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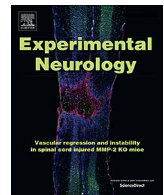

Research Paper

Roles of Cdc42 and Rac in Bergmann glia during cerebellar corticogenesis

Isao Sakamoto[a,1], Takehiko Ueyama[a,*,1], Masakazu Hayashibe[a], Takashi Nakamura[a], Hiroaki Mohri[a], Hiroshi Kiyonari[b], Michiko Shigyo[c], Chihiro Tohda[c], Naoaki Saito[a,*]

[a] Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, Kobe 657-8501, Japan

[b] Animal Resource Development Unit and Genetic Engineering Team, RIKEN Center for Life Science Technologies, Kobe 650-0047, Japan

[c] Division of Neuromedical Science, Department of Bioscience, Institute of Natural Medicine, University of Toyama, Toyama 930-0194, Japan

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ABSTRACT

Bergmann glia (BG) are important in the inward type of radial migration of cerebellar granule neurons (CGNs). However, details regarding the functions of Cdc42 and Rac in BG for radial migration of CGN are unknown. To examine the roles of Cdc42 and Rac in BG during cerebellar corticogenesis, mice with a single deletion of Cdc42 or Rac1 and those with double deletions of Cdc42 and Rac1 under control of the glial fibrillary acidic protein (GFAP) promoter: *GFAP-Cre*;*Cdc42*$^{flox/flox}$ (Cdc42-KO), *GFAP–Cre*;*Rac1*$^{flox/flox}$ (Rac1-KO), and *GFAP-Cre*; *Cdc42*$^{flox/flox}$;*Rac1*$^{flox/flox}$ (Cdc42/Rac1-DKO) mice, were generated. Both Cdc42-KO and Rac1-KO mice, but more obviously Cdc42-KO mice, had disturbed alignment of BG in the Purkinje cell layer (PCL). We found that Cdc42-KO, but not Rac1-KO, induced impaired radial migration of CGNs in the late phase of radial migration, leading to retention of CGNs in the lower half of the molecular layer (ML). Cdc42-KO, but not Rac1-KO, mice also showed aberrantly aligned Purkinje cells (PCs). These phenotypes were exacerbated in Cdc42/Rac1-DKO mice. Alignment of BG radial fibers in the ML and BG endfeet at the pial surface of the cerebellum evaluated by GFAP staining was disturbed and weak in Cdc42/Rac1-DKO mice, respectively. Our data indicate that Cdc42 and Rac, but predominantly Cdc42, in BG play important roles during the late phase of radial migration of CGNs. We also report here that Cdc42 is involved in gliophilic migration of CGNs, in contrast to Rac, which is more closely connected to regulating neurophilic migration.

1. Introduction

Astrocytes play important roles in the establishment and maintenance of numerous brain functions, including control of the blood-brain barrier; regulation of blood flow; supply of energy metabolites to neurons; synaptic function; extracellular balance of ions, fluids, and transmitters (Pekny et al., 2016). Astrocytes also play pivotal roles in corticogenesis/lamination of the cerebrum (Borrell and Gotz, 2014) and cerebellum during development (Buffo and Rossi, 2013), when they exist as radial glia (RG) and Bergmann glia (BG). RG function as progenitor cells giving rise to both neurons and glial cells, but also exert scaffolding activity for migrating neurons during corticogenesis (Borrell and Gotz, 2014). BG derive directly from morphological transformation of RG and are unique astrocytes in the cerebellum that play essential roles in cerebellar corticogenesis (Buffo and Rossi, 2013).

The cerebellar cortex consists of three layers: the molecular layer (ML), the Purkinje cell layer (PCL), and the internal granule layer (IGL) (Chedotal, 2010). BG are located at the PCL and extend their radial processes (also referred to as BG fibers or BG radial fibers) across the ML. These processes terminate at the pial surface of the cerebellum in endfeet (Yamada and Watanabe, 2002), and are involved in the development of the IGL and the maturation of Purkinje cells (PCs) (Buffo and Rossi, 2013; Xu et al., 2013). Cerebellar granule neurons (CGNs) proliferating and differentiating in the EGL begin to inwardly and radially migrate to form the IGL. This is a characteristic phenomenon of cerebellar corticogenesis (Chedotal, 2010). This inward type of radial migration begins at birth and is completed by P20 in mice (Hatten et al., 1997). This process peaks between P7 and P12 (Komuro et al., 2001). In contrast, PCs originate at the ventricular zone at E12, outwardly migrate to the PCL from E13 to E17, and establish monolayer alignment by P7 (Yamada and Watanabe, 2002; Yuasa et al., 1991). BG are reported to be involved in the inward type of radial migration (Rakic, 1971). However, the detailed functional mechanisms of BG during radial migration of CGNs are still unclear.

