Biochemical and Biophysical Research Communications 390 (2009) 585–590

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Adrm1 interacts with Atp6v0d2 and regulates osteoclast differentiation

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ARTICLE INFO

Article history:
Received 29 September 2009
Available online 8 October 2009

Keywords:
Osteoclast
Atp6v0d2
Adrm1
Cell fusion
Differentiation
Yeast two-hybrid screen

ABSTRACT

Bone homeostasis is tightly regulated by matrix-producing osteoblasts and bone-resorbing osteoclasts. During osteoclast development, mononuclear preosteoclasts derived from myeloid cells fuse together to form multinucleated, giant cells. Previously, we reported that the d2 isoform of the vacuolar (H^+) ATPase V0 domain (Atp6v0d2) plays an important role in osteoclast maturation and bone formation. To understand how Atp6v0d2 controls osteoclast maturation, we have performed a yeast two-hybrid screen using full-length Atp6v0d2 as the bait, and identified adhesion-regulating molecule 1 protein (Adrm1) as a potential functional partner of Atp6v0d2. The interaction between Atp6v0d2 and Adrm1 was confirmed in yeast and *in vivo* using immunoprecipitation assays. We also show that Adrm1 is required for cell migration and osteoclast maturation.

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Introduction

Bone homeostasis is maintained through a dynamic balance between bone-resorbing osteoclasts and matrix-producing osteoblasts. Disruption of this balance may lead to bone diseases, such as osteoporosis. Osteoclasts—giant multinucleated cells (MNCs) derived from hematopoietic macrophage/monocyte lineage precursor cells—are formed as mononuclear preosteoclasts fuse together. Bone resorption is multistep process, which includes secretion of such lysosomal enzymes as tartrate-resistant acid phosphatase (TRAP), cathepsin K, and several matrix metalloproteases, as well as extracellular acidification at the ruffled border [1–3]. This extracellular acidification is mediated by vacuolar type H^+-ATPase complex (V-ATPase). V-ATPases are composed of at least 13 distinct subunits, and several of these subunits have multiple isoforms [4]. The expression of these isoforms is regulated in a cell type- and tissue-specific manner. Two isoforms of V-ATPase V0 subunit d, d1 and d2, have been identified in mice and humans. Both of these isoforms are more abundantly expressed in osteoclasts than in other tissues [5–7]. In particular, the expression of Atp6v0d2 is more specific than that of Atp6v0d1 during osteoclast differentiation, which is thought to highlight the importance of Atp6v0d2 for osteoclastogenesis, although the exact function of Atp6v0d2 has not been elucidated [8]. Previously, we reported that genetic inactivation of Atp6v0d2 in mice results in markedly increased bone mass due to defective osteoclasts. Interestingly, Atp6v0d2 deficiency did not affect the differentiation of osteoclasts or V-ATPase activity in this cell population. Rather, Atp6v0d2 was required for efficient osteoclast maturation [9].

In this study, we have further investigated the functions of Atp6v0d2 during osteoclast differentiation by searching for Atp6v0d2-interacting proteins. We screened a human bone marrow cDNA-derived two-hybrid library using Atp6v0d2 as a bait, and identified adhesion-regulating molecule 1 (Adrm1) as an Atp6v0d2-binding partner. Adrm1 was previously identified due to its upregulated expression levels in metastatic tumor cells, and its involvement in cell adhesion and cell migration [10–13]. Although initially described as a cell membrane glycoprotein, Adrm1 is not glycosylated, is intracellularly localized, and probably has no direct role in cell adhesion [11,14,15]. Furthermore, recent studies suggested that Adrm1 is a proteasome subunit that functions as novel ubiquitin receptor [16–19]. To elucidate the role of Adrm1 during osteoclast differentiation, we performed retrovirus-mediated RNA interference (RNAi) knockdown of Adrm1 expression during *in vitro* osteoclast differentiation. We also examined the effects of Adrm1 on fusion efficiency, cell adhesion, and cell migration.

Abbreviations: Adrm1, adhesion-regulating molecule 1 protein; Atp6v0d2, d2 subunit of the vacuolar (H^+) ATPase V0 domain; RANKL, receptor activator of nuclear factor-κB ligand; M-CSF, macrophage-colony stimulating factor.

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doi:10.1016/j.bbrc.2009.10.010

Materials and methods

Cell culture. Murine preosteoclasts were prepared from the bone marrow cells of 6- to 8-week-old mice as described previously [9]. To generate osteoclasts, bone marrow-derived monocytes/macrophages (BMMs; 1×10^4 cells/well in 96-well plates) were cultured with macrophage-colony stimulating factor (M-CSF, 50 ng/ml) and soluble receptor of nuclear factor-κB (RANKL, 150 ng/ml) for 3–4 days.

