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Binding of PICK1 PDZ domain with calcineurin B regulates osteoclast differentiation

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ABSTRACT

The calcineurin/nuclear factor of activated T cell (NFAT) signaling pathway plays a major role in osteoclast differentiation; however, the proteins that react with the calcineurin-NFAT complex in osteoclasts to regulate osteoclastogenesis remain unclear. Here, we present evidence that PICK1 also positively regulates calcineurin B in osteoclasts to activate NFAT to promote osteoclastogenesis. mRNA and protein expression of PICK1 in murine primary bone marrow macrophages (BMMs) was significantly increased during RANKL-induced osteoclast differentiation. The interaction of PICK1 with calcineurin B in BMMs was confirmed by co-immunoprecipitation. An inhibitor of the PICK1 PDZ domain significantly decreased osteoclastogenesis marker gene expression and the number of TRAP-positive multinucleated cells among RAW264.7 osteoclast progenitor cells. Overexpression of *PICK1* in RAW264.7 cells significantly increased the number of TRAP-positive mature osteoclasts. Increased NFAT activation with transcriptional activation of *PICK1* during RAW264.7 osteoclastogenesis was also confirmed in a tetracycline-controlled *PICK1* expression system. These results suggest that the PDZ domain of PICK1 directly interacts with calcineurin B in osteoclast progenitor cells and promotes osteoclast differentiation through activation of calcineurin-NFAT signaling.

1. Introduction

Bone resorption occurs through a distinctive function of osteoclasts, and current anti-resorption therapy has thus targeted this cell type [1]. Further understanding of molecular regulation during osteoclast differentiation is expected to provide unique molecular targets to control osteoclast activity in osteoporosis and bone regenerative therapies.

Osteoclasts are specialized cells derived from the monocyte/macrophage hematopoietic lineage, and their differentiation is primarily induced by macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL). RANKL is supplied by osteoblasts, activated T cells, and bone marrow stromal cells [1], and it binds to its specific membrane-bound receptor RANK on the precursor cells, which activates downstream signaling pathways to control osteoclastogenesis. The signaling pathways contain cascades mediated by protein kinases and calcium signaling. These signals finally trigger the transcriptional activation of the nuclear factor of activated T cells (NFAT) c1 (also known as NFAT2), which is the master transcription factor of osteoclast differentiation [2].

In the molecular signaling cascades occurring during osteoclastogenesis, calcineurin (also known as protein phosphatase 2B) is a crucial downstream effector of the RANKL-induced signal transduction pathway to induce differentiation [3]. Calcineurin consists of calcineurin A, a heterodimer of a calmodulin-binding catalytic subunit, and calcineurin B, a Ca^{2+}-binding regulatory subunit. Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase that plays an important role in the coupling of Ca^{2+} signals to cellular behavior [4]. Calcineurin is

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highly expressed in osteoclasts [5]; however, it is not clear how the distinct calcineurin-dependent events are coordinated.

We previously identified protein interacting with C kinase 1 (PICK1) as a new calcineurin B-binding protein that regulates NFAT activity in neuron-like PC12 cells [6]. PICK1 is a ~47 kDa membrane-associated protein and has been found to be expressed in many tissues, with the highest expression found in the brain, followed by the testis [7]. In neurons, PICK1 plays a crucial role in synaptic plasticity by regulating the trafficking of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor [8] and post-translational modification of its interacting proteins, the dysfunction of which is associated with many neurological disorders [9]. PICK1 is also associated with complete infertility, as PICK1 knockout male mice show abnormal spermatogenesis in the testis [10]. Involvement of PICK1 in heart failure, progression of human breast cancer [7], and bone metastasis in prostate cancer [11] has also been reported.

PICK1 has a distinctive molecular structure, containing a PSD95/DlgA/Zo-1 (PDZ) protein-protein interaction domain. The PDZ domain is promiscuous and has been shown to interact with more than 60 proteins [12], including the consensus C-terminal sequence of calcineurin B [6]. These observations prompted us to explore the possibility that the PDZ domain of PICK1 binds to calcineurin B to regulate osteoclast differentiation via activation of NFAT. The objective of this study was to investigate the involvement of PICK1 in calcineurin-NFAT-regulation during osteoclastogenesis.

