Available online at www.sciencedirect.com
ScienceDirect
Neuroscience Research 56 (2006) 391–399
Neuroscience Research
www.elsevier.com/locate/neures

Intracellular binding of fukutin and α-dystroglycan: Relation to glycosylation of α-dystroglycan

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Received 21 January 2006; accepted 17 August 2006
Available online 26 September 2006

Abstract

The functions of fukutin, a gene product responsible for Fukuyama type congenital muscular dystrophy, still remain unclear, although a relation to the glycosylation of α-dystroglycan is presumed. To investigate the functions of fukutin, immunohistochemistry, examination using cultured astrocytes, enzyme-linked immunosorbent assay (ELISA)-based binding assay and immunoprecipitation were performed using control muscle and central nervous system tissues. Immunohistochemistry showed that α-dystroglycan and fukutin were co-expressed, especially in the glial cytoplasm and glia limitans of the central nervous system. An anti-fukutin antibody added to the culture medium did not bring about any changes in the astrocytes cultured on laminin-coated dishes. Together with the immunohistochemical results, the intracellular function of fukutin is considered. ELISA-based binding assay and immunoprecipitation may suggest the direct binding of fukutin and α-dystroglycan, at least in part. Fukutin seems to bind to both the hypoglycosylated and fully glycosylated form of α-dystroglycan, and seems bind to the core area rather than the sugar chain of α-dystroglycan. Fukutin may directly interact with α-dystroglycan during glycosylation, but further examinations are needed to confirm these details.

Keywords: Fukutin; α-Dystroglycan; Glycosylation; Intracellular; Binding; Muscular dystrophy

1. Introduction

Recent studies have revealed the importance of glycosylation of α-dystroglycan (α-DG) for the genesis of some muscular dystrophies. α-DG is a heavily glycosylated protein, and is one of the components of the dystrophin–glycoprotein complex (DGC), which is involved in the basement membrane formation, linking intracellular and extracellular proteins (Michele and Campbell, 2003; Montanaro and Carbonetto, 2003; Muntoni et al., 2002). Congenital muscular dystrophies exhibiting hypoglycosylation of α-DG include Fukuyama type congenital muscular dystrophy (FCMD), muscle–eye–brain disease (MEB) and Walker–Warburg syndrome (WWS) (Hayashi et al., 2001; Jiménez-Mallebrera et al., 2003; Kano et al., 2002). These diseases are autosomal recessive diseases and exhibit muscular dystrophy, central nervous system (CNS) lesions and eye anomalies (Dobyns, 1997; Osawa et al., 1997; Pihko and Santavuori, 1997). An abnormal basement membrane has been reported in the skeletal muscle and CNS of FCMD (Ishii et al., 1997; Saito et al., 1999; Yamamoto et al., 1997) and in the skeletal muscle of WWS (Sabatelli et al., 2003; Vajsar et al., 2000), electron microscopically.

Protein O-linked mannose β1,2-*N*-acetylglucosaminyltransferase (POMGnT1), which is an enzyme implicated in glycosylation of α-DG, is responsible for MEB (Takahashi et al., 2001; Yoshida et al., 2001). Mutated POMGnT1 shows a reduced enzymatic activity (Manya et al., 2003). Hypoglycosylation of α-DG has been reported in some WWS patients having mutations in protein-O-mannosyltransferase 1 (POMT1), which is also an enzyme involved in the glycosylation of α-DG (Beltrán-Valero de Bernabé et al., 2002; Kim et al., 2004). Protein O-mannosylation is defective in mutated POMT1 (Akasaka-Manya et al., 2004). Hypofunction of POMGnT1 or POMT1 may result in the

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0168-0102/$ – see front matter
doi:10.1016/j.neures.2006.08.009

hypoglycosylation of α-DG, and an abnormal basement membrane may be formed (Kano et al., 2002; Michele and Campbell, 2003; Montanaro and Carbonetto, 2003; Muntoni et al., 2002; Sabatelli et al., 2003). Fukutin, a gene product responsible for FCMD, may also be related to the glycosylation of α-DG, like POMGnT1 and POMT1, but its functions still remain unclear. To clarify whether fukutin is involved in the glycosylation of α-DG, immunohistochemistry, examination using cultured astrocytes, enzyme-linked immunosorbent assay (ELISA)-based binding assay and immunoprecipitation were performed.

