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Research Article

β-Taxilin participates in differentiation of C2C12 myoblasts into myotubes

Hiroshi Sakane [a,1], Tomohiko Makiyama [a], Satoru Nogami [a,2], Yukimi Horii [a], Kenji Akasaki [b], Hiromichi Shirataki [a,*]

[a] Department of Molecular and Cell Biology, Graduate school of Medicine, Dokkyo Medical University, 880 Kitakobayashi, Mibu-town, Tochigi 321-0293, Japan
[b] Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan

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ABSTRACT

Myogenesis is required for the development of skeletal muscle. Accumulating evidence indicates that the expression of several genes are upregulated during myogenesis and these genes play pivotal roles in myogenesis. However, the molecular mechanism underlying myogenesis is not fully understood. In this study, we found that β-taxilin, which is specifically expressed in the skeletal muscle and heart tissues, was progressively expressed during differentiation of C2C12 myoblasts into myotubes, prompting us to investigate the role of β-taxilin in myogenesis. In C2C12 cells, knockdown of β-taxilin impaired the fusion of myoblasts into myotubes, and decreased the diameter of myotubes. We also found that β-taxilin interacted with dysbindin, a coiled-coil-containing protein. Knockdown of dysbindin conversely promoted the fusion of myoblasts into myotubes and increased the diameter of myotubes in C2C12 cells. Furthermore, knockdown of dysbindin attenuated the inhibitory effect of β-taxilin depletion on myotube formation of C2C12 cells. These results demonstrate that β-taxilin participates in myogenesis through suppressing the function of dysbindin to inhibit the differentiation of C2C12 myoblasts into myotubes.

1. Introduction

Skeletal muscle, an organ that is required for locomotion and energy metabolism, is composed of multinucleated myofibers [1], and myogenesis is required for the development of skeletal muscle. During myogenesis, mononucleated myoblasts exit from the cell cycle and express muscle-specific genes [1–3]. Then, mononucleated myoblasts fuse to form multinucleated myofibers [2–5]. To understand the molecular mechanism of myogenesis, the profile of gene expression during differentiation of C2C12 mouse skeletal myoblasts has been investigated [6–8], and a variety of genes upregulated in the process of myogenesis have been emerged. The following studies have shown that these upregulated molecules play pivotal roles in myogenesis [9], but the molecular mechanism underlying myogenesis is not fully understood.

β-Taxilin, which was originally identified as MDP77 [10,11], is a member of the taxilin family composed of at least three proteins (α-, β- and γ-taxilin) which interacts with syntaxin proteins [12,13]. β-Taxilin is specifically expressed in the skeletal muscle and heart tissues [10,11], while other taxilin family members are ubiquitously expressed [10,11]. We have recently found that α-taxilin is involved in recycling of TfnR and interacts with SNX4 that is required for recycling of TfnR [14,15]. Therefore, it is possible that members of the taxilin family are involved in the regulation of intracellular vesicle trafficking. However, the function of β-taxilin in the skeletal muscle and heart tissues is less understood.

Dysbindin is a ubiquitously expressed coiled-coil-containing protein that was identified as a binding protein of dystrobrevin [16,17]. Knockdown of dysbindin impairs degradation of the internalized D2 dopamine receptor [18], suggesting that dysbindin plays a role in sorting internalized receptors to lysosomes. It has been also reported that dysbindin is involved in cardiomyocyte hypertrophy [17].

In this study, we found that β-taxilin interacts with dysbindin, and demonstrate that β-taxilin and dysbindin conversely regulate differentiation of myoblasts into myotubes. Our findings shed light on a novel function of β-taxilin in myogenesis and provide useful information for understanding the molecular mechanism underlying myogenesis.

Abbreviations: TfnR, transferrin receptor; SNX, sorting nexin; MHC, myosin heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; DM, differentiation medium; siRNA, small interfering RNA; RT, reverse transcription; Vamp, vesicle-associated membrane protein

* Corresponding author.
E-mail address: hiro-sh@dokkyomed.ac.jp (H. Shirataki).
[1] Present address: Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan.
[2] Present address: Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan.

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2. Materials and methods

2.1. Antibodies

An anti-β-taxilin antibody (1 ng/ml for western blotting) was prepared as described previously [13]. Anti-clathrin heavy chain (610499, 1:5000 dilution for western blotting), anti-MHC (MAB4470, 1:5000 dilution for western blotting and 1:500 dilution for immunocytochemistry), and anti-myogenin (sc-12732, 1:100 dilution for western blotting) antibodies were purchased from BD Biosciences (San Jose, CA), R&D Systems (Minneapolis, MN), and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Anti-myc (562, 1:5000 dilution for western blotting) and anti-GAPDH antibodies (171–3, 1:5000 dilution for western blotting) were purchased from MBL (Nagoya, Japan). An anti-α-tubulin antibody (ab15246, 1:100000 dilution for western blotting) was purchased from Abcam (Cambridge, UK). Anti-TfnR (136800, 1:5000 dilution for western blotting) and anti-GFP (A6455, 1:10000 dilution for western blotting) antibodies were purchased from Invitrogen (Carlsbad, CA). An anti-GFP antibody (11814460001) used for immunoprecipitation was purchased from Roche Diagnostics (Basal, Switzerland).

