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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.

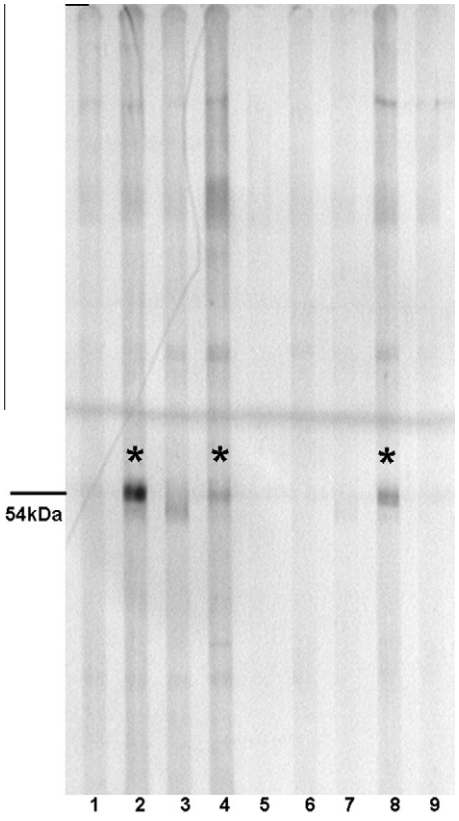

Fig. 1. Three mouse antisera recognize a 54 kDa protein in human muscle. An extract of normal human muscle was subjected to SDS–PAGE on a 3–12.5% gradient gel as a strip and antisera (0.07 ml of 1/100 in PBS) were applied in the lanes of a miniblotter. The position of a 54 kD marker is indicated and 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

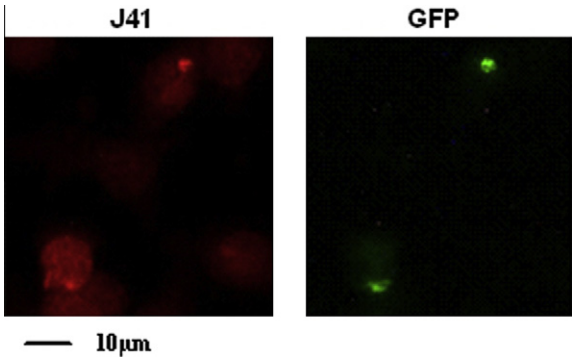

Fig. 2. Antibody staining co-localizes with GFP at the Golgi of GFP-fukutin-transfected HeLa cells. A HeLa cell-line expressing GFP-fukutin was grown on coverslips. After fixing with acetone-methanol, the cells were incubated with mouse antibody against fukutin (mouse number J41) followed by TRITC anti-(mouse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

Table 1
Twelve monoclonal antibodies against fukutin. Column 1: Antibody name and clone number; column 2: intensity of western blot staining in Fig. 3; Column 3: name given to the mouse used for hybridoma production, *GST2* (mix) was immunized with the mixture of four fukutin fragments and the other three mice (*pETF2*, *G70* and *J41*) with full-length fukutin; Column 4: phage mapping: the epitope group allocated and amino-acid sequence of the epitope from Fig. 4; Column 5: HeLa-GFP IMF: reactivity at the Golgi in the *IMF* (immunofluorescence microscopy) experiment shown in Fig. 2, rated as either "*good*" (similar to Fig. 2) or "*poor*".

Antibody	Western Blot	Origin (name of mouse)	Phage mapping	HeLa-GFP IMF
MANFU1 7A2	++	GST2 (mix)	Group 6, clone	Poor
MANFU2 10F9	++	PETF2	Group 4 mapped AA243–246	Poor
MANFU3 4E6	+++	PETF2	Group 4 mapped AA243–246	Good
MANFU4 3C7	++	PETF2	Group 1, clone	Good
MANFU5 7H2	++	PETF2	No clones	Good
MANFU6 4H8	(w+)	PETF2	Group 5, clone	Poor
MANFU7 1B5	++	G70	Group 3 mapped AA180–187	Poor
MANFU8 2F5	++	G70	Group 3 mapped AA180–187	Good
MANFU9 4E12	++	G70	Group 3 mapped AA180–187	Good
MANFU10 5H10	++	G70	Group 3 mapped AA180–187	Poor
MANFU11 3E4	++	J41	Group 2 mapped AA452–461	Good
MANFU12 6B5	+++	J41	Group 2 mapped AA452–461	Good

