Note

ISG15 Regulates RANKL-Induced Osteoclastogenic Differentiation of RAW264 Cells

Tomoharu Takeuchi,*[a] Genki Shimakawa,[a] Mayumi Tamura,[a] Hideyoshi Yokosawa,[b] and Yoichiro Arata[a]

[a] *Faculty of Pharmaceutical Sciences, Josai University; 1–1 Keyakidai, Sakado, Saitama 350–0295, Japan: and* [b] *School of Pharmacy, Aichi Gakuin University; 1–100 Kusumoto-cho, Chikusa-ku, Nagoya 464–8650, Japan.*
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Interferon-stimulated gene 15 kDa (ISG15) is a protein upregulated by interferon-*β* that negatively regulates osteoclastogenesis. We investigated the role of ISG15 in receptor activator of nuclear factor-*κ*B ligand (RANKL)-induced osteoclastogenic differentiation of murine RAW264 cells. RANKL stimulation induced ISG15 expression in RAW264 cells at both the mRNA and protein levels. Overexpression of ISG15 in RAW264 cells resulted in suppression of cell fusion in RANKL-stimulated cells as well as the reduced expression of *ATP6v0d2*, a gene essential for cell fusion in osteoclastogenic differentiation. These results suggest that ISG15 suppresses RANKL-induced osteoclastogenesis, at least in part, through inhibition of *ATP6v0d2* expression.

Key words interferon-stimulated gene 15 kDa (ISG15); osteoclast; interferon; receptor activator of nuclear factor-*κ*B ligand (RANKL); RAW264; *ATP6v0d2*

Interferon-*α*/*β* is a multifunctional cytokine that exerts various biological functions in immunity, cell differentiation, and so on *via* the induction of downstream genes called interferon-stimulated genes (ISGs).[1,2] Interferon-stimulated gene 15 kDa (ISG15), a ubiquitin-like protein, is one of the proteins that are most upregulated by interferon stimulation.[3,4] Upon stimulation of the cells with interferon or pathogenic materials such as lipopolysaccharides, the expression of ISG15 and several other enzymes that catalyze protein modification with ISG15 (ISGylation) is induced, followed by the ISGylation of various cellular proteins. ISGylation affects the nuclear factor-kappa B (NF-*κ*B) signaling pathway as well as the activity of double-stranded RNA-activated protein kinase,[5,6] both of which have important roles in osteoclastogenic differentiation.[7,8]

Osteoclasts are tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells derived from monocyte/macrophage lineage cells.[7,9] Receptor activator of nuclear factor-*κ*B ligand (RANKL) is essential for the induction of osteoclastogenesis.[10] The murine RAW264 cell line is a useful model of osteoclastogenesis as, upon stimulation with RANKL, RAW264 cells differentiate into TRAP-positive mononuclear cells, which subsequently undergo cell fusion to form TRAP-positive multinuclear osteoclasts. *In vivo*, osteoclastogenesis is tightly controlled by factors produced from cells such as osteoblasts and osteocytes, and deregulation of osteoclastogenesis results in various diseases such as osteoporosis.[7,9,11,12] One important factor involved in this process is interferon-*β*, whose production from osteoclast precursor cells is induced by RANKL stimulation and which is also secreted by osteocytes.[13,14] Although it has been reported that interferon-*β* negatively regulates osteoclastogenesis *via* reduction of c-Fos protein expression and induction of inducible nitric oxide synthase expression,[13,15] the roles of ISGs in osteoclastogenesis largely remain to be clarified. In the present study, we found that RANKL stimulation induces ISG15 expression in murine RAW264 cells and that ISG15 overexpression in RAW264 cells suppresses RANKL-induced osteoclastogenic differentiation, probably *via* the inhibition of *ATP6v0d2* expression.

MATERIALS AND METHODS

Cell Culture The mouse macrophage-like cell line RAW264 was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and cultivated in MEM*α* medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (BioWest, Nuaille, France) and 1× penicillin/streptomycin/amphotericin B (Wako Pure Chemical Industries, Ltd.) at 37°C under 5% CO_2.

