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Receptor Activator of Nuclear Factor-κB Ligand Activates Nuclear Factor-κB in Osteoclast Precursors*

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ABSTRACT

Receptor activator of nuclear factor-κB ligand [RANK ligand (RANK-L)] stimulates mature osteoclasts to resorb bone, a process associated with NF-κB activation. RANK-L also prompts macrophages to develop the osteoclast phenotype. Although NF-κB is essential for osteoclast differentiation, it is not known whether RANK-L activates this transcription complex in osteoclast precursors. We report that RANK-L rapidly induces NF-κB activation in both authentic osteoclast precursors, namely bone marrow macrophages, and RAW 264.7 cells, a murine macrophage line also capable of RANK-L-mediated osteoclastogenesis. Supershift studies reveal the RANK-L-induced DNA binding moiety contains p50/p65, the most common NF-κB complex. Subcellular translocation of p50 and p65 subunits is confirmed by Western blots and immunofluorescence analysis. RANK-L activates NF-κB in both bone marrow macrophages and RAW 264.7 cells by serine phosphorylation of IκBα within 5 min, resulting in rapid IκBα degradation and resynthesis. Attesting to function, RANK-L treatment of RAW 264.7 cells transiently transfected with a plasmid containing NF-κB consensus elements linked to luciferase greatly enhances reporter activity. Our data suggest that activation of the NF-κB pathway is an integral component of RANK-L-induced osteoclast differentiation. (*Endocrinology* **142:** 1290–1295, 2001)

OSTEOCLASTS, CELLS that play an essential role in controlling bone morphogenesis and remodeling, arise by the proliferation and differentiation of precursors in the hemopoietic lineage (1–3). Commitment of mononuclear precursors to mature osteoclasts involves transcription factors such as c-Fos and PU.1 (4–6). Similarly, mice lacking both p50 and p52 subunits of nuclear factor-κB (NF-κB) develop osteopetrosis because of failed osteoclastogenesis (7, 8).

Recent developments have made it possible to generate both human and murine osteoclasts by treating purified myeloid precursors with the cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANK-L) (9–11). Although M-CSF is a well characterized cytokine that supports the survival and proliferation of cells in the macrophage lineage (12), the identity and function of RANK-L were determined only recently (9, 10, 13, 14). RANK-L is a member of the tumor necrosis factor (TNF) cytokine superfamily and functions as an osteoclast-specific protein that exists in both soluble and membrane-bound forms (9, 10). RANK, the surface receptor for RANK-L, initiates osteoclastogenic signal transduction after ligation with RANK-L. The proximal RANK-derived signals include binding of TRAF family members such as TRAF2, TRAF3, TRAF5, and TRAF6, which, in turn, initiates a cascade of kinases. Two critical distal events in RANK signaling are activation of the NF-κB complex and the transcription factor, activator protein-1 (15).

RANK-L activates NF-κB in mature osteoclasts (16, 17). It is not known, however, whether this transcription complex is activated by RANK-L in osteoclast precursors. For example, it has been previously reported that NF-κB activation is not detected in RANK-L-treated RAW 264.7 cells, a murine myelomonocytic cell line that differentiates into osteoclasts under the influence of the cytokine (18). Given that RAW 264.7 cells represent an immortalized line, uncertainty exists as to whether RANK-L-induced NF-κB is a component of the initial stage of osteoclastogenesis.

We found that RANK-L, via serine 32/36 phosphorylation and degradation of IκBα, readily activates the most common NF-κB dimer, p50/p65, in both bone marrow macrophages (BMMs) and RAW 264.7 cells. Furthermore, RANK-L promotes the expression of an NF-κB-dependent reporter plasmid transiently transfected into RAW cells. Thus, RANK-L-stimulated osteoclastogenesis is associated with NF-κB activation.