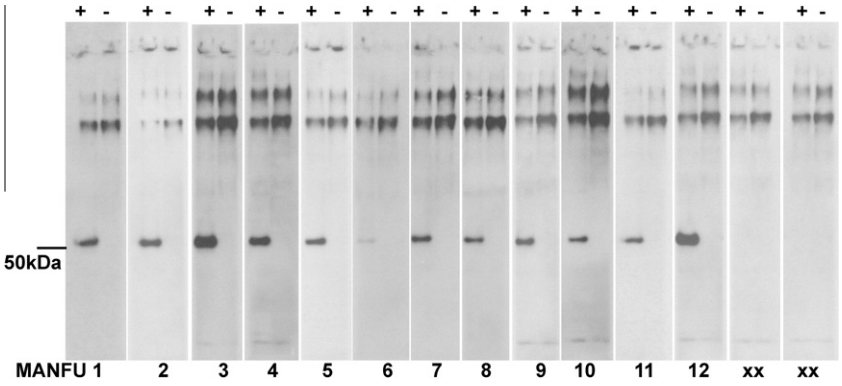

Fig. 3. Twelve mAbs recognize endogenous fukutin from HeLa cells after enrichment by immunoprecitation. HeLa cell extracts were immunoprecipitated with either a goat anti-fukutin serum (+) or with pre-immune control serum (−) and these were loaded in pairs for SDS–PAGE and western blotting with 14 different hybridoma culture supernatants adjusted to 1 μg/ml mouse Ig concn. Each lane of the gel contained all the protein immunoprecipitated from two 100 mm culture dishes of HeLa monolayers. The supernatants were MANFU1 to 12 (see Table 1) plus two negative hybridoma supernatants from the same fusions (XX). The fukutin band runs at the expected size, just above the 50 kDa Mr. marker.

Fig. 4. Epitope mapping of eight mAbs to specific binding sites on fukutin. The mAbs were attached to Petri dishes coated with anti-(mouse Ig) and used to select phage displaying random 15-mer peptides on their surface [15]. After determining the mAb specificity of each selected phage clone, the DNA was sequenced to determine the amino-acid sequence of the peptide displayed. The complete 461aa sequence of fukutin is shown, together with the 15-mer phage peptide sequences that matched it. In each case, the peptides were recognized specifically by the named mAbs only and not by other mAbs in the panel. Where several peptides are shown alongside several mAbs (e.g. MANFU7-10), ALL the mAbs recognized ALL the peptides.

may have an enzymatic function, but the alternative possibility that it has an essential supporting role in dystroglycan glycosylation by modifying the localization or activity of other proteins has not been ruled out.

Detection of endogenous fukutin by mAbs required pre-enrichment of cell extracts (Fig 3), but the 54 kD band was confidently identifiable as authentic fukutin because it was recognized by mAbs against five different fukutin epitopes. Only monoclonal antibodies against different epitopes on fukutin make it possible to distinguish with confidence between endogenous fukutin and a more abundant cross-reacting protein on western blots, as we have shown for another low-abundance antigen, DMPK [17]. Although polyclonal mouse antisera were able to detect a 54 kD protein on western blots of total cell extracts (Fig. 1), we are unable to prove that this is authentic fukutin.

The localization of over-expressed fukutin very specifically to the Golgi (Fig. 2) and its detection at this site by mAbs against five different fukutin epitopes (Table 1) suggests that low endogenous protein levels can explain the failure of mAbs to detect fukutin in untransfected cells. The alternative possibility that epitope masking at the Golgi prevents antibody access to endogenous fukutin appears less likely when mAbs against five different fukutin epitopes give the same result.

We have shown that at least six or seven different epitopes are recognized by the panel of 12 mAbs (Table 1) and four regions of the fukutin amino-acid sequence have been shown to contain binding sites for 9 mAbs using phage-displayed peptide libraries (Fig. 4). The two mAbs against the extreme C-terminus of fukutin may be especially valuable for further functional studies. The remaining three mAbs may recognize conformational epitopes that either select no clones from the peptide library (MANFU5) or select clones that do not match the linear sequence of fukutin (MANFU1 and 6). Table 1 shows that epitope diversity was increased by using spleens from four different mice.

The mAbs described in this study are freely available for academic research from the MDA Monoclonal Antibody Resource (www.glennmorris.org/mabs.htm).

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