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Detection of the dystroglycanopathy protein, fukutin, using a new panel of site-specific monoclonal antibodies

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ABSTRACT

Mutations in the gene encoding fukutin protein cause Fukuyama muscular dystrophy, a severe congenital disorder that occurs mainly in Japan. A major consequence of the mutation is reduced glycosylation of alpha-dystroglycan, which is also a feature of other forms of congenital and limb-girdle muscular dystrophy. Immunodetection of endogenous fukutin in cells and tissues has been difficult and this has hampered progress in understanding fukutin function and disease pathogenesis. Using a new panel of monoclonal antibodies which bind to different defined sites on the fukutin molecule, we now show that fukutin has the predicted size for a protein without extensive glycosylation and is present at the Golgi apparatus at very low levels. These antibodies should enable more rapid future progress in understanding the molecular function of fukutin.

1. Introduction

Fukuyama-type congenital muscular dystrophy (FCMD: MIM 253800), one of the most prevalent autosomal recessive disorders in the Japanese population, was originally described clinically as a muscular dystrophy combined with cortical dysgenesis (micropolygyria) and ocular abnormalities [1]. The gene responsible for FCMD was identified on chromosome 9q31 by linkage analysis and positional cloning and was named fukutin [2,3]. A 3-kb retrotransposal insertion in the 3' non-coding region of the fukutin gene is the most common mutation in Japan, but other fukutin mutations occur outside Japan and cause various phenotypes, including Walker–Warburg syndrome (WWS: MIM 236670) and limb-girdle muscular dystrophy (LGMD) [4–6]. It is clear, therefore, that partial or complete loss of fukutin function can give rise to a wide spectrum of phenotypes with different severities.

Mutations in fukutin cause abnormal glycosylation of cell surface α-dystroglycan which in turn reduces its laminin-binding activity [7], but a direct catalytic function for fukutin has not been established. Transfected fukutin is targeted to the Golgi apparatus, where glycosylation events usually occur, by an amino-terminal transmembrane domain. Fukutin also binds directly to the enzyme POMGnT1 (*O*-mannose-β-1,2-*N*-acetylglucosaminyltransferase1) and the transmembrane domain is required for this interaction [8]. This suggests that fukutin mutations may affect αDG glycosylation by their influence on POMGnT1 [8], mutations in which are responsible for a related dystroglycanopathy, muscle-eye-brain disease (MEB: MIM 253280) [9]. Both POMGnT1 and the POMT1/2 complex have glycosyltransferase activities directly involved in synthesis of *O*-mannosyl sugar chains on α-DG [9,10]. Yoshida-Moriguchi and colleagues [11] reported that a phosphodiester-linked moiety on *O*-mannose of α-DG is defective in fukutin-deficient and other dystroglycanopathies and that this specific modification is necessary for laminin binding activity. The enzyme that carries out this modification has not been identified. An indirect role for fukutin in α-DG glycosylation remains a possibility. Thus, Tachikawa et al. [12] found that four pathogenic missense mutants of fukutin caused mis-localization to the endoplasmic reticulum (ER) instead of the Golgi, rather than having a direct effect on α-DG glycosylation. These mutants were able to localize correctly when mis-folding was inhibited. Zebrafish studies [13] have suggested that fukutin may also have a role in maintaining the Unfolded Protein Response (UPR) and this may contribute to the unique clinical features of Fukuyama MD.

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2. Materials and methods

A fukutin cDNA fragment (bases 60–1,494) containing the open reading frame was subcloned by PCR into the vector pEGFP-N1 (Clontech) and into pET vectors for bacterial expression [3]. Subfragments were cloned into pGEX vectors for bacterial expression as GST-fusion proteins. Monoclonal antibody production [14] and epitope mapping with phage-displayed peptide libraries [15] were performed as previously described. HeLa cells were grown as monolayers on tissue culture plastic Petri dishes or glass coverslips in DMEM with 10% fetal bovine serum. Immunoprecipitation and western blotting were performed as previously described [16]. Secondary antibodies were from DAKOpatts, Copenhagen.

3. Results

Balb/c mice were immunized either with full-length recombinant human fukutin or with a mixture of 4 GST-fusion proteins containing fukutin fragments (amino-acids 26–58, 177–220, 233–268 and 415–461). Sera from 3 out of 8 mice recognized a 54 kDa band on western blots of human muscle (Fig. 1). This is the predicted size for the unmodified amino-acid sequence of fukutin. However, experience with rabbit and goat antisera against fukutin (our unpublished data) showed that protein bands of this size on western blots are not always authentic fukutin. To select mice for hybridoma fusions, we therefore used an additional criterion: recognition of over-expressed GFP-fukutin at the Golgi apparatus (Fig. 2) to show that their sera contained anti-fukutin antibodies.