Materials and Methods

Reagents

Polyclonal and monoclonal anti-IκBα and polyclonal anti-p50NF-κB antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antiphospho-IκBα was obtained from New England Biolabs, Inc. (Beverly, MA). Polyclonal anti-p65NF-κB was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-p50NF-κB for electrophoretic mobility shift assay (EMSA) was obtained from Geneka (Montréal, Canada). The bicinchoninic acid kit for protein determination and enhanced chemiluminescence kits were obtained from Pierce Chemical Co. (Rockford, IL). Recombinant murine M-CSF was purchased from R&D Systems, Inc. (Minneapolis, MN). Murine RANK-L was expressed in our laboratory as described previously (19). All other chemicals were obtained from Sigma (St. Louis, MO).

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Cell culture

BMMs were isolated from whole bone marrow of 4- to 6-week-old C3H/Hen males (Harlan Industries, Indianapolis, IN) and incubated in tissue culture dishes at 37 C in 5% CO_2 in the presence of recombinant mouse M-CSF (5 ng/ml). After 24 h in culture, the nonadherent cells were collected and layered on a Ficoll-Hypaque gradient, and the cells at the gradient interface were collected. The cells were replated at 65,000/cm^2 in αMEM, supplemented with 10% heat-inactivated FBS at 37 C in 5% CO_2 in the presence of M-CSF (5 ng/ml). RAW 264.7 cells, obtained from American Type Culture Collection (Manassas, VA), were grown in DMEM supplemented with10% heat-inactivated FBS.

Immunoblotting

Untreated or RANK-L-stimulated monolayers of BMMs or RAW cells were washed twice with ice-cold PBS. Cells were lifted from the dish after treatment with 5 mM EDTA and 5 mM EGTA in PBS. Cells were then resuspended in hypotonic lysis buffer A [10 mM HEPES (pH 7.8), 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 μg/ml leupeptin] and incubated on ice for 15 min, and Nonidet P-40 was added to a final concentration of 6.4%. Nuclei were pelleted, and both these and cytosolic fractions were carefully transferred to fresh tubes. Nuclei were washed and then resuspended in nuclear extraction buffer B [20 mM HEPES (pH 7.8), 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin] and rotated for 30 min at 4 C. The samples were then centrifuged, and nuclear proteins in the supernatant were transferred to fresh tubes.

Nuclear or cytosolic extracts were boiled in the presence of SDS sample buffer [0.5 M Tris-HCl (pH 6.8), 10% (wt/vol) SDS, 10% glycerol, and 0.05% (wt/vol) bromophenol blue] for 5 min and subjected to electrophoresis on 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using a semidry blotter (Bio-Rad Laboratories, Inc., Richmond, CA) and incubated in blocking solution (5% nonfat dry milk or 3% BSA prepared in PBS containing 0.1% Tween-20) for 1 h to reduce nonspecific binding. Membranes were then exposed to primary antibodies (1 h at room temperature or overnight at 4 C), washed four times, and incubated with secondary goat antimouse or rabbit IgG horseradish peroxidase-conjugated antibody for 1 h. Membranes were washed extensively, and an enhanced chemiluminescence detection assay was performed following the manufacturer's directions.

Immunofluorescence analysis

BMMs grown on chamber slides were treated with or without RANK-L (100 ng/ml) for the indicated times, fixed with methanol, permeabilized with 0.5% Triton X-100 for 30 min, and then blocked with 20% normal serum in PBS for 20 min at room temperature. Polyclonal antibodies to NF-κB p50 and p65 were applied overnight at 4 C, followed by a 45-min incubation at room temperature with fluorescein-conjugated goat antirabbit IgG. The cells were washed and mounted with an antifade kit (Molecular Probes, Inc., Eugene, OR). Immunofluorescence analysis was performed with a Nikon E-800 microscope (Nikon, Melville, NY) and a Microradiance confocal system (Bio-Rad Laboratories, Inc.). Data were collected and analyzed with LaserSharp NT-2000 software.