RNA Preparation and Reverse Transcription (RT)-Polymerase Chain Reaction (PCR) Cells were seeded into 6-well plates at a density of 10000 cells/well. After 1 d, cells were stimulated with 500 ng/mL sRANKL (a soluble form of RANKL; Oriental Yeast, Tokyo, Japan) and allowed to differentiate. Total RNA was isolated from the cells using an RNeasy® Mini Kit (Qiagen, Hilden, Germany) and an RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. Total RNA was reverse-transcribed using the GoScript™ Reverse Transcription System (Promega, WI, U.S.A.) with oligo(dT)15 primers. The cDNA mixture thus obtained was used for PCR analysis. The primers used for PCR were as follows: *ISG15*, 5′-GTC CCA GCA GCA CAG TGA TG-3′ and 5′-GAT GGG GGC CTT AGG CAC AC-3′; *TRAP*, 5′-AAA TCA CTC TTT AAG ACC AG-3′ and 5′-TTA TTG AAT AGC AGT GAC AG-3′; *GAPDH*, 5′-CAC CAC CAT GGA GAA GGC TG-3′ and 5′-ATG ATG TTC TGG GCA GCC CC-3′; *NFATc1*, 5′-CTC GAA AGA CAG CAC TGG AGC AT-3′ and 5′-CGG CTG CCT TCC GTC TCA TAG-3′; *DC-STAMP*, 5′-TGG AAG TTC ACT TGA AAC TAC GTG-3′ and 5′-CTC GGT TTC CCG TCA GCC TCT CTC-3′; and *ATP6v0d2*, 5′-TCA GAT CTC TTC AAG GCT GTG CTG-3′ and 5′-GTG CCA AAT GAG TTC AGA GTG ATG-3′. The above mentioned primers for *NFATc1*, *DC-STAMP*, and *ATP6v0d2* were designed according to the method described by Kim *et al.*[16]

Western Blotting Cells were washed with phosphate-

* To whom correspondence should be addressed. e-mail: t-take@josai.ac.jp

buffered saline (PBS) and lysed in 200 μL of sample buffer (50 mM Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate, 10% glycerol, 0.01% bromophenol blue, and 2% 2-mercaptoethanol) *via* sonication. After boiling and centrifugation, the resulting supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the separated proteins were transferred onto nitrocellulose membranes (Bio-Rad, CA, U.S.A.). The membranes were immunoblotted with anti-mouse ISG15 antibody (Santa Cruz, Dallas, TX, U.S.A.) and subsequently incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin antibody (Bio-Rad), followed by detection with enhanced chemiluminescence immunoblotting detection reagents (GE Healthcare, Little Chalfont, U.K.).

Plasmid Construction A truncated version of the mouse *ISG15* open reading frame that lacked the C-terminal region found in the precursor form of ISG15 was amplified by PCR from the cDNA mixture prepared from RAW264 cells. A forward primer containing an *Eco*RI site and the initiation codon ATG, 5′-GAA TTC ATG GCC TGG GAC CTA AAG-3′, and a reverse primer containing a stop codon and a *Sal*I site, 5′-GTC GAC TTA CCC ACC CCT CAG GCG CAA ATG-3′, were used for the amplification. The PCR fragment was then cloned into the *Eco*RI and *Sal*I sites of the pCI-*neo*-3FLAG vector[17] to produce the pCI-*neo*-3FLAG-ISG15 mammalian expression plasmid.

Generation of ISG15 Stable Transfectants The pCI-*neo*-3FLAG-ISG15 and pCI-*neo*-3FLAG (mock) vectors were transfected into RAW264 cells using MultiFectam (Promega) according to the manufacturer's instructions. Stable transfectants were selected using the medium containing 300 μg/mL G418 (Sigma-Aldrich, MO, U.S.A.) to establish the FLAG-ISG15 expressing cell lines RAW-ISG15 #1 and #2 and the negative control cell lines RAW-Mock #1 and #2. The expression of FLAG-ISG15 was confirmed by Western blotting (Suppl. Fig. 1A).

