Cellular Signalling 23 (2011) 389–397

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Expression of $G\alpha_z$ in C2C12 cells restrains myogenic differentiation

Hua Mei, Maurice K.C. Ho, Lisa Y. Yung, Zhenguo Wu, Nancy Y. Ip, Yung H. Wong *

Section of Biochemistry and Cell Biology, Division of Life Science, Biotechnology Research Institute, Molecular Neuroscience Center, The State Key Laboratory of Molecular Neuroscience, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

ARTICLE INFO

Article history:
Received 9 August 2010
Received in revised form 16 September 2010
Accepted 1 October 2010
Available online 12 October 2010

Keywords:
Heterotrimeric G protein
G_z
Myogenesis
Reporter gene assay

ABSTRACT

The recent identification of $G\alpha_z$ expression in C2C12 myoblasts and its demonstrated interaction with the transcription factor Eya2 inferred an unanticipated role of $G\alpha_z$ in muscle development. In the present study, endogenous $G\alpha_z$ mRNA and protein expressions in C2C12 cells increased upon commencement of myogenesis and peaked at around 4–6 days after induction but were undetectable in adult skeletal muscle. Surprisingly, stable expression of recombinant $G\alpha_z$ in C2C12 myoblasts strongly suppressed myotube formation upon serum deprivation, and the constitutively active mutant $G\alpha_z$QL exerted more pronounced effects. Transcriptional activities of reporter genes responsive to early (MyoD, MEF2 and myogenin) and late (muscle creatine kinase and myosin heavy chain) myogenic markers were reduced by transiently expressed $G\alpha_z$QL. Membrane attachment of $G\alpha_z$ was apparently required for the suppressive effects because a fatty acylation-deficient $G\alpha_z$ mutant could not inhibit myogenin expression. Introduction of siRNA against $G\alpha_z$ enhanced myogenin-driven luciferase activity and increased myosin heavy chain expression. Immunostaining of C2C12 cells over-expressing $G\alpha_z$ showed delayed nuclear expression of myogenin and severe myotube deformation. $G\alpha_z$ expression was accompanied by reduced levels of Rock2, RhoA and RhoGAP, enhanced expression of Rnd3, and a reduction of serum-responsive factor-driven reporter activity. These results support a novel role of $G\alpha_z$ in restraining myogenic differentiation through the disruption of Rho signaling.

1. Introduction

Heterotrimeric G proteins are crucial components in signal transduction that modulate a wide spectrum of cellular events, including complex processes such as cell proliferation and differentiation. For myogenesis, several G protein subunits (e.g., $G\alpha_s$ and $G\beta\gamma$) participate in the development of both skeletal and smooth muscles [1–3]. Myogenic differentiation is primarily regulated by two families of transcription factors: myogenic regulatory factors (MRFs such as MyoD and myogenin) and myocyte enhancer factor 2 (MEF2) [4]. Their transcriptional activities are enhanced by other signaling molecules such as the p38 mitogen-activated protein kinase (MAPK) [5] which can be regulated by many G protein-coupled receptors (GPCRs). GPCR signals are likely to modulate myogenic differentiation since their expression in myoblasts decreases upon the formation of multinucleated myotubes [6]. Indeed, several GPCRs including such as thrombin can inhibit the process of myotube formation [7].

Some GPCRs expressed in skeletal muscle can interact with $G\alpha_z$ [8]. $G\alpha_z$ is a unique member of the $G\alpha_i$ family with extremely slow GTP hydrolysis rate [9]. A recent study has implicated the involvement of $G\alpha_z$ in regulating the function of transcription cofactor Eya2 [10]. Eya2 associates and cooperates with the homeodomain-containing Six1/4 to activate the expressions of myogenin and other MEF3-containing genes [11]. Interaction with $G\alpha_z$ prevents Eya2 from binding to Six1/4 and may therefore inhibit muscle differentiation. Alternatively, since $G\alpha_z$ can inhibit the transcriptional activity of serum responsive factor (SRF) by disrupting RhoA signaling [12], it may suppress SRF-induced MyoD expression. Despite its predominant expression in neuronal tissues [13], $G\alpha_z$ is endogenously expressed in mouse C2C12 myoblasts and can be rapidly targeted to the membrane upon stimulation by neuregulin [14]. Here, we attempted to elucidate the role of $G\alpha_z$ in myogenesis of C2C12 myoblasts by over-expressing or suppressing $G\alpha_z$, and then examining their effects on the expressions of myogenic markers and myotube morphology.