EMSA

Nuclear extracts (1.5–3 μg) were incubated with an end-labeled double stranded oligonucleotide probe containing the sequence 5′-AAACAGGGGGCTTTCCCTCCTC-3′ derived from the κB3 site of the TNF promoter (20) (GenBank accession no. U68415, bases 481–502) for BMMs and 5′-AGTTGAGGGGACTTTCCCAGCC-3′ (Santa Cruz Biotechnology, Inc.) for RAW 264.7 cells. The reaction was performed in a total of 20 μl binding buffer [20 mM HEPES (pH 7.8), 100 mM NaCl, 0.5 mM dithiothreitol, 1 μl of poly(dI-dC), and 10% glycerol] for 20 min at room temperature. For supershift assay, the nuclear extract was incubated with specific antibodies for an additional 30 min. The samples were fractionated on a 4–20% TBE gel (Novex, San Diego, CA) and visualized by exposing dried gel to film.

Transfection and reporter gene assays

RAW 264.7 cells were transiently transfected with a plasmid containing a luciferase reporter gene driven by two repeats of human immunodeficiency virus type 1 κB enhancer (provided by Dr. David V. Goeddel, Tularik, Inc, South San Francisco, CA) and a plasmid containing cytomegalovirus β-galactosidase using SuperFect transfection reagent (QIAGEN, Valencia, CA). Transfected cells were lysed in reporter lysis buffer (Promega Corp., Madison, WI) and mixed with luciferase assay reagent (Promega Corp.), and luciferase activity was measured in a luminometer (MGM Instruments, Hamden, CT). β-Galactosidase activity was determined by mixing the same amount of cell lysate and assay 2 × buffer (Promega Corp.) and reading the absorbance at 420 nm after 30-min incubation at 37 C.

Results

RANK-L induces serine phosphorylation and subsequent degradation of IκBα in osteoclast precursors

Activation of NF-κB is an event required for osteoclast differentiation *in vivo* (7, 8). To determine whether NF-κB is activated by RANK-L in osteoclast precursors, in the form of BMMs, as well as in RAW 264.7 cells, a line capable of forming osteoclasts in response to RANK-L (18), cells were treated with the cytokine, and cytosolic extracts were subjected to Western blotting. We found that RANK-L treatment of both BMMs and RAW 264.7 cells causes rapid serine phosphorylation of IκBα at 5 min, an event followed by degradation and resynthesis of the protein (Fig. 1). Consistent with the known capacity of NF-κB to transactivate the IκBα gene (21) cytosolic levels of the inhibitory protein gradually increase.

In most cell types, mobilization of NF-κB involves phosphorylation of IκBα serine residues 32/36, resulting in ubiquitination and rapid proteosomal degradation of the phosphorylated inhibitory protein (22–25). We previously reported that activation of NF-κB in BMMs by another osteoclastogenic cytokine, TNFα, is mediated by c-Src-dependent tyrosine phosphorylation of IκBα without degradation of the protein (26). TNFα and RANK-L are both members of the TNF superfamily, raising the possibility that each cytokine prompts tyrosine phosphorylation of IκBα. Tyrosine phosphorylation of IκBα is not detectable within 1 h of RANK-L treatment (data not shown), suggesting that NF-κB activation in BMMs induced by RANK-L involves the clas-

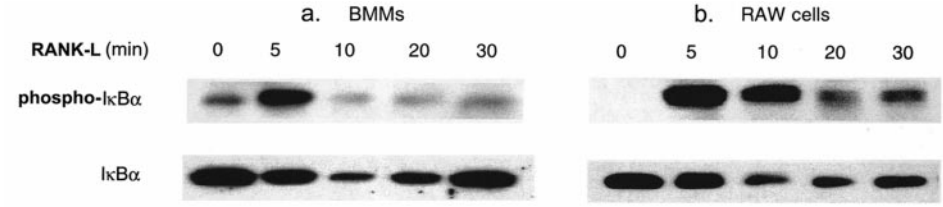

FIG. 1. RANK-L induces serine phosphorylation and subsequent degradation of IκBα. BMMs (a) and RAW 264.7 cells (b) were treated with RANK-L (100 ng/ml) for the indicated time, and cytosolic extracts were electrophoresed and analyzed by Western blotting with antibodies against phospho-IκBα (serine 32) and IκBα.

