EXPERIMENTAL CELL RESEARCH 316 (2010) 477–490

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr

Research Article

M-Ras is activated by bone morphogenetic protein-2 and participates in osteoblastic determination, differentiation, and transdifferentiation

*Haruko Watanabe-Takano, Kazunori Takano, Etsuko Keduka, Takeshi Endo**

Department of Biology, Graduate School of Science, Chiba University, 1-33 Yayoicho, Inageku, Chiba, Chiba 263-8522, Japan

ARTICLE INFORMATION

Article Chronology:
Received 20 July 2009
Revised version received
26 September 2009
Accepted 28 September 2009
Available online 2 October 2009

Keywords:
M-Ras
Osteoblast differentiation
Transdifferentiation
BMP-2
p38 MAPK
JNK

ABSTRACT

The small GTPase M-Ras is highly expressed in the central nervous system and plays essential roles in neuronal differentiation. However, its other cellular and physiological functions remain to be elucidated. Here, we clarify the novel functions of M-Ras in osteogenesis. M-Ras was prominently expressed in developing mouse bones particularly in osteoblasts and hypertrophic chondrocytes. Its expression was elevated in C3H/10T1/2 (10T1/2) mesenchymal cells and in MC3T3-E1 preosteoblasts during differentiation into osteoblasts. Treatment of C2C12 skeletal muscle myoblasts with bone morphogenetic protein-2 (BMP-2) to bring about transdifferentiation into osteoblasts also induced M-Ras mRNA and protein expression. Moreover, the BMP-2 treatment activated the M-Ras protein. Stable expression of the constitutively active M-Ras(G22V) in 10T1/2 cells facilitated osteoblast differentiation. M-Ras(G22V) also induced transdifferentiation of C2C12 cells into osteoblasts. In contrast, knockdown of endogenous M-Ras by RNAi interfered with osteoblast differentiation in 10T1/2 and MC3T3-E1 cells. Osteoblast differentiation in M-Ras(G22V)-expressing C2C12 cells was inhibited by treatment with inhibitors of p38 MAP kinase (MAPK) and c-Jun N-terminal kinase (JNK) but not by inhibitors of MAPK and ERK kinase (MEK) or phosphatidylinositol 3-kinase. These results imply that M-Ras, induced and activated by BMP-2 signaling, participates in the osteoblastic determination, differentiation, and transdifferentiation under p38 MAPK and JNK regulation.

Introduction

The Ras family of small GTPases performs crucial roles in a variety of cellular functions, including cell proliferation, differentiation, survival, and transformation, as well as physiological and pathological functions [1,2]. The family comprises as many as 36 genes in human [3]. However, each member seems to possess specific functions. M-Ras was first cloned from mouse C2C12 myoblast and rat brain cDNA libraries and is highly expressed in the central nervous system [4,5]. Although M-Ras is phylogenetically relatively close to the classical Ras (H-Ras, K-Ras, and N-Ras) and R-Ras subfamilies, it evolved independently of classical Ras, R-Ras, and the other members of the family in metazoans [6]. This is consistent with the fact that M-Ras has specific amino acids in the extended effector domains in addition to throughout the whole sequence, as compared with the other members [4,6].

Classical Ras has multiple effector proteins including Raf (A-Raf, B-Raf, and C-Raf), phosphatidylinositol 3-kinase (PI3K) and Ral guanine nucleotide exchange factors (GEFs) [7]. Constitutively activated oncogenic Ras causes cellular transformation through the activation of these effector proteins. M-Ras shares several effector proteins with classical Ras and possesses specific effectors as well [8–12]. Neuronal differentiation in PC12 cells induced by nerve growth factor (NGF) requires sustained activation of the

* *Corresponding author.* Fax: +81 43 290 3911.
E-mail address: t.endo@faculty.chiba-u.jp (T. Endo).

0014-4827/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2009.09.028

extracellular signal-regulated kinase (ERK) pathway (Raf–MEK–ERK cascade) [13,14]. M-Ras activated by NGF signaling is responsible for this sustained activation of the ERK pathway, resulting in the neuronal differentiation, whereas classical Ras activates the ERK pathway only transiently [5]. M-Ras also participates in dendrite formation in rat cortical neurons by activating the ERK pathway [15]. On the other hand, M-Ras activated by tumor necrosis factor-α (TNF-α) activates Rap1, leading to lymphocyte adhesion [16]. Other cellular or physiological functions of endogenous M-Ras and the signaling mechanisms involved remain to be elucidated, although exogenously expressed constitutively active M-Ras exerts several cellular functions, including the determination of particular cell lineage from hematopoietic stem cells [17–20].