TRAP Assay RAW264 cells were seeded into a 96-well plate (1000 cells/well) and cultured for 1 d. Subsequently, the medium was changed to that containing 500 ng/mL sRANKL. After 5 d, the cells were washed with PBS and lysed with 100 μL of TRAP buffer (50 mM sodium tartrate, 50 mM sodium acetate, 150 mM KCl, 0.1% TritonX-100, 1 mM sodium ascorbate, and 0.1 mM $FeCl_3$, pH 5.2) for 10 min at 4°C. Then, the prepared cell extract (10 μL) was added to 100 μL of TRAP buffer containing 2.5 mM *p*-nitrophenyl phosphate (Thermo Fisher Scientific, MA, U.S.A.) as a TRAP substrate, and the reaction mixture was incubated for 1 h at 37°C. After 50 μL of 0.9 M NaOH was added to the mixture to stop the reaction, the absorbance at 405 nm was measured using a SpectraMax M5 microplate reader (Molecular Devices, CA, U.S.A.).

TRAP Staining RAW264 cells were seeded into a 6-well plate (10000 cells/well), treated with 500 ng/mL sRANKL, and allowed to differentiate for 5 d. Differentiated cells were washed with PBS and then treated with 4% paraformaldehyde solution for 10 min at room temperature. After being washed again with PBS, the cells were treated with PBS containing 0.1% TritonX-100 and then stained with a TRAP staining solution containing 50 mM sodium tartrate, 45 mM sodium acetate, pH 5.2, 0.1 mg/mL naphthol AS-MX phosphate (Sigma-Aldrich), and 0.6 mg/mL fast red violet LB (Sigma-Aldrich) for 1 h at room temperature. The cells were viewed under a TC5400 microscope (Meiji Techno, Saitama, Japan) equipped with Moticom 2000 (Shimadzu, Kyoto, Japan), and TRAP-positive cells containing 3 or more nuclei were counted. Photographs were taken with a 10× objective.