Abbreviations: AC, adenylyl cyclase; PBS-CMF, Ca^{2+}/Mg^{2+} free phosphate-buffered saline; DM, differentiation medium; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; GST, glutathione S-transferase; GM, growth medium; MAPK, mitogen-activated protein kinase; MCK, muscle creatine kinase; MEF2s, myocyte enhancer factor 2 s; MRFs, myogenic regulatory factors; Myog, myogenin; MHC, myosin heavy chain; RBD, Rho binding domain; Rock2, Rho-associated, coiled-coil containing protein kinase 2; SRF, serum responsive factor.

* Corresponding author. Section of Biochemistry and Cell Biology, Division of Life Science, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China. Tel.: +852 2358 7328; fax: +852 2358 1552.

E-mail addresses: janemei@ust.hk (H. Mei), bokcmho@ust.hk (M.K.C. Ho), bolisa@ust.hk (L.Y. Yung), bczgwu@ust.hk (Z. Wu), boip@ust.hk (N.Y. Ip), boyung@ust.hk (Y.H. Wong).

0898-6568/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2010.10.009

2. Materials and methods

2.1. Materials

Plasmid encoding glutathione S-transferase (GST)-fused Rhotekin-Rho binding domain (RBD) was kindly donated by Xiangdong Ren (Department of Dermatology, State University of New York at Stony Brook). C2C12 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Antisera against specific targets were obtained from: $G\alpha_z$ from Calbiochem (San Diego, CA, USA) as well as Hermann Ammer (Department of Veterinary Sciences, University of Munich, Germany), myogenin (F5D) and myosin heavy chain (MF20) from Developmental Studies Hybridoma Bank (DSHB; Iowa City, IA, USA), Rho (clone 55), p190RhoGAP (clone D2D6) and Rnd3 (clone 4) from Millipore (Billerica, MA, USA), Rock2 from ECM Biosciences (Versailles, KY, USA). Reagents for cell culture, transfection, total RNA extraction, cDNA synthesis and quantitative PCR, as well as gene-specific primers and siRNAs for $G\alpha_z$ knock-down (Gnaz Stealth™ Select RNAi: siRNA1 — MSS247236 and siRNA2 — MSS247235) were purchased from Life Technologies (Carlsbad, CA, USA). One-step reverse transcription-PCR reagent was obtained from Promega (Madison, WI, USA). The luciferin substrate kit was from Roche Applied Science (Mannheim, Germany). Glutathione Sepharose 4B was from GE Healthcare (Piscataway, NJ, USA). Other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Cell culture and transfection

C2C12 cells were maintained in the Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) (growth medium, GM), and the cells were grown at 37 °C in an environment of 5% CO_2. Cells were seeded into 12-well plates at a density of 4×10^4 cells/well. After one day incubation in GM, C2C12 cells were transfected with various plasmids using LipofectAMINE™ PLUS™ reagents (Life Technologies). After 6–10 h incubation, the transfection mixture was replaced with fresh GM. For the introduction of siRNAs or combinations of siRNAs and DNAs, C2C12 cells were seeded into 12-well plates at a density of 3.5×10^4 cells/well. After one day incubation in GM, C2C12 cells were transfected using LipofectAMINE™ 2000 reagents. The transfection mixture was replaced with fresh GM 4–6 h later. Transfectants were allowed to grow for 20 h before incubation in DMEM containing 2% horse serum (differentiation medium, DM). The cells were harvested 24 h later for luciferase assays.

2.3. Luciferase assay

Cell lysates were prepared from transfectants using the luciferase reporter gene assay kit (Roche Applied Science). 25 μl of cell lysates was loaded into 96-well plates and the luciferase activity was determined using a microplate luminometer LB96V (EG&G Berthold, Germany). Injector M connected to lysis buffer and injector P connected to the luciferin substrate were set to inject 25 μl of each component into each well. A 1.6-s delay time followed by a 2-s measuring time period was assigned to injector M, whereas injector P was measured for 10 s after the luciferin was introduced into the well. Results were collected by WinGlow version 1.24 and expressed as relative luminescence units. Statistical calculation was performed using GraphPad Prism 3.