Yeast two-hybrid screen. The yeast two-hybrid Matchmaker 3 system (Clontech Laboratories, Palo Alto, California, USA) was used to screen for potential Atp6v0d2-interacting proteins. A human bone marrow cDNA library cloned into pGAD-Rec such that it contained sequences encoding an amino-terminal GAL4 activation domain and a hemagglutinin tag was pretransformed into the Y187 yeast strain. This library was then screened after mating the transformants with AH109 yeast containing the human Atp6v0d2 bait construct pGBKT7-d2, which encoded full-length human Atp6v0d2 fused to a GAL4 DNA-binding domain. The mated yeast cells were grown on agar plates containing Trp, Leu, and His dropout nutritional selection media in the presence of 12 mM 3-amino-1,2,4-triazole. To reduce the false positives after selection, the selected yeast colonies were transferred to Trp, Leu, His, and Ade dropout nutritional selection media and assayed for lacZ activity. Prey plasmids encoding human bone marrow proteins that interacted with Atp6v0d2 in the lacZ-positive yeast clones were isolated by transforming them into *Escherichia coli* and selecting the bacteria on carbenicillin-containing culture plates. Isolated plasmids were then subjected to DNA sequence analysis.

Coimmunoprecipitation and Western blotting. Western blotting and immunoprecipitation analyses were performed as described previously [20]. Briefly, cells were incubated in buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 10% glycerol, and protease and phosphatase inhibitors for 30 min on ice and scraped into microcentrifuge tubes. The whole-cell extracts were vigorously vortexed and then microcentrifuged for 20 min at 10,000*g*. Protein concentrations in the supernatants were determined using a DC Protein Assay Kit (Bio-Rad), and 10–20 μg of the cellular proteins were separated by SDS–PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was probed with a 1:1000 dilution of the antibodies described in the figure legends. For immunoprecipitation, 300–500 μg of the cellular proteins were mixed with 5 μg of the antibodies described in the figure legends for 4–6 h at 4 °C. This mixture was incubated with 50 μl of protein G-Sepharose beads for 2 h at 4 °C. The beads were washed five times in lysis buffer and subjected to Western blotting.

RNAi expression vectors, retroviral infection, and analysis of osteoclasts. RNAi oligonucleotides (Adrm1, 5′-GAA AGA CGA AGA AGA AGA TAT-3′; GFP, 5′-CAT GGA TGA ACT ATA CAA A-3′) were synthesized by Integrated DNA Technologies and cloned into the retroviral small interference RNA (siRNA) vector pSuper-retro-Puro (OligoEngine). BMMs were infected with retroviruses and cultured with M-CSF (50 ng/ml) and RANKL (150 ng/ml) for 3–5 days to generate osteoclasts as described previously [20]. For osteoclast analyses, osteoclasts were fixed with 10% formalin and stained for TRAP activity, whereas bone slices were stained with 0.5% toluidine blue as described previously [9].

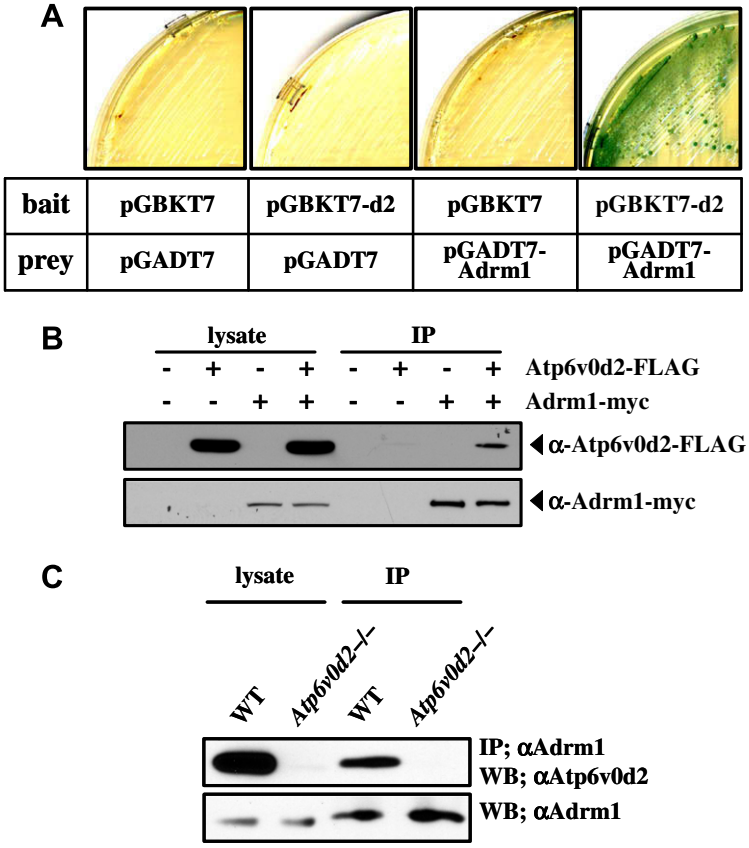

Fig. 1. Adrm1 interacts with ATP6v0d2. (A) Yeast strain AH109 was transformed with pGBKT7 or pGBKT7-d2 (bait) along with pGADT7 or pGADT7-Adrm1 (prey). Transformed yeast cells were then spread on Trp, Leu, His, and Ade dropout nutritional selection media and assayed for lacZ activity. (B) 293T cells were transfected with the indicated plasmids. Cell lysates were prepared and immunoprecipitation was performed with anti-myc antibodies. The precipitates were subjected to Western blotting with anti-Myc or anti-FLAG antibodies. (C) Cell lysates were prepared from osteoclasts derived from the bone marrow of wild-type or Atp6v0d2-deficient mice, and immunoprecipitation was performed with anti-Adrm1 antibodies. The precipitates were subjected to Western blotting with anti-ATP6v0d2 or anti-Adrm1 antibodies.

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