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RANKL-induced schlafen2 is a positive regulator of osteoclastogenesis

Na Kyung Lee [a,1], Han Kyung Choi [a], Hyun Joo Yoo [a], Jihye Shin [a], Soo Young Lee [a,b,*]

[a] Division of Life and Pharmaceutical Sciences, Center for Cell Signaling and Drug Discovery Research, Ewha Womans University, Seoul 120-750, Republic of Korea
[b] Department of Life Science, College of Natural Sciences, Ewha Womans University, Seoul 120-750, Republic of Korea

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ABSTRACT

Osteoclasts are hematopoietic lineage derived-multinucleated cells that resorb bone. Their activity in balance with that of osteoblast is essential for bone homeostasis. Receptor activator of NF-κB ligand (RANKL) is known as an essential cytokine for the osteoclastogenesis, and c-Jun signaling in cooperation with NFAT family is crucial for RANKL-regulated osteoclastogenesis. We show here that schlafen2 (Slfn2), a member of a new family of growth regulatory genes involved in thymocyte development, is critical for osteoclastogenesis. RANKL selectively induces Slfn2 expression in osteoclast precursors via Rac1 signaling pathway. Targeted inhibition of Slfn2 by small interfering RNAs (siRNAs) markedly inhibits the formation of osteoclasts by diminishing the activation of c-Jun and the expression of c-Jun and NFATc1. In contrast, the overexpression of Slfn2 markedly increased phosphorylation and transactivation of c-Jun by RANKL. Together, these results indicate that Slfn2 has an essential role in osteoclastogenesis, functioning upstream of c-Jun and NFATc1.

1. Introduction

Bone remodeling is controlled by the balanced action of osteoblastic bone formation and osteoclastic bone resorption [1]. Osteoclasts differentiate from cells of the monocyte/macrophage lineage to become multinuclear, tartrate-resistant acid phosphatase (TRAP)-positive cells capable of resorbing bone [2]. A key factor responsible for initiating this differentiation process is a receptor activator of NF-κB (RANK) ligand (RANKL; also known as TRANCE/OPGL/ODF), a member of the tumor necrosis factor superfamily, expressed in stromal cells/osteoblasts and activated T cells [3]. The binding of RANKL to the cell surface receptor called RANK, expressed in osteoclast precursors, leads to recruitment of TNF receptor-associated factor (TRAF)s thereby causing the activation of NF-κB [4], mitogen-activated protein kinases (MAPKs) [5], and Src kinase [6]. During the differentiation process of macrophages/monocytes to osteoclasts with bone-resorbing activity, transcription factors such as c-Fos, NF-κB, MITF, NFATc1, and CREB play critical and specific roles [3,7–9]. All of these transcription factors function downstream of RANKL or M-CSF signaling, and there are genetic evidences that each of them is involved at distinct steps of osteoclast differentiation [10–14]. From a study utilizing mice carrying a mutant of c-Jun phosphorylation sites (JunAA/JunAA) and mice lacking JNK1 ($Jnk1^{-/-}$), it was recently reported that c-Jun is also essential for efficient osteoclastogenesis in bone marrow-derived monocyte/macrophages (BMMs) [15]. Moreover, osteoclastogenic activities of NFATc1 were regulated by c-Jun activity [16].

To characterize proteins that are upregulated by RANKL and play essential roles for osteoclast differentiation, two-dimensional electrophoresis (2-DE) and MALDI-TOF analysis were performed, and schlafen2 (Slfn2) was identified as one of the proteins highly induced by RANKL. The Slfn family has originally been discovered in the context of the development of the immune system [17]. Members of the Slfn protein family have been implicated in the regulation of cell differentiation [18]. However, the nature of their involvement in bone remodeling, wherein RANKL-induced osteoclast differentiation plays a central role, is yet unknown. Here, we show for the first time that Slfn2 is critically involved in the osteoclastogenesis through increasing c-Jun activation and NFATc1 expression.