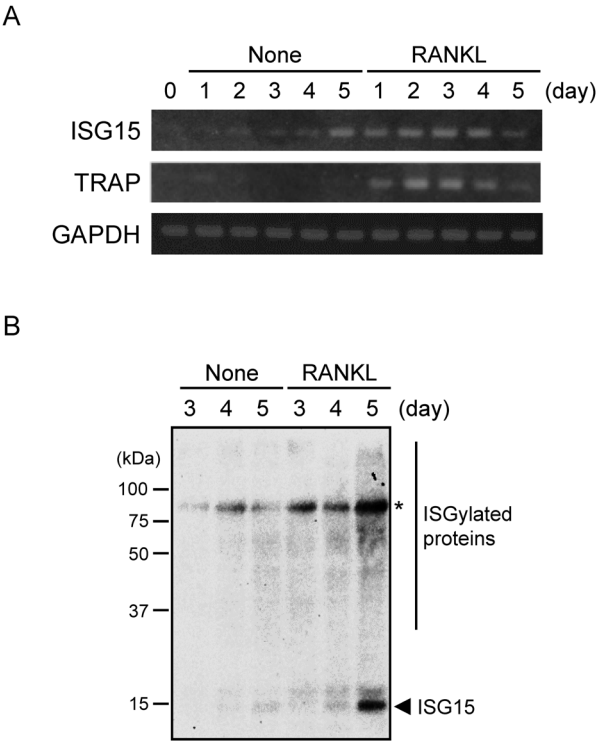

Fig. 1. RANKL Induces *ISG15* Expression

(A) Total RNA was isolated from RAW264 cells at the indicated days after treatment with or without RANKL and then subjected to RT-PCR to analyze the expression of *ISG15*, *TRAP*, and *GAPDH* (control) mRNAs. (B) Extracts were prepared from RAW264 cells at the indicated days after treatment with or without RANKL and subjected to Western blotting with an anti-ISG15 antibody. The bands indicated by an asterisk seems to be nonspecific because these bands were detected even in the absence of free ISG15 and therefore were not considered to be ISGylated proteins. Since these bands increased upon RANKL stimulation, we assume that these are proteins upregulated by RANKL-stimulation and non-specifically bound to the anti-ISG15 antibody used. The gel was stained with Coomassie brilliant blue to confirm equal protein loading (data not shown).

RESULTS AND DISCUSSION

RANKL Induces ISG15 Expression In order to clarify the role of ISG15 in osteoclastogenic differentiation, we first examined whether RANKL induces ISG15 expression in RAW264 cells. Using RT-PCR (Fig. 1A), we found that RANKL induces mRNA expression for *ISG15* as well as for *TRAP*, a marker for osteoclastogenic differentiation. Furthermore, Western blotting with an anti-ISG15 antibody (Fig. 1B) showed that a protein band corresponding to ISG15 could be detected 5 d after RANKL treatment. In the latter experiment, smeared bands of proteins that ran more slowly than free ISG15 were also detected, implying that several proteins had been modified with ISG15 (see Fig. 1B, bands designated "ISGylated proteins"). It should be noted that the expression of *ISG15* mRNA was detected 1 d after RANKL stimulation (Fig. 1A), while the band corresponding to free ISG15 protein was not detected until 5 d after RANKL stimulation (Fig. 1B). Although the above results seem somewhat inconsistent, we speculate that it is due to the sensitivity of the Western blotting. Thus, we assume that not only *ISG15* mRNA but also ISG15 protein is expressed by RAW264 cells in the

early phase of osteoclastogenic differentiation in response to RANKL stimulation.

ISG15 Suppresses RANKL-Induced Formation of Multinuclear Osteoclasts but Not Expression of TRAP Activity We next examined whether ISG15 affects osteoclastogenesis. To determine this, we established 4 stable RAW264 cell lines: 2 in which FLAG-tagged ISG15 was overexpressed (RAW-ISG #1 and #2), and 2 which harbored the empty expression plasmid (RAW-Mock #1 and #2). The cells from these lines were stimulated with RANKL, and then, 5 d after stimulation, their TRAP activities were measured (Fig. 2A), as the expression of TRAP activity is characteristic of osteoclastogenic differentiation.[7,9] The levels of TRAP activities in RAW-ISG #1 and #2 were higher than those in RAW-Mock #1 and #2, suggesting that ISG15 plays a role in osteoclastogenic differentiation.

To clarify further the role of ISG15 in osteoclastogenic differentiation, we next examined the effect of RANKL on cell fusion by determining the numbers of TRAP-positive multinuclear osteoclasts formed by RAW-ISG and RAW-Mock cells after RANKL stimulation (Figs. 2B, C). As expected, RAW-Mock cells successfully differentiated into TRAP-positive multinuclear cells following RANKL stimulation. However, although RAW-ISG cells differentiated into TRAP-positive mononuclear cells after RANKL stimulation (see Fig. 2B, latter panel), the number of TRAP-positive multinuclear cells produced by RAW-ISG cells was less than that produced by RAW-Mock cells (Fig. 2C). These results indicate that ISG15 inhibits cell fusion during osteoclastogenesis.