Cdc42 and Rac (Rac1, Rac2, and Rac3) are the best characterized members of the Rho family of small guanosine triphosphatases

* Corresponding authors at: 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan.
E-mail addresses: tueyama@kobe-u.ac.jp (T. Ueyama), naosaito@kobe-u.ac.jp (N. Saito).

[1] These authors contributed equally to this work.

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(GTPases), which play fundamental roles in a wide variety of cellular processes, including establishment/maintenance of actin-based cytoskeleton and neuronal polarity (Bosco et al., 2009; Takano et al., 2015). We recently described the functions of Rac in CGNs during cerebellar corticogenesis using CGN-specific Rac1/Rac3 double-knockout (DKO) mice, which show severely impaired radial migration of CGNs during the early phase and have defects in the IGL (Nakamura et al., 2017). However, the roles and functions of Cdc42 and Rac in BG during cerebellar corticogenesis remain unknown.

To examine the roles of Cdc42 and Rac in BG during cerebellar corticogenesis (radial migration of CGNs during cerebellar development), we generated mice with deletions of Cdc42, Rac1, or Cdc42 and Rac1 under control of the glial fibrillary acidic protein (GFAP) promoter: *GFAP–Cre*;*Cdc42$^{flox/flox}$* (Cdc42-KO), *GFAP–Cre*;*Rac1$^{flox/flox}$* (Rac1-KO), and *GFAP–Cre*;*Cdc42 $^{flox/flox}$*;*Rac1$^{flox/flox}$* (Cdc42/Rac1-DKO). Here, we report that Cdc42-KO, but not Rac1-KO, mice have impaired radial migration of CGNs during the late phase of the radial migration of CGNs, which leads to the retention of CGNs in the inferior half of the ML.

2. Materials and methods

2.1. Animals

All animal experiments were conducted in accordance with the guidelines of Kobe University and RIKEN. The *Cdc42^{flox}* (Ueyama et al., 2014) and *GFAP-Cre*;*Rac1^{flox}* (Ishii et al., 2016) mice have been described previously. The *GFAP-Cre*;*Cdc42$^{flox/+}$* progeny of *Cdc42$^{flox/flox}$* and *GFAP-Cre* mice (Bajenaru et al., 2002) were back-crossed with *Cdc42$^{flox/flox}$* mice to obtain *GFAP-Cre*;*Cdc42$^{flox/flox}$* (hereafter referred to as Cdc42-KO) mice. These mice were in turn backcrossed to *Cdc42$^{flox/flox}$* mice to generate experimental animals. *GFAP-Cre*;*Rac1$^{flox/flox}$* (hereafter referred to as Rac1-KO) mice were backcrossed with *Rac1$^{flox/flox}$* mice to generate experimental animals. The *GFAP-Cre*;*Cdc42$^{flox/+}$*;*Rac1$^{flox/+}$* progeny of *GFAP-Cre*;*Cdc42$^{flox/flox}$* and *Rac1$^{flox/flox}$* mice were back-crossed with *Cdc42$^{flox/flox}$*;*Rac1$^{flox/flox}$* mice to obtain *GFAP-Cre*;*Cdc42$^{flox/flox}$*;*Rac1$^{flox/flox}$* (hereafter referred to as Cdc42/Rac1-DKO) mice. These mice were in turn backcrossed with *Cdc42$^{flox/flox}$*;*Rac1$^{flox/flox}$* mice to generate experimental animals. *CAG-STOP^{flox}-tdTomato* (Ai9) reporter mice were purchased from the Jackson Laboratory and backcrossed with *GFAP-Cre* mice to generate *GFAP-Cre*;*tdTomato* mice, which were then used to examine the effect of the GFAP promoter. The offspring of these mice were genotyped by polymerase chain reaction (PCR) using the following primers: 5′-ACTCCTTCATAAAGCCCTCG-3′ (forward) and 5′-ATCACTCGTTGCATCGACCG-3′ (reverse) for *GFAP-Cre*, 5′-ATCGGTCACTGTTCTACTTTG-3′ and 5′-TACTGCTATGACTGAAAACCTC-3′ for Cdc42^{flox}, 5′-ATTTTCTAGATTCCACTTGTGAAC-3′ and 5′-ATCCCTACTTCCTTCCAACTC-3′ for *Rac1^{flox}*, and 5′-GGCATTAAAGCAGCGTATCC-3′ and 5′-CTGTTCCTGTACGGCATGG-3′ for *tdTomato*. Wild type (WT) C57BL/6 mice were purchased from Clea Japan.