2. Materials and methods

2.1. Materials

Human cerebra, spinal cords and diaphragms obtained from autopsy cases were used for immunohistochemical staining. Human cerebra and diaphragms obtained from autopsy cases were used for ELISA-based binding assay and immunoprecipitation. These cases were selected from the records of Tokyo Women's Medical University, and did not include neuromuscular diseases. Each autopsy was performed after the family members granted informed consent in accordance with the Ethical Guideline of Tokyo Women's Medial University and the Helsinki Declaration of 1996.

A primary culture of astrocytes obtained from 1 to 2-day-old Wistar rat pups (Kawaguchi et al., 2005) was used for immunohistochemistry and examination using cultured astrocytes. Cells were positive for an astrocyte-specific marker, glial fibrillary acidic protein (GFAP). The expression of fukutin was confirmed by the reverse transcriptase-polymerase chain reaction (RT-PCR) using primers of mouse fukutin cDNA, and by sequencing of the RT-PCR product (data not shown). The experiment was approved by the Animal Experiment Ethical Committee of the Tokyo Women's Medical University.

2.2. Construction of fusion proteins

Fusion proteins of fukutin-cellulose binding domain (CBD) and α-DG-CBD were prepared in a previously described manner (Yamamoto et al., 2002), and used for ELISA-based binding assay and immunoprecipitation. Briefly, PCR using Takara Ex Taq™ (Takara, Tokyo, Japan) was performed, after an incubation of RNA with M-MLV reverse transcriptase (Life Technologies, Rockville, MD, USA). Oligonucleotide primers covering the open reading frame (112–1497 bp) of fukutin cDNA (GenBank: AB008226) and covering the mucin domain (1340–1789 bp) of α-DG (GenBank: XM_018223) were designed according to the instructions of pET-35 Xa/LIC vector kit (Novagen, Madison, WI, USA), and synthesized by Takara. The sequence of the primers for fukutin was 5′-GGTATTGAGGGTCGCATGAGTAGAATCAATAA-GAACGTGGTTTTG-3′ (coding sense) and 5′-AGAGGAGAGTTAGAGCCT-CAATATAACTGGATAACCT-3′ (anticoding sense), and for α-DG was 5′-GGTATTGAGGGTCGCGCTACACCCACACCTGTCAC-3′ (coding sense) and 5′-AGAGGAGAGTTAGAGCC TTTGGTGGTGACCCGGGGCA-3′ (anticoding sense). The reaction mixture was amplified for 35 cycles in a Zymoreactor thermo-cycler (ATTO Co. Tokyo, Japan). The amplification profiles consisted of denaturing at 94 °C for 1.5 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. After electrophoresis of the PCR products using 1.2% agarose gel, gels containing DNA of 1386 bp (fukutin) or 450 bp (α-DG) were cut and the DNA was purified using a BANDPURE DNA purification kit (Progen, Australia). The purified fukutin cDNA or α-DG cDNA was ligated to the pET-35 Xa/LIC vector following the instructions of the pET-35 Xa/LIC vector kit (Novagen). The plasmid containing fukutin cDNA was transfected to *Epicurian coli* BL21-CodonPlus™ (DE3) competent cells according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA), and a fusion protein (20 mg/mL in 20 mM Tris–Cl, pH7.4, 100 mM NaCl) was obtained. Purification of fusion protein was performed using Protein Refolding kit according to the manufacturer's instructions (Novagen). For the control, a CBD protein was also synthesized (Yamamoto et al., 2002).