Bone morphogenetic proteins (BMPs) are involved in almost all processes associated with skeletogenesis [21,22]. Various BMPs, such as BMP-2, -4, and -7, bring about the determination of mesenchymal progenitor cells to osteoblastic lineage cells (preosteoblasts), differentiation of the preosteoblasts into osteoblasts, maturation of the differentiated osteoblasts, and apoptosis as a result of maturation. In addition, they regulate chondrocyte differentiation and chondrogenesis and play direct and indirect roles in osteoclastogenesis during skeletal development. BMP signaling is mediated by receptor-regulated R-Smad transcription factors, Smad1/5/8, and a common-mediator Co-Smad, Smad4. Although the Smad pathway is a well-characterized BMP signaling pathway, BMPs also induce other signaling pathways. Several lines of evidence indicate that BMPs activate multiple MAP kinase (MAPK) signaling pathways: ERK, p38 MAPK, and JNK pathways [23,24]. BMP signaling induces Runx2 and osterix (Osx), transcription factors essential for osteogenesis. Runx2 directs multipotent mesenchymal cells to an osteoblastic lineage and differentiation of preosteoblasts to osteoblasts [25–27], whereas Osx is required for osteoblast differentiation and maturation represented by calcification [28]. The p38 MAPK and JNK pathways, activated through BMP signaling, are involved in osteoblast differentiation in cooperation with BMP–Smad signaling by activating Runx2 and Osx [29–31]. However, the signaling pathways linking the BMP receptor to the p38 MAPK or JNK pathway and those connecting Smads, p38 MAPK, or JNK to Runx2 or Osx remain obscure. In addition, the involvement of other unidentified signaling pathways that regulate osteoblast differentiation in cooperation with BMP signaling cannot be ruled out.

We found that M-Ras was prominently expressed in developing mouse bones and that its expression was induced during osteoblast differentiation. In addition, M-Ras was activated by BMP-2 stimulation. Thus, we examined the role of M-Ras in osteogenesis. Forced expression and knockdown of *Mras* revealed that M-Ras is involved in multiple steps of osteogenesis, i.e., osteoblastic determination, differentiation, and maturation. M-Ras also has the potential to cause transdifferentiation of myoblasts into osteoblasts. The M-Ras-induced osteogenesis was mediated by the activation of p38 MAPK and JNK.

Materials and methods

Cell culture

Mouse C3H/10T1/2 (10T1/2) mesenchymal progenitor cells [32] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS [10T1/2 growth medium (GM)]. To induce osteoblast differentiation, 1.5×10^5 cells were cultured on a 35-mm dish for 48 h in the growth medium and then cultured in growth medium containing 50 μM L-ascorbic acid and 10 mM β-glycerophosphate (Ost DM) for 8 days by replacing the medium every 48 h. Recombinant human BMP-2 was provided by Astellas Pharma Inc. The same number of cells cultured in the growth medium was transferred to DMEM supplemented with 5% FBS and 0.1 μg/ml BMP-2 and cultured for 3 days by replacing the medium every 24 h. To induce adipocyte differentiation, the same number of cells cultured in the growth medium was cultured for 48 h in growth medium containing 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1 mM indomethacin, and 1 μM dexamethasone, and then for 24 h in growth medium containing 10 μg/ml insulin. This cycle of replacing the medium was repeated thrice. Mouse MC3T3-E1 (MC3T3) preosteoblasts [33] were obtained from RIKEN BRC Cell Bank and were maintained in the alpha modification of minimum essential medium (α-MEM) supplemented with 10% FBS (MC3T3 GM). Mouse C2C12 myoblasts [34,35] were maintained in DMEM supplemented with 10% FBS (C2 GM). To induce osteoblast or adipocyte differentiation in MC3T3 or C2C12 cells, the cells were treated in a similar manner as were the 10T1/2 cells.

To determine signaling pathways involved in osteoblast differentiation, the following kinase inhibitors were added to Ost DM at final concentrations of 10 μM: MEK inhibitor U0126 (Promega), PI3K inhibitor LY294002 (Promega), p38 MAPK inhibitor SB202190 (Sigma), and JNK inhibitor SP600125 (Sigma).

Detection of differentiated state

To detect alkaline phosphatase (ALP) activity cytochemically, cells were fixed in 4% paraformaldehyde in PBS containing 2 mM $MgCl_2$ for 15 min, left in ALP buffer (100 mM NaCl, 50 mM $MgCl_2$, and 100 mM Tris–HCl, pH 9.5) for 15 min, incubated in ALP buffer containing 0.1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and 1 mg/ml nitro blue tetrazolium chloride for 15–60 min, and then washed three times with water. Biochemical determination of ALP activity was conducted with LabAssay ALP (Wako Pure Chemical Industries). Protein concentration was determined by the method of Bradford. To detect calcification by von Kossa staining, formalin-fixed cells were incubated in 5% $AgNO_3$, irradiated with ultraviolet light for 60 min, and treated with 5% Na_2SO_3 for 2 min. To detect adipocyte differentiation, formalin-fixed cells were stained with 3% Oil Red O in 60% isopropanol for 10 min.

Immunoblotting

Anti-rat M-Ras polyclonal antibody (pAb) [5] was absorbed with H-Ras protein and affinity-purified with M-Ras through Formyl-Cellulofine (Seikagaku Corp.). Cultured cells were washed with PBS and lysed with a lysis buffer (1% Nonidet P-40, 5% glycerol, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 μg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml pepstatin A, 10 mM Na_3VO_4, and 10 mM NaF). These samples were subjected to SDS-PAGE, and immunoblotting was carried out