ISG15 Suppresses *ATP6v0d2* Expression Cell fusion is a complicated process that involves multiple factors.[18,19] Of the multiple candidate factors, we next focused on DC-STAMP and ATP6v0d2 because both factors have been reported to be involved in osteoclast fusion[16] and are downstream of NFATc1, a master transcription factor for osteoclastogenesis.[20] Using RT-PCR, we found that the levels of *NFATc1*, *TRAP*, and *DC-STAMP* expression in RAW-ISG cells in response to RANKL treatment were somewhat higher than those in RAW-Mock cells under the same conditions (Fig. 3). This result is consistent with that obtained from the TRAP assay (Fig. 2A). In contrast, *ATP6v0d2* expression was less in TRAP-treated RAW-ISG cells than in TRAP-treated RAW-Mock cells, which suggests that ISG15 overexpression affects cell fusion *via* the inhibition of *ATP6v0d2* expression. This result is somewhat enigmatic, as the expression of both *DC-STAMP* and *ATP6v0d2* is regulated by the same transcription factor NFATc1.[21,22] However, it is possible that ISG15 affects the factor(s) responsible for the regulation of *ATP6v0d2* expression.

To our knowledge, this is the first report revealing that overexpression of ISG15 inhibits osteoclastogenesis. Since RANKL induces the expression of interferon-β that inhibits excess osteoclastogenesis,[13] ISG15 may assist interferon-β in regulating osteoclastogenesis as one of the interferon-stimulated gene products. Recently, it was reported that, although ISG15 knockout mice display low bone mass because of a defect in osteoblastic differentiation, macrophages isolated from the knockout mice were able to undergo osteoclastogenic differentiation *in vitro*.[23] This result indicates that ISG15 is not essential for osteoclastogenesis. In contrast, as osteocytes, which were not included in the above *in vitro* differentiation experiment, secrete interferon-β,[14] it is possible that interferon-β secreted by osteocytes stimulates the expression of ISG15 in osteoclastic precursor cells, leading to the suppression of cell fusion during osteoclastogenic differentiation. Thus, we speculate that ISG15 has a regulatory, but not es-