2. Materials and methods

2.1. Cell culture and reagents

RAW264.7 monocyte/macrophage cell line was maintained at 37 °C in 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). RAW264.7 cells stably transfected with a cDNA encoding a dominant negative mutant of Rac1, RacN17 (RAW-RacN17) and 293-RANK cells expressing RANK by tetracycline induction were previously described [19–21]. Recombinant M-CSF was purchased from R & D Systems.

* Corresponding author. Division of Life and Pharmaceutical Sciences, Center for Cell Signaling and Drug Discovery Research, Department of Life Science, College of Natural Sciences, Ewha Womans University, Seoul 120-750, Republic of Korea. Tel.: +82 2 3277 3770; fax: +82 2 3277 3760.
E-mail address: leesy@ewha.ac.kr (S.Y. Lee).
[1] Present address: Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA.

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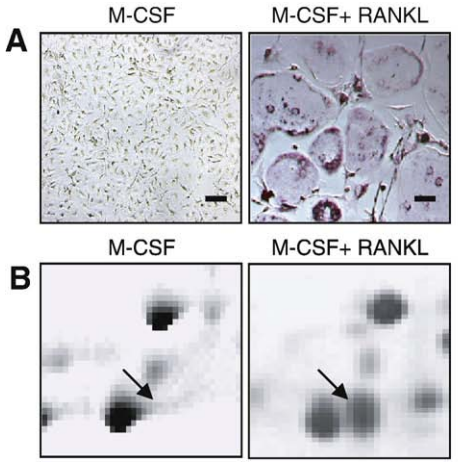

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Identified protein	Accession#*	*Mr*(Da) / p*I***	Sequence *** coverage (%)
Schlafen2	gi:3983150	42438 / 6.0	38%

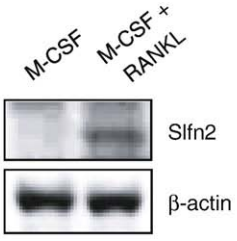

Fig. 1. Identification of Slfn2 by proteomics analysis. (A) BMMs were incubated with or without RANKL (100 ng/ml) in the presence of M-CSF (20 ng/ml) for 3 days. These cells were stained for TRAP as described in Experimental procedures. Bar, 100 μm. (B) Cells as in (A) were lysed and cellular proteins were resolved by 2-DE. The gels were silver-stained and scanned. The protein spot indicated by arrow was subjected to trypsin digestion and mass spectrometry was performed using MALDI-TOF. (C) The protein spot indicated by arrow in (B) was identified as Slfn2. *, Accession number in 'GeneInfo Identifier' sequence identification number; **, Theoretical *Mr* (Da) and p*I*; ***, Sequence coverage % (Mass data). (D) The increased expression of Slfn2 in RANKL-stimulated cells was confirmed by Western blotting. Whole cell lysates (50 μg each) were immunoblotted with anti-Slfn2 Ab and reprobed with anti-actin Ab.

Soluble RANKL was from Peprotech. A synthetic Slfn2 peptide corresponding to the EKDRKKMKNSHLRKC was conjugated to keyhole limpet hemocyanin (KLH) and immunized to rabbits using a standard protocol. The antibody (Ab) was affinity purified from pooled antiserum. Monoclonal antibodies (mAbs) specific for myc and HA epitopes were from BIOMOL Research Laboratories and Roche Diagnosis, respectively, and polyclonal antibodies specific for c-Jun, phosphorylated c-Jun, and NFATc1 were from Santa Cruz Biotechnology. All Abs against MAP kinases and phospho-IκB were from Cell Signaling. All other chemicals and Flag epitope (M2) were from Sigma-Aldrich.