2.2. Antibodies

The following specific antibodies (Abs) were used (monoclonal unless indicated): anti-Cdc42 (44/CDC42, BD Biosciences, 1/500); anti-Rac1 (23A8, Millipore, 1/500); polyclonal anti-GFAP (Z0334, DAKO, 1/1000); anti-Cre recombinase (2D8, Millipore, 1/1000); polyclonal anti-calbindin (AB1778, Millipore, 1/2000); polyclonal anti-PKCγ (sc-211, Santa Cruz Biosciences, 1/2000); anti-NeuN (A60, Millipore, 1/100); polyclonal anti-BLBP (BLBP-Rb-Af400-1, Frontier Institute Co., Ltd., Japan, 1/200); polyclonal anti-active caspase-3 (G7481, Promega, 1/250), anti-EGFR (D38B1, Cell Signaling Technology (CST), 1/1000), anti-ErbB2 (D8F12, CST, 1/500), anti-FGFR1 (D8E4, CST, 1/1000), anti-FGFR3 (EPR2304(3), Abcam, 1/1000), polyclonal anti-neuregulin 1 type III (ab23248, Abcam, 1/200), polyclonal anti-Notch3 (ab23426, Abcam, 1/200), and anti-glutamine synthetase (GS, GS-6, Millipore, 1/100). Alexa488- and Alexa564-conjugated secondary Abs were obtained from Invitrogen. Horseradish peroxidase (HRP)-conjugated anti-tubulin-α Abs were obtained from MBL International (Japan).

2.3. Cell culturing and RNA interference

Primary astrocyte cultures were prepared from mouse cerebral cortex at postnatal day 1 or 2 (P1–2), as previously described (Ishii et al., 2016). Briefly, dissected cerebral cortices were dissociated using a neuron dissociation solution (Wako Pure Chemical Industries, Japan), and cultured in Eagle's Minimum Essential Medium (Wako) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Japan) and penicillin-streptomycin (PS) solution (Wako) in 25-cm^2 flasks (2 brains per flask) (Corning Inc.). After 5–7 days, the flasks were subjected to 1 h of continuous shaking to obtain purified astrocytes. Cell lysates were used for immunoblotting experiments and DNA microarray analysis.

U-87 MG astrocytic cells (ATCC) were maintained in DMEM (Wako) containing 10% FBS and PS. The validated RNAi sequences for *RAC1* (siRAC1-618: 5′-CCTTTGTACGCTTTGCTCA-3′ (Ishii et al., 2016; Ueyama et al., 2006)) and *CDC42* (siCDC42-197: 5′-GATTACGACCGCTGAGTTA-3′ (Qin et al., 2010; Ueyama et al., 2014)) have been previously described. The siRNAs for *RAC1*, *CDC42*, and a control siRNA (MISSION Universal Negative Control) were purchased from Sigma-Aldrich. The siRNAs were transfected into U87 cells using RNAiMAX (Invitrogen), and the cells were used for experiments 48 h after transfection.

MDCK cells with stable expression of the short hairpin RNA (shRNA) expression plasmid (*pSUPER*, OrigoEngine; MDCK^{cont}) or the shRNA expression plasmid containing a *Cdc42*-targeting sequence (MDCK$^{Cdc42\text{-}KD}$) were established as described previously (Ueyama et al., 2014), and maintained in DMEM containing 10% FBS, PS, and 0.3 mg/ml G418 solution (Wako).

All cells were maintained in a 5% CO_2 humidified incubator at 37 °C.

2.4. Reverse transcriptase PCR

Reverse transcriptase PCR (RT-PCR) was performed using 1 μg of total RNA obtained from the primary astrocytes of WT mice using SuperScript III reverse transcriptase (Invitrogen) and random primers. The following primer pairs were used for PCR (30 cycles): 5′-GCAGACAGACGTGTTCTTAATTTGC-3′ and 5′-TGTAACAAAAACTTGGCATCAAATGCG-3′ for *Rac1* (454 bp), 5′-CCCACACACACCCATCCTTC-3′ and 5′-TGGAGCTATATCCCAGAAAAAGGAG-3′ for *Rac3* (440 bp), and 5′-GAAATGCAGACAATTAAGTGTGTTGTTG-3′ and 5′-TCATAGCAGCACACACCTGCGGCT-3'for *Cdc42* (579 bp).

2.5. Immunoblotting

Cells were lysed in homogenizing buffer (Ueyama et al., 2007) by sonication in the presence of a protease inhibitor cocktail, a protein phosphatase inhibitor cocktail (Nacalai Tesque, Japan), and 1% Triton X-100. Total lysates were centrifuged at 800 × *g* for 5 min at 4 °C, and the supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting for 16 h at 4 °C using primary Abs diluted in phosphate buffered saline (PBS) containing 0.03% Triton X-100 (PBS-0.03T). The bound primary Abs were detected using secondary Ab-HRP conjugates using the ECL detection system (Bio-Rad Labolatories).

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