2. Materials and methods

2.1. Osteoclast progenitor cells

The protocol for animal experiments in this study was approved by the Institutional Animal Care and Use Committee of Osaka University Graduate School of Dentistry (approval number: 19-056-1). Osteoclast progenitor bone marrow macrophages (BMMs) and murine macrophage-like RAW264.7 osteoclast progenitor cells were prepared according to the method described in our previous report [13]. Briefly, BMMs from male wild-type ddY mice were cultured in growth medium, which consisted of α-MEM medium (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 50 ng/ml human recombinant M-CSF (Leukoprol, Kyowa Hakko, Tokyo, Japan), 2 mM L-glutamine (Wako), 100 units/ml penicillin, and 100 μg/ml streptomycin (Wako). RAW264.7 cells were maintained in growth medium, which consisted of α-MEM medium containing 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.2. Establishment of PICK-overexpressing RAW264.7 cells

Full length cDNA of mouse *PICK1* was PCR-amplified from a mouse cDNA library (Clontech, Mountain View, CA) and cloned into the pENTR/D-TOPO vector using the pENTR Directional TOPO cloning kit (Thermo Fisher Scientific, Carlsbad, CA) as described previously [14], and subcloned into the pLenti 6.3-GW vector (pLenti 6.3-GW/PICK1) using the Gateway System. 293FT cells were cultured in a 10 cm^2 culture dish and transfected with 10 μg of pLenti 6.3-GW/PICK1vector (*PICK1* expression vector) or pLenti 6.3-GW/EmGFP vector (control *GFP* expression vector), 6 μg of Lipofectamine 2000 (Thermo Fisher Scientific), and 6 μg of packaging mix (Thermo Fisher Scientific) to produce *PICK1*- and *GFP*-expressing lentivirus, respectively. The *PICK1* and *GFP* lentiviruses were used for establishing *PICK1*-overexpressing RAW264.7 cells (PICK1/RAW264.7) and *GFP*-overexpressing RAWs (GFP/RAW264.7), respectively. After transduction, 10 μg/ml blasticidin S was used to select transformants.

2.3. Establishment of tetracycline (tet)-controlled PICK1-expressing/NFAT-reporter RAW264.7 cells

The pENTR/D-TOPO vector containing *PICK1* cDNA was subcloned into the pLenti6.3/TO vector (Thermo Fisher Scientific) to construct the pLenti6.3/TO/PICK1 vector. pNFAT/Luc-RAW246.7 cells, which stably express the NFAT response element/luciferase reporter gene [13], were transduced with the pLenti6.3/TO/PICK1 vector and pLenti3.3/TR vector (Thermo Fisher Scientific), and then cloned using 100 μg/ml geneticin (G418 Sulfate; Thermo Fisher Scientific) and 10 μg/ml blasticidin S, which resulted in establishment of tet-controlled *PICK1* expressing/NFAT-reporter RAW264.7 cells (tet-PICK1/pNFAT/Luc-RAW264.7 cells).

2.4. Induction of osteoclastogenesis

For the osteoclastogenic culture, BMMs and RAW264.7 cells were seeded in a 96-well plate in growth medium at 5×10^6 and 5×10^3 cells/well, respectively. After 24 h, the medium was changed to osteoclastogenic induction medium [13], which consisted of α-MEM medium containing 10% FBS, 100 ng/ml soluble RANKL (sRANKL, Oriental yeast, Osaka, Japan), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin for RAW264.7, PICK1/RAW264.7, GFP/RAW264.7, and tet-PICK1/pNFAT/Luc-RAW264.7 cells. sRANKL (100 ng/ml) was also added to BMMs for osteoclastogenic induction with α-MEM medium containing 10% FBS, 50 ng/ml M-CSF, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained up to 6 days and the medium was changed every other day.

The inhibition assay for the PDZ domain of PICK1 in RAW264.7 cells was also performed by adding 6-100 μM PICK1 PDZ domain inhibitor (FSC231: Calbiochem, La Jolla, CA) [15] to the induction medium.

Standard tartrate resistant acid phosphatase (TRAP) staining [13] was performed to evaluate osteoclast formation. TRAP-positive dark red cells with more than three nuclei were counted as multinucleated osteoclasts under light microscopy.

2.5. Detection of NFAT activity (luciferase measurement)

For luciferase measurement, tet-PICK1/NFAT/Luc-RAW264.7 cells were seeded in opaque white 96-well plates. To induce *PICK1* expression, tet-PICK1/NFAT/Luc-RAW264.7 cells were cultured in induction medium supplemented with 10-1000 ng/ml doxycycline (Dox: Thermo Fisher Scientific) for 48 h, followed by measurement of luciferase activity using the ONE-GloTM luciferase assay system (Promega, Madison, WI) and a microplate reader (GloMax-Multi Detection System, Promega) [13].

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

RT-PCR analysis was performed according to the method described in our previous report [13]. The PCR conditions and primer pairs used are given in Table 1. To determine the tissue distribution of *PICK1* mRNA expression, first-strand cDNA derived from the brain, skin, liver, lung, heart, stomach, muscle, testis, thymus, bone (epiphysis and femur) tissues of adult mice (GenoStaff, Tokyo, Japan) were used. Quantitative TaqMan real-time PCR analysis for expression of *PICK1* and *NFATc1* was performed using the AB 7300 real-time PCR system (Thermo Fisher Scientific). The expression of *GAPDH* mRNA was used as an internal control. The TaqMan primer and probe sets used were Mm00501103_ml (*PICK1*), Mm00479445_m1 (*NFATc1*) and 4352339E (*GAPDH*).

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