained around nuclei with the innermost part of calnexin staining (green). B, C: FKRP and GM130 staining in mouse C2C12 myotubes. FKRP (green) was stained in the inner aspect of GM130 localization (red). Treatment with brefeldin A induced the dissociation of the Golgi apparatus, but FKRP remained localized around nuclei (C). D: FKRP and emerin staining in human skeletal myotubes. FKRP (green) was stained on the outer aspect of emerin staining (red). E, F: Immunostaining of transfected fukutin and GM130 in C2C12 myotubes. The merged image shows the co-localization of transfected fukutin and GM130. Treatment with brefeldin A dispersed both stainings into the cytosol (F). G: Spatial relationship between FKRP and fukutin. Endogenous FKRP (green) and transfected fukutin (red) in C2C12 myotubes showed different localizations. Bars denote 10 μm.

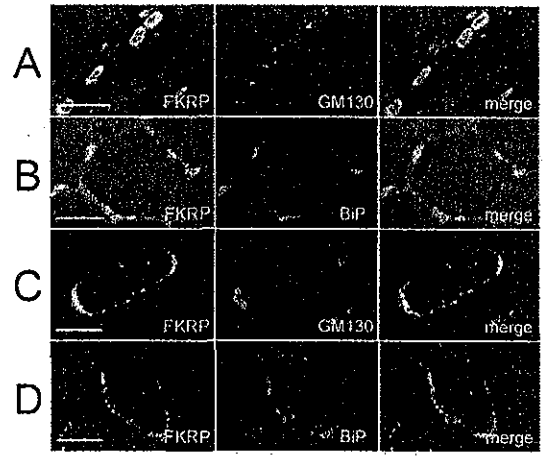

Fig. 3. **Immunohistochemical staining of FKRP in skeletal muscle.** The anti-FKRP antibody stained continuously around nuclei (A–D, green), while the anti-GM130 antibody stained nuclear poles and granular concentrates in cytosol (A, C, red). On higher magnifications, FKRP and GM130 are seen to overlap partially at both nuclear poles (C). BiP staining merges with FKRP in muscles (D). Bars denote 50 μm (A, B) and 5 μm (C, D).

RESULTS

Characterization of Newly Raised Antibodies against FKRP and Fukutin—Polyclonal antibodies against FKRP and fukutin were raised using recombinant fragments comprising the carboxyl-terminal 112 and 121 amino acids, respectively; the fragments share no homology to each other. Western blot analysis of a homogenate from cultured human skeletal muscle cells using the anti-FKRP antibody detected a band migrating at approximately 60 kDa (lane 2 in Fig. 1A), which matches the size of transiently expressed myc-FKRP in COS cells (lane 1).

The anti-fukutin antibody was not adequately sensitive to detect endogenous fukutin in skeletal muscle or in cultured muscle cell homogenates. In C2C12 muscle cells, in which myc-fukutin was transiently expressed, the anti-fukutin antibody detected two adjacent bands migrating at 60 kDa (lane 1 in Fig. 1B). The anti-myc antibody also detected two same sized bands (lane 2), suggesting that some posttranslational modification, such as glycosylation, generates the two bands. The specificity of each antibody was also examined by cross Western blot experiments in which the anti-FKRP antibody was allowed to react with the recombinant fukutin protein expressed in C2C12 cells, and the anti-fukutin antibody to react with the recombinant FKRP expressed in COS cells. No band was detected in either blotting, demonstrating the specificity of each antibody.

Subcellular Localization of FKRP and Fukutin in Differentiated Myocytes—To determine the subcellular localization of FKRP and fukutin in differentiated myocytes, human myocytes or C2C12 cells were immunostained with the two antibodies (Fig. 2). FKRP staining was detected surrounding the nuclei only in myotubes, but not in myoblasts. We compared this localization with those of organelle marker proteins against calnexin, a chaperone protein in rough ER (*10*). Calnexin localizes around the nuclei and merges with FKRP staining (Fig. 2A), although calnexin is also diffusely distributed in the cytosol. Double staining with antibodies for GM130, a matrix protein in the *cis*-Golgi compartment (*11*) or emerin, an inner nuclear membranous protein, showed that FKRP co-localizes with neither of them, the inner aspect of GM130 and outer aspect of emerin (Fig. 2B, D). After treating of the cells with brefeldin A, which induces the dissociation of the Golgi apparatus (*12*), GM130 staining was dispersed in the cytoplasm while FKRP staining was unaffected (Fig. 2C). These results suggest that FKRP is not localized in the Golgi or inner nuclear membrane but in the rough ER.