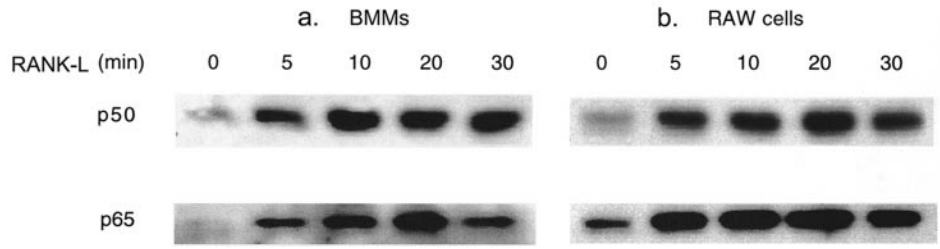

FIG. 2. RANK-L induces nuclear translocation of NF-κB. BMMs (a) and RAW 264.7 cells (b) were treated with RANK-L (100 ng/ml) for the indicated time, and nuclear extracts were electrophoresed and analyzed by Western blotting with antibodies against p50 or p65.

sical pathway involving IκBα serine phosphorylation and degradation.

RANK-L induces nuclear translocation of p50/p65

Treatment of both osteoclast precursors and RAW 264.7 cells with RANK-L leads to degradation of IκBα, the protein that retains NF-κB members in the cytosol. Thus, the disappearance of IκBα is likely to be followed by nuclear translocation of NF-κB, which comprises dimers of specific subunits. To confirm subcellular translocation of NF-κB and to identify the subunits contained in the NF-κB complex in response to RANK-L, Western blots were performed using nuclear extracts from both BMMs and RAW 264.7 cells. As shown in Fig 2, RANK-L promoted rapid appearance of p50 and p65 in nucleus in both cell types. In parallel studies, intact cells treated under the same experimental conditions were processed for immunofluorescence and analyzed using anti-p50 and anti-p65 antibodies. Both p50 and p65 were predominantly localized in cytoplasm in naive cells, whereas treatment with RANK-L led to their nuclear accumulation (Fig. 3).

RANK-L induces NF-κB activation

Although the data in Figs. 2 and 3 demonstrate that RANK-L treatment of osteoclast precursors leads to translocation of the p50/p65 complex, they do not provide direct evidence that the event is followed by DNA binding and activation of transcription. To examine these questions, both BMMs and RAW 264.7 cells were treated with RANK-L, and nuclear extracts were subjected to EMSA.

As shown in Fig. 4A, a complex recognizing the κB3 sequence of the TNF promoter (TNF-κB3), appears after 5 min of RANK-L treatment of BMMs and persists for at least 30 min. The specificity of the EMSA probe was established by competition with excess unlabeled TNF-κB3 and a consensus binding site for NF-κB (Santa Cruz Biotechnology, Inc.). In contrast, mutated forms of the consensus oligonucleotides fail to compete with TNF-κB3 (Fig. 4B). Use of the same probe to identify nuclear-residing NF-κB in RANK-L-treated RAW cells yielded positive, but less dramatic, results (data not shown). On the other hand, when an NF-κB consensus oligonucleotide (Santa Cruz Biotechnology, Inc.) was used, specific RANK-L-stimulated NF-κB nuclear translocation was also clearly evident in RAW cells (Fig. 4, C and D).

To identify the subunits contained in the RANK-L-activated NF-κB complex, we performed EMSA, using TNF-κB3 as a probe for BMMs and an NF-κB consensus oligonucleotide (Santa Cruz Biotechnology, Inc.) for RAW cells in the absence or presence of anti-p50 and p65 antibodies. Addition