2.2. cDNA construct

Full-length cDNA of human dysbindin was isolated from a yeast two-hybrid library (630471, Clontech, Palo Alto, CA) as a binding partner of β-taxilin according to the manufacturer's protocol. Full-length cDNA of dysbindin was introduced into the BglII and EcoRI sites of a pEGFP-C1 vector. cDNA fragments of dysbindin corresponding to amino acids 94–351, 94–181, and 181–351 were amplified by PCR and introduced into the XhoI and EcoRI sites of a pEGFP-C3 vector.

2.3. Cell culture and transfection

C2C12 myoblasts were cultured in DMEM supplemented with 20% fetal bovine serum (growth medium) at 37 °C in a 5% CO_2 incubator. To induce differentiation into myotubes, the culture medium of C2C12 myoblasts was replaced with DMEM supplemented with 2% horse serum (hereafter referred to as DM). Transfection of expression vectors into COS7 cells or C2C12 myoblasts was performed using Lipofectamine 2000 or 3000 (Invitrogen) according to the manufacturer's protocols, respectively. To knockdown β-taxilin, C2C12 myoblasts were transfected with 10 nM siRNA using RNAi max (Invitrogen) according to the manufacturer's protocol. Negative control (12935-300, Invitrogen), β-taxilin #1 (CCUGAACAAGCUGCAAGCACCUGAA), β-taxilin #2 (CAACGGAAAGACAGCUGGCAGUGAA), dysbindin #1 (CCGAAGUACUCUGCUGGACUAGAAU) and dysbindin #2 (GAGGAAGGAGCUUGAAGCCUUCAAA) stealth siRNAs were purchased from Invitrogen.

2.4. Western blotting

Cells were lysed in lysis buffer [1% NP-40, 20 mM Tris–HCl (pH 8.0), 137 mM NaCl, and 10% glycerol]. The protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and then western blotting. Immunoreactive bands were detected using Clarity Western ECL Substrate (Bio-Rad) or ECL prime (GE healthcare, Little Chalfont, Buckinghamshire, UK).

2.5. Mouse tissue preparation

Mouse tissue was prepared as described previously [19]. Briefly, adult mice were perfused through the left ventricle with PBS. PBS perfusion was performed to remove circulating blood cells. Then, the tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use. All experiments were performed in accordance with the National Institutes of Health Guide for the care and use of laboratory animals and approved by the Animal Research Committee of Dokkyo Medical University. All efforts were made to minimize animal suffering and the number of animals used in this study.

2.6. Immunocytochemistry

Cells grown on coverslips were fixed in PBS containing 3.7% formaldehyde and then permeabilized with PBS containing 0.2% (w/v) Triton X-100, 0.2% bovine serum albumin, and 0.05% NaN_3. The cells were treated with the indicated antibodies for 1 h. After washing three times with PBS, the cells were treated with the secondary antibody for 1 h. Cells were observed using an FV10i confocal laser-scanning fluorescence microscope (Olympus, Tokyo, Japan). To measure the diameter of C2C12 myotubes, four fields were chosen randomly, and at least 9 myotubes per field were selected to measure the diameter. For each myotube, 10 measurements along the myotube were performed using FV10-ASW 3.0 software. To measure the number of nuclei in MHC-positive C2C12 cells, three fields were chosen randomly, and the number of nuclei in MHC-positive cells was counted. The diameter of C2C12 myotubes and the number of nuclei in MHC-positive C2C12 cells were evaluated independently by three of the authors. The results were expressed as the percentages of cells containing one, 2–4 or >5 nuclei in the total number of MHC-positive cells.

2.7. Immunoprecipitation

Transfected COS7 cells were lysed in 1 ml of the lysis buffer, and then 450 μl of the cell lysate was immunoprecipitated with 1 μg nonimmune IgG or an anti-GFP antibody using protein G sepharose (GE healthcare) at 4 °C for 2 h. The immunoprecipitates were washed three times with the lysis buffer and applied to SDS-polyacrylamide gel electrophoresis analysis.

2.8. RT-PCR

Total RNA was isolated from C2C12 cells using NucleoSpin RNA II (TaKaRa, Shiga, Japan). An RNA sample (2 μg) was reverse transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) in a total volume of 20 μl. Quantitative RT-PCR was performed using a light cycler nano (Roche Diagnostics). Aliquots (0.5 μl) of the reverse transcription products were amplified in a reaction mixture (20 μl) containing FastStart Essential DNA Green Master (Roche Diagnostics) and 0.5 μM of each primer. Forward and reverse primers were as follows: mouse *β-TAXILIN*, GCACCTGAAGAAAAGCTTGATT and GGAGCTTCAGTTTCTTTTGCTC; mouse *GAPDH*, ACCACAGTCCATGCCATCAC and CACCACCCTGTTGCTGTAGCC; mouse *MHC IIb*, CCGAGCAAGAGCTACTGGA and TGTTGATGAGGCTGGTGTTC; mouse *DYSBINDIN*, TGAAGGAGCGGCAGAAGT and GTAGCCCGTGGACAGGTACT.

2.9. Subcellular fractionation

Subcellular fractionation was performed as described previously [20]. Briefly, cells were suspended in buffer A [10 mM Tris–HCl (pH 7.5), 1 mM EDTA, and 0.25 M sucrose] and then homogenized with 15 strokes in a ball homogenizer (clearance: 0.012 mm). The homogenate was centrifuged at 1000 g for 10 min at 4 °C, and the supernatant was used as the post-nuclear supernatant. The post-nuclear supernatant was centrifuged further at 100,000 g for 1 h at 4 °C to separate the cytosolic fraction

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