2.3. Antibodies

An anti-α-DG antibody (VIA4-1, monoclonal, Upstate Biotechnology, Lake Placid, NY, USA) was used for immunohistochemistry, ELISA-based binding assay and immunoprecipitation. An anti-α-DG antibody (IIH6C4, monoclonal, Upstate) was used for examination using cultured astrocytes, and an anti-α-DG antibody (α-DG-p; polyclonal, kindly provided by Dr. Voit, Herrmann et al., 2000) was for immunohistochemistry. Anti-fukutin antisera (anti-C1 and anti-N1; polyclonal, kindly provided by Dr. Saito, Saito et al., 2000) were used for the ELISA-based binding assay and immunoprecipitation. The anti-N1 fukutin antisera was made against a peptide corresponding to residues 37–49 and anti-C1 antisera was to residues 448–461 (Saito et al., 2000). An anti-fukutin antibody (polyclonal, Yamamoto et al., 2002) was used for immunohistochemistry and examination using cultured astrocytes. Anti-GFAP antibodies (polyclonal and monoclonal, DakoCytomation, Denmark) were used for immunohistochemistry.

2.4. Immunohistochemistry

Tissues were fixed in 2% paraformaldehyde/phosphate-buffered saline (PBS) at RT, for 2 h, and immersed in 30% sucrose at 4 °C, overnight. Then, frozen sections of 6 μm were made. Cultured astrocytes were fixed in 95% ethanol for 5 min at RT. For immunofluorescence staining, sections were incubated with the mixture of two primary antibodies at 4 °C, overnight. The combinations of primary antibodies were anti-fukutin (1:100) and anti-α-DG (VIA4-1, 1:30), anti-α-DG (VIA4-1, 1:30) and anti-GFAP (polyclonal, 1:500) or anti-fukutin (1:100) and anti-GFAP (monoclonal, 1:400). After washing in PBS, sections were incubated with Cy3-conjugated donkey anti-rabbit IgG (1:200, Jackson Immunoresearch Laboratory, West Grove, PA, USA) and FITC-conjugated donkey anti-mouse IgG (1:200, Jackson Immunoresearch) for 6 h. Specimens were examined with using a fluorescence microscope (Axiovert S100, Carl Zeiss, Jena, Germany) or a laser-scanning confocal microscope (TCS-SL, Leica, Heidelberg, Germany).

Some tissue sections were stained with anti-fukutin (1:100) or anti-α-DG-p (1:500) antibody, using an avidin–biotin complex method. The tissue was incubated overnight at 4 °C with the primary antibody. After washing, the tissue was treated with biotinylated anti-rabbit IgG or biotinylated anti-sheep IgG at RT for 30 min and then with avidin–biotin complex at RT for 30 min. The color was developed with chromogen diaminobenzidine tetrahydrochloride and the slides were counterstained by hematoxylin.

Experiments omitting the primary antibodies served as the negative controls.

2.5. Examination using cultured astrocytes

Astrocytes in confluent growth were trypsinized and inoculated on 35 mm laminin-coated dishes (BD Bioscience, Bedford, MA, USA) with Dulbecco's modified Eagle medium (Gibco BRL, Bethesda, MD, USA) supplemented with 10% fetal bovine serum, 10% Ham's F12, 100 units/mL penicillin and 1 μg/mL streptomycin. The anti-α-DG antibody (IIH6C4, 1:100) or anti-fukutin antibody (1:50 and 1:100) was added at the same time of the inoculation of astrocytes, and astrocytes were cultured for 2 h, at 37 °C. Dishes without added antibodies served as the negative controls.

2.6. ELISA-based binding assay

Fresh frozen tissues stored at −70 °C were homogenized with 50 mM Tris–Cl (pH 7.4) with 150 mM NaCl, 1 mM ethylenediaminetetraacetate (EDTA), 1% triton X-100, 5 μg/mL leupeptin, 2 μg/mL aprotinin and 0.25 M phenylmethylsulfonylfluoride (PMSF) and incubated at 4 °C for 60 min. After centrifugation at 12,500 × *g*, 4 °C, the supernatant was mixed with agarose wheat germ agglutinin (WGA, Vector Laboratories, Burlingame, CA) and incubated at 4 °C, overnight. After washing, the glucoconjugates were eluted with 0.3 M N-acetyl-D-glucosamine in Tris-buffered saline (TBS, 100 mM Tris–Cl, pH 7.4, 150 mM NaCl) and used as tissue extracts.

For the ELISA-based binding assay using tissue extracts, microtiter plates pretreated with 1 mg/well of fukutin-CBD or CBD at 37 °C for 2 h were incubated with tissue extracts of various dilutions at 37 °C for 2 h. After