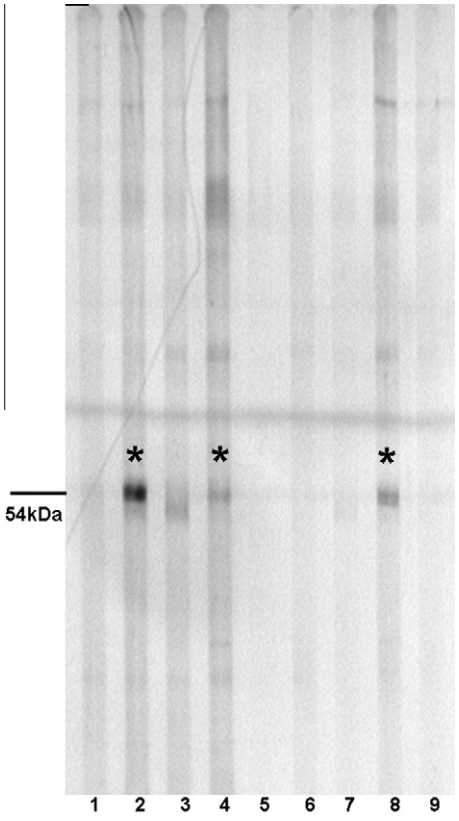

Fig. 1. Three mouse antisera recognize a 54 kDa protein in human muscle. An extract of normal human muscle was subjected to SDS–PAGE on a 3–12.5% gradient gel as a strip and antisera (0.07 ml of 1/100 in PBS) were applied in the lanes of a miniblotter. The position of a 54 kD marker is indicated and the three positive antisera are marked with an asterix. The lanes are as follows: (lanes 1–4), four mice immunized with mixture of GST-fukutin fragments, mice "*GST1 to 4*"; (lane 5) PBS control, (lane 6) normal mouse serum control, (lanes 7–9) three mice immunized with full-length fukutin, mice "*pETF1 to 3*". *GST2* and *pETF2* were used for hybridoma fusions (see Table 1).

Four hybridoma fusions were performed using spleens from three mice (*pETF2, G70 and J41*) that responded to full-length protein and one mouse (*GST2*) that responded to the fragment mixture. Twelve mAbs that recognized recombinant fukutin in both ELISA and western blot are shown in Table 1; all except one were from the immunizations with full-length protein. None of the mAbs recognized endogenous 54 kDa fukutin on western blots. Seven of them did, however, recognize over-expressed GFP-fukutin at the Golgi apparatus (Table 1).

Because it seemed likely that endogenous fukutin was present at too low concentration in muscle for detection, we concentrated fukutin from HeLa cells by immunoprecipitation with a goat polyclonal antibody [16] and tested 14 mAbs by western blotting of this highly-enriched extract. Eleven of them reacted well with a single 54 kDa protein band, while three reacted only very weakly or not at all (Fig. 3). All mAbs were used at a concentration of about 1 μg/ml and immuno-precipitations using pre-immune serum as negative control showed that only the 54 kDa band is fukutin-specific (Fig. 3).

Epitope mapping of the binding sites on fukutin for the mAb panel was performed using phage-displayed random 15-mer peptide libraries [15]. In this method, only the amino-acids within the 15-mer peptide which are important for mAb binding match with the target sequence. Reactivity with phage-displayed peptides revealed six mapping groups, plus MANFU5 which would not react with any phage colonies (possibly a conformational epitope). Groups 1 (amino-acids 223–231: MANFU4), 2 (amino-acids 452–461: MANFU11 and 12) and 3 (amino-acids 182–187: MANFU7-10 inclusive) were mapped with confidence by matching 3 or 4 different peptides from the random library to the fukutin sequence (Fig. 4). Group 4 (MANFU2 and 3) reacted with a single peptide only, but with four sequential amino-acids matched (PHSR: amino-acids 243–246), this was unlikely to have occurred by chance. It was possible to place other mAbs into Groups 1, 5 or 6 by their reactivity with different phage peptides, even though it was not possible to match the phage peptides with the fukutin sequence in these cases, possibly because the epitope is conformation-dependent.

4. Discussion

The results are consistent with the view that fukutin is a very low abundance protein, required for glycosylation of α-dystroglycan at the Golgi, though not substantially-glycosylated itself. It

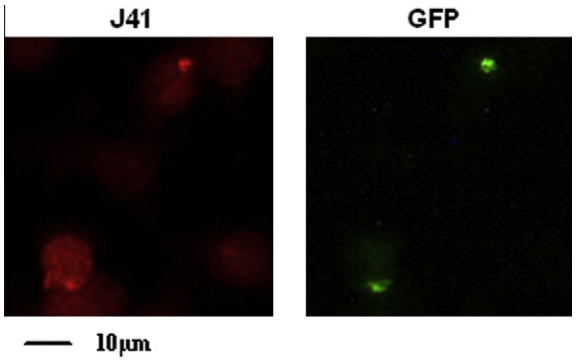

Fig. 2. Antibody staining co-localizes with GFP at the Golgi of GFP-fukutin-transfected HeLa cells. A HeLa cell-line expressing GFP-fukutin was grown on coverslips. After fixing with acetone-methanol, the cells were incubated with mouse antibody against fukutin (mouse number J41) followed by TRITC anti-(mouse Ig).