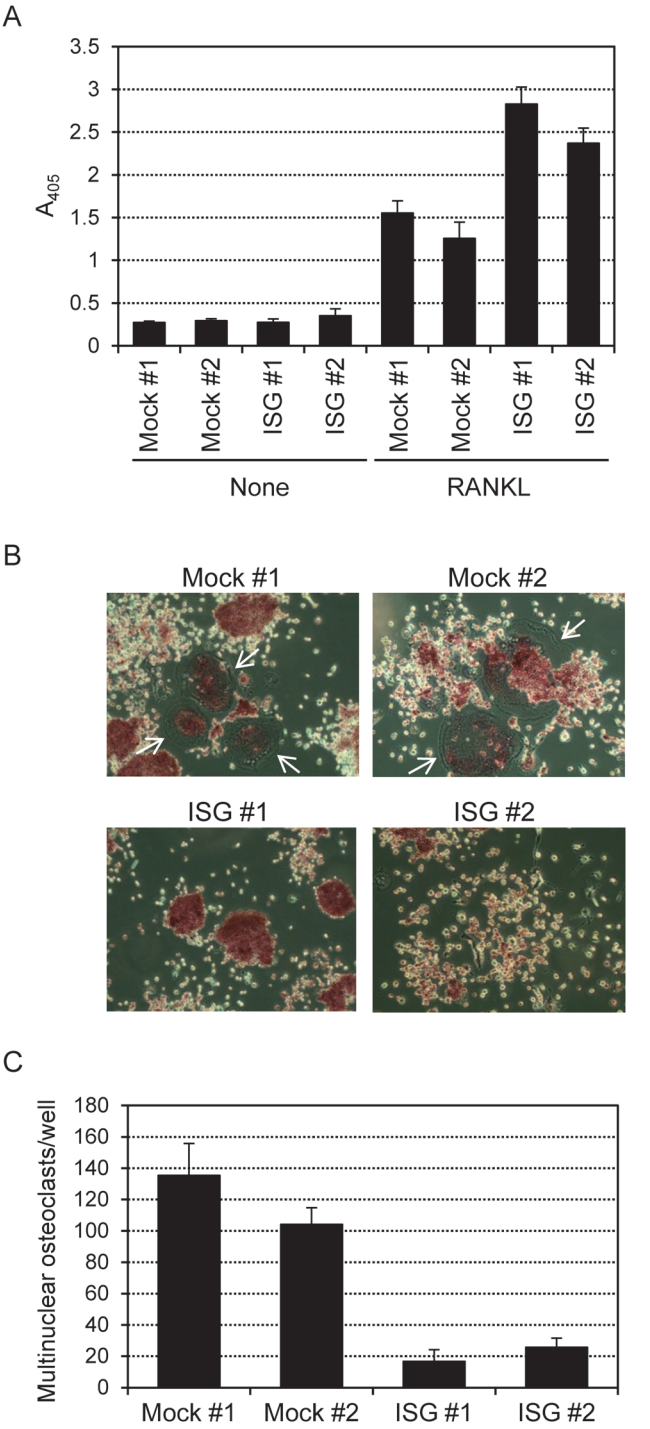

Fig. 2. ISG15 Overexpression Suppresses RANKL-Induced Formation of Multinuclear Osteoclasts but Not Expression of TRAP Activity

(A) Two RAW264 cell lines stably expressing FLAG-ISG15 (RAW-ISG #1 and #2) and 2 lines expressing the empty pCI-*neo*-3FLAG vector (RAW-Mock #1 and #2) were treated with or without RANKL for 5 d. The cells were then lysed, and their TRAP activities were assayed by measuring the absorbance at 405 nm. Data are expressed as means±S.D. (n=3). (B) Five days after cells from RAW-ISG #1 and #2 and RAW-Mock #1 and #2 were stimulated with RANKL, they were stained with the TRAP staining solution. The white arrows in the upper panel indicate TRAP-positive multinuclear osteoclasts. (C) The number of TRAP-stained multinuclear osteoclasts containing 3 or more nuclei in (B) was counted. Data are expressed as means±S.D. (n=3).

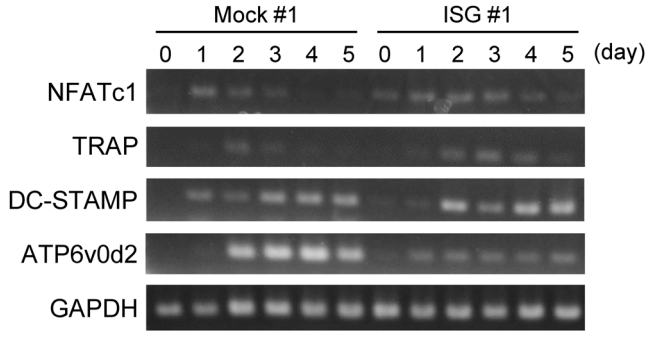

Fig. 3. ISG15 Inhibits *ATP6v0d2* Expression

Total RNA was isolated from a RAW264 cell line stably expressing FLAG-ISG15 (RAW-ISG #1) and a cell line expressing the empty pCI-*neo*-3FLAG vector (RAW-Mock #1), respectively, at the indicated days after RANKL treatment and then subjected to RT-PCR to analyze the expression of *NFATc1*, *TRAP*, *DC-STAMP*, *ATP6v0d2*, and *GAPDH* (control) mRNAs.

sential, role in osteoclastogenesis. In summary, the results of the present study suggest that ISG15 acts in osteoclastogenesis *via* the downregulation of *ATP6v0d2*. With respect to its other effects on osteoclastogenesis, ISG15 has recently been shown to disrupt actin architecture that is important for osteoclast fusion.[24,25] Such a line of study supports our finding that ISG15 has a role in osteoclastogenesis. On the other hand, an intracellular free-form of ISG15 and extracellular ISG15 are reported to have important biological functions.[26,27] We detected both forms of ISG15 in RAW-ISG cell extracts and the conditioned medium, respectively (Suppl. Figs. 1A, B). Therefore it is possible that the intracellular free-form of ISG15 or extracellular ISG15 but not ISGylation may play an important role in the suppression of osteoclastogenesis.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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