2.2. Plasmids

The c-Jun (pFA-Jun) pathway-specific transactivators and the GAL4 UAS-containing luciferase reporter (pFR-Luc) plasmid were from Stratagene. Retroviral vectors, pMX-IRES-EGFP was provided by Dr. T. Kitamura (University of Tokyo, Tokyo) and pCIN-4-Slfn2 [17] was

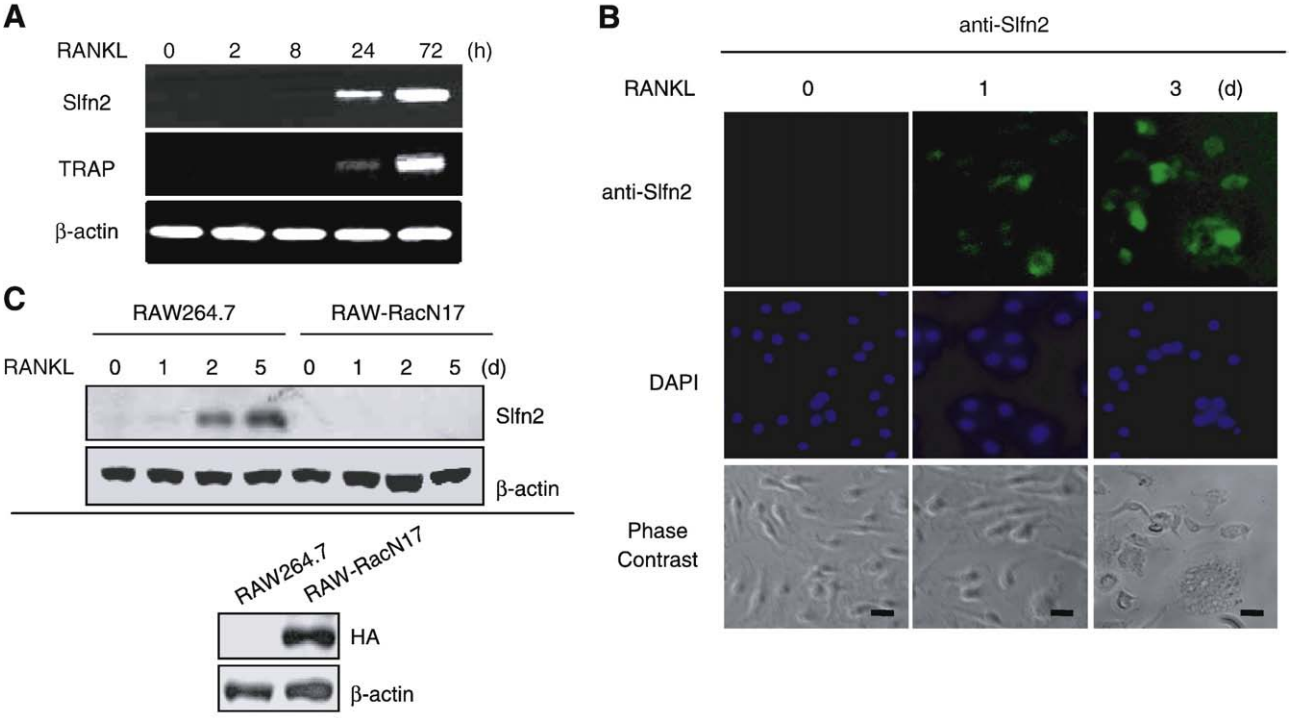

Fig. 2. Expression of Slfn2 by RANKL during osteoclast differentiation. (A) RT-PCR analysis on the time course of Slfn2 and TRAP induction in BMMs following RANKL (100 ng/ml) and M-CSF (20 ng/ml) treatment for the indicated times. (B) BMMs were stimulated with RANKL and M-CSF as the indicated periods. The cells were immunostained by anti-Slfn2 Ab. The nuclei were stained with DAPI. Bar, 50 μm. (C) Expression of Slfn2 protein in RAW264.7 and RAW-RacN17 cells stimulated with RANKL for the indicated periods. Whole cell lysates (50 μg each) were immunoblotted with anti-Slfn2 Ab and reprobed with anti-actin Ab. Characterization of RAW-RacN17 cells (lower panel). Immunoblotting confirmed the stable expression of HA-tagged RacN17 protein in RAW-RacN17 cells.