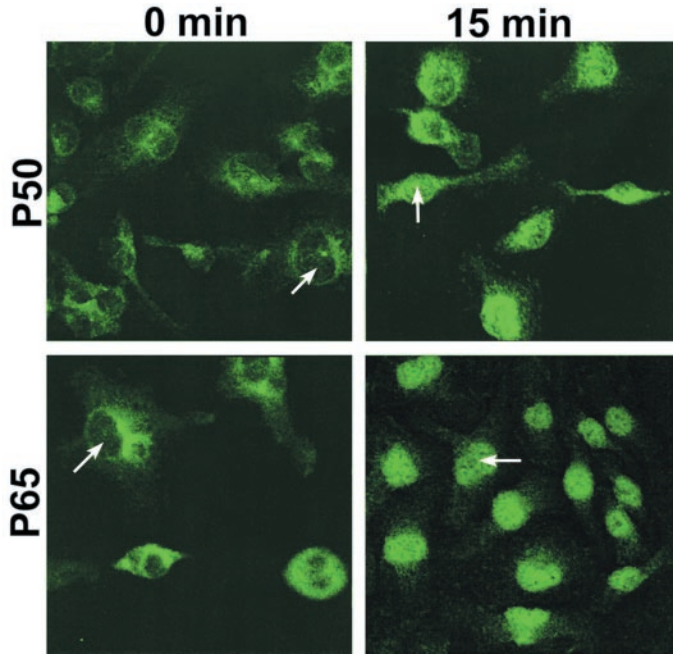

FIG. 3. RANKL induces subcellular translocation of p50/p65. BMMs treated without or with RANK-L (100 ng/ml, 15 min) were fixed and incubated with antibodies against p50 or p65, followed by FITC-conjugated second antibodies. The subcellular localization of fluorescein isothiocyanate-labeled proteins was visualized by confocal laser microscopy. *Arrows* identify cell nuclei.

of anti-p50, anti-p65, or both antibodies led to supershift of the specific DNA-bound protein in both cell types, suggesting that the RANK-L-induced DNA binding moiety consists largely of p50/p65, the most common NF-κB heterodimer (Fig. 5).

RANK-L stimulates an NF-κB-responsive reporter in RAW 264.7 cells

Nuclear translocation of NF-κB typically results in activation of genes containing one or more consensus binding sequences to which the heterodimeric complex can bind. To determine whether RANK-L induced nuclear translocation of the p50/p65 is functionally important, we transiently transfected RAW 264.7 cells with an NF-κB-responsive reporter construct. RANK-L stimulated luciferase expression by NF-κB-dependent reporter plasmid containing cells in a dose-dependent manner (Fig. 6).

Discussion

Osteoclasts are multinucleated cells of the macrophage lineage that form by fusion of mononuclear precursors under