The staining of transfected C2C12 cells with antibodies against fukutin and its introduced tag showed the same distribution in the perinuclear area in a discontinuous dot-like pattern under confocal microscopy (data not shown). When transfected C2C12 cells were stained for fukutin and GM130, the two stains precisely overlapped (Fig. 2E), and treatment with brefeldin A dispersed both proteins (Fig. 2F). These phenomena indicate that fukutin is localized in the *cis*-Golgi in differentiated myocytes.

We compared the localization of FKRP and fukutin in C2C12 cells in which myc-fukutin was transiently expressed. FKRP and myc-fukutin displayed different localizations in myocytes (Fig. 2G).

Localization of FKRP in human skeletal muscle—In skeletal muscle biopsy specimens, FKRP was detected continuously around the nuclei of skeletal muscle fibers (Fig. 3A–D, green). After absorption of the FKRP antibody with recombinant FKRP protein, no staining was observed (data not shown). We compared this localization with those of organelle marker proteins against BiP, a chaperone protein in rough ER (*10*), and GM130. BiP was localized around the nuclei and merged with FKRP staining (Fig. 3B, D). GM130 was present at the poles of the nuclei and, in addition, a few concentrated granules were present within the cytosol and did not overlap with FKRP (Fig. 3A, C). These findings suggest that FKRP is localized in rough ER *in vivo*. Interestingly, in some nuclear poles, GM130 and FKRP staining was found to be partly merged (Fig. 3C). It is important to note that ER and the Golgi apparatus form an essentially continuous structure in the intracellular transport system, and that this is a possible explanation for the partial co-localization of FKRP and GM130.

DISCUSSION

The localization of FKRP is distinctly different from that of fukutin. Recently, Esapa et al. reported the subcellular localization of FKRP and fukutin in the medial-Golgi apparatus using rat kidney fibroblasts (*13*). Our data do not support the localization of FKRP in the Golgi apparatus, although immunohistochemical staining of skeletal muscle biopsy sample showed the partial incorporation of FKRP into other Golgi resident proteins. We assume that this difference is a result of the different cell types used in these two studies because FKRP colocalized with fukutin in CHO cells in which FKRP and myc-fukutin were transiently expressed (data not shown). In our experi-

ments using C2C12 cells, endogenous FKRP was seen only in differentiated myotubes, and the localization was different from those of GM130 and fukutin. Thus, we believe during muscle cell development, FKRP is expressed and functions in the rough ER after myotube formation. In addition, preliminary observations by immunoelectron microscopy demonstrated FKRP localization on the outer nuclear membrane or in transporting vesicles between the nuclear membrane and the Golgi apparatus, close to the distribution of ribosomes (unpublished data). It is unlikely that the localization of extrinsic fukutin is an artefact due to overexpression of this protein, because the extrinsic fukutin localizes only in the *cis*-Golgi apparatus in all transfected cells despite of its variable expression level.

Newly synthesized α-DG in the rough ER is thought to be modified by a series of glycosyltransferases, finally forming unique sugar chains consisting of *O*-mannosyl tetrasaccharide (Siaα2–3Galβ1–4GlcNAcβ1–2Man-*O*-protein) (*14*, *15*). The first *O*-mannosylation step is catalyzed by protein *O*-mannosyltransferase 1 (POMT1) (*16*), and this step may occur in rough ER. In fact, in yeast, several O-mannosyltransferases and their donor substrate, dolichyl-*P*-mannose, are indeed localized in rough ER (*17*). A mutation in *POMT1* causes Walker-Warburg syndrome, the most severe form of congenital muscular dystrophy with brain involvement, and it is also known that skeletal muscle biopsy samples from patients with Walker-Warburg syndrome show markedly reduced levels of α-DG (*16*, *18*). Our observations predict that FKRP localizes in rough ER, suggesting that FKRP may play a role in the first *O*-mannosylation step of α-DG with POMT1. Further analyses of the relationship between POMT1 and FKRP may clarify the role of FKRP in the *O*-mannosylation of α-DG.

In this study, we have clarified the precise subcellular localization of fukutin and FKRP in myocytes. The localization of both proteins suggests that they are involved in the glycosylation of α-DG, but in different manners. The biological characterization of fukutin and FKRP as glycosyltransferases and structural analyses of the α-DG sugar chains in patients are necessary to gain a complete understanding of the glycosylation process of α-DG, and, hence, the possible elucidation of the pathomechanism of FCMD and MDC1C.

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