2.4. PCR-based quantitation of mRNA expression

Total RNAs were extracted from C2C12 cells by TRIzol (Life Technologies) using manufacturer's protocol and reconstituted in 1.0 μg/μl with nuclease-free water. For semi-quantitative reverse transcription-PCR, 2 μg of total RNA and 0.6 μM of each gene-specific primer were used in each 50 μl reaction of AccessQuick™ RT-PCR system (Promega). Reverse transcription was firstly performed at 45 °C for 45 min, then proceeded to PCR with conditions as: 95 °C for 2 min (1 cycle); 95 °C for 30 s, 50 °C for 45 s and 72 °C for 30 s (42–47 cycles); 72 °C for 5 min (1 cycle). 10 μl of the products were loaded onto agarose gels for quantification. Gene-specific primers used were Gnaz-F: GGGCATTGTGGAGAACAAGT, Gnaz-R: TGTCACTGCGTCAAACA-CAA, Myog-F: GAAAGTGAATGAGGCCTTCG, Myog-R: ACGATGGACG-TAAGGGAGTG, Actb-F: GAGAGGTATCCTGACCCTGAAGTA, Actb-R: TGTTGAAGGTCTCAAACATGATCT (F and R meant forward and reversed primers, respectively). For quantitative PCR analysis of RhoA-related genes, 2.5 μg of total RNA extracted from transfected C2C12 cells were reverse-transcribed into cDNA using SuperScript® III First-Strand Synthesis System (Life Technologies) following the manufacturer's protocol. 0.25–0.50 μl of cDNA samples were used in quantitative PCR using SYBR® GreenER™ qPCR SuperMixes Universal kit (Life Technologies) and the reactions were performed in the Applied Biosystems 7500 Fast Real-Time PCR System using the standard mode with 10 μl reaction volume. Gene-specific primers used were RhoA-F: ACAAGCGTCCTCAGCG, RhoA-R: GTCTTACCACAAGCTCCATC, Rnd3-F: GCTTCCCAGAAAATTACGTCC, Rnd3-R: GAGTCTCTGGTCTACTGATGT, Rock2-F: GAAATGGGTTAGTCGGTTGG, Rock2-R: ACGATCACCTTCAA-TAACTGC, RhoGAP-F: GGAAGCTCACAAAATCAACG, RhoGAP-R: AGAAG-CAGATGGACAGGT, GAPDH-F: CCCACTCTTCCACCTTCG, GAPDH-R: TCCTTGGAGGCCATGTAGGCCAT, Myog-F(qPCR): CAGGAGAT-CATTTGCTCG, Myog-R(qPCR): GGGCATGGTTTCGTCTGG.

2.5. Preparation of total cell extracts and crude membrane fractions

C2C12 cells were washed with Ca^{2+}/Mg^{2+} -free phosphate-buffered saline (PBS-CMF) and harvested with PBS-CMF containing 10 mM EDTA at 4 °C. After centrifugation at 200 ×*g*, cell pellets were resuspended in total cell lysis buffer (50 mM HEPES, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 1% Triton X-100, 0.1% SDS, 10% glycerol, 1 mM sodium pyrophosphate and EDTA-free Complete protease inhibitor cocktail (Roche Applied Science)) and vigorously shaken on the vortex mixer for 15 min. Cell debris was spun down at 14,000 rpm for 10 min, and the supernatant was retained as the total cell extract. For membrane protein preparations, C2C12 cells were washed with PBS-CMF and harvested with PBS-CMF containing 10 mM EDTA at 4 °C. After pelleting, cells were resuspended in hypotonic lysis buffer (50 mM Tris–HCl, pH 7.5, 2.5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, 1 mM dithiothreitol) and homogenized by 10 passages through a 27-gauge needle. Nuclei were removed by low-speed centrifugation (3200 rpm, 5 min), and the supernatants were retained. The membrane fractions were collected by centrifugation at 14,000 rpm for 10 min.

2.6. Immunostaining

For immunostaining of myosin heavy chain, C2C12 cells were grown on cover slips coated with 0.2% gelatin, fixed by 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized by 0.2% Triton X-100 in PBS for 15 min. After blocking with 5% BSA in PBS for 1 h, the cells were incubated overnight at 4 °C with the indicated primary antibodies followed by 1 h of incubation with FITC- or Alexa Fluor® dye-conjugated anti-mouse or rabbit secondary antibodies (1:1000; GE Biosciences/Life Technologies). After washing with PBS, the cover slips were mounted with MOWIOL® 4-88 (EMD Chemicals) or antifading polyvinyl alcohol mounting medium (Fluka #10981). The cells were observed with a Nikon Eclipse TE2000 or Olympus IX81 inverted fluorescence microscopes. Pictures were taken by monochrome low noise cooled CCD camera SPOT-RT (Diagnostic Instruments, Sterling Heights, MI, USA) controlled by MetaMorph imaging acquisition software (Universal Imaging, West Chester, PA, USA), or DP30BW (Olympus, Tokyo, Japan) controlled by the bundled software DP-BSW.