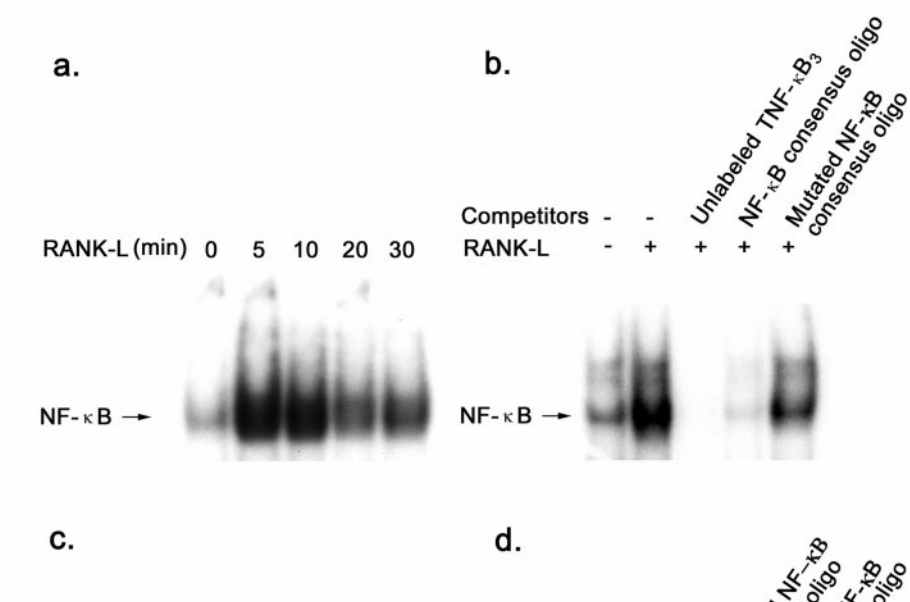

FIG. 4. RANK-L induces activation of p50/p65. a, BMMs were exposed to 100 ng/ml RANK-L for the indicated length of time. Nuclear proteins from BMMs were extracted, and EMSA was performed using the TNF-κB3 sequence as a probe. b, EMSA was performed with RANK-L-untreated and -treated (10 min) BMMs in the absence or presence of a 50-fold excess of unlabeled TNF-κB3 or a consensus binding site for NF-κB and its mutated form. c, RAW 264.7 cells were exposed to 100 ng/ml RANK-L for the indicated length of time. Nuclear proteins were extracted, and EMSA was performed using an NF-κB consensus oligonucleotide (Santa Cruz Biotechnology, Inc.) as a probe. d, EMSA was performed with RANK-L-untreated and -treated (15 min) RAW cells in the absence or presence of a 50-fold excess of unlabeled oligonucleotide or its mutated form.

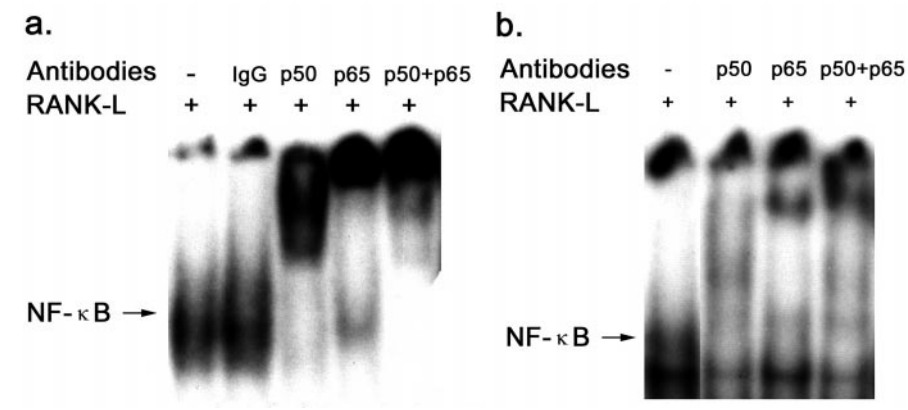

FIG. 5. RANK-L-activated NF-κB includes p50/p65 heterodimer. Nuclear extracts were prepared from RANK-L-exposed BMMs (100 ng/ml, 10 min; a) and RAW 264.7 cells (100 ng/ml, 15 min; b) and subjected to EMSA in the absence or presence of antibodies to p50 or/and p65.

control of the two key cytokines, RANK-L and M-CSF (1–3). An unresolved issue concerns the identity of transcriptional events activated by RANK-L during differentiation of mononuclear precursors. The first step in characterization of these target genes involves defining the transcription factors that are activated after exposure of precursors to osteoclastogenic cytokines.

Differentiation along the macrophage/osteoclast lineage requires the transcription factors, c-Fos, which activates the c-Jun N-terminal kinase (JNK) signaling pathway (4, 5), and NF-κB (7, 8). Although RANK-L is central to the osteoclastogenic process, whether it does so via NF-κB and/or JNK is controversial. Thus, Wong *et al.* found that RANK-L treatment of murine osteoclasts fails to activate JNK (17). In contrast, Jimi and his colleagues demonstrated a time- and dose-dependent increase in JNK activation in osteoclasts following exposure to RANK-L (16). Although their studies do not address osteoclastogenesis, Hsu *et al.* reported that RANK-L stimulated JNK, but not NF-κB, in RAW 264.7 cells (18). This latter study stands in contrast to ours, which establishes RANK-L-induced NF-κB activation in this osteoclastogenic line as well as authentic osteoclast precursors. We also found that RANK-L-induced NF-κB nuclear translocation is RAW 264.7 clone dependent (data not shown). We posit, therefore,

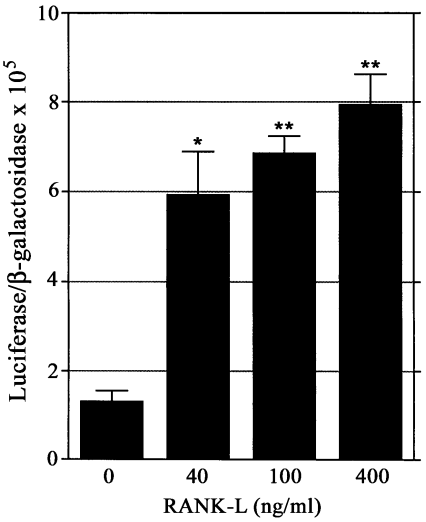

FIG. 6. RANK-L promotes NF-κB-dependent gene expression. RAW 264.7 cells were transiently cotransfected with a plasmid containing a luciferase reporter gene driven by two repeats of the human immunodeficiency type 1 (HIV-1) κB enhancer and a plasmid containing cytomegalovirus β-galactosidase. Twenty-four hours after transfection, cells were treated without or with 40, 100, or 400 ng/ml RANK-L for 6 h. Luciferase activities were measured and normalized by β-galactosidase expression. The values shown are the mean ± SD of triplicate determinations. *, $P < 0.005$; **, $P < 0.0001$ (*vs.* untreated cells, by unpaired *t* test).

that the inconsistency between Hsu's data and ours reflex their use of a nonresponsive clone.

The NF-κB family has been implicated in osteoclast formation, function, and survival (22). Thus, mice lacking p50 have fewer osteoclasts, whereas neither mature osteoclasts nor tartrate-resistant acid phosphatase-positive precursors are present in p50/p52 null mice (7). On the other hand, a recent report suggests that NF-κB is involved in activation, but not survival, of osteoclasts (27), diverging from the posture that interleukin-1 (IL-1) promotes osteoclast survival (28) through activation of an NF-κB complex containing p50 and p65 (29).

NF-κB is activated by numerous agonists, including inflammatory cytokines such as TNFα, phorbol myristyl acetate, DNA-damaging agents, double stranded RNA, and viruses, including human immunodeficiency virus type 1 (22–25). We now report that RANK-L activates NF-κB during the process of osteoclast differentiation. Treatment of primary osteoclast precursors, in the form of BMMs, and RAW 264.7 cells results in rapid nuclear translocation of p50 and p65, the two most common subunits of the NF-κB family, where they associate with a consensus DNA-binding sequence. The capacity of RANK-L to transactivate an NF-κB reporter construct in RAW cells establishes that the nuclear translocated transcription complex is functional. Importantly, we found that M-CSF fails to activate NF-κB in BMMs (data not shown), confirming the specificity and importance of RANK-L in this process.

We have previously shown that TNFα activates NF-κB in BMMs by c-Src tyrosine phosphorylation of IκBα (26). To determine whether such is the case for RANK-L, we examined the phosphorylation status of IκBα in BMMs and RAW 264.7 cells after exposure to the cytokine. We found that the inhibitory protein is rapidly phosphorylated at serine 32 by RANK-L, degraded, and then resynthesized. In contrast to TNFα, tyrosine phosphorylation of IκBα does not occur in BMMs in response to RANK-L.

The fact that NF-κB activation is required for osteoclast formation and function may be central to the pathogenesis of postmenopausal osteoporosis and rheumatoid arthritis, in which osteoclastogenesis is accelerated. In addition to RANK-L, increased generation and/or activity of osteoclasts can be mediated by IL-1 and TNFα (30–32), which are abundant in rheumatoid synovium and are potent activators of NF-κB. Interleukin-1 targets stromal cells and/or osteoblasts, leading to the release of RANK-L (33). In addition, the cytokine acts directly on preosteoclasts generated in a stroma coculture system (and hence differentiated with RANK-L) to stimulate their fusion and survival (34). Additionally, IL-1 enhances the survival and activation of mature osteoclasts (28). The fusogenic, survival, and activation functions of IL-1 are accompanied by activation of NF-κB (28, 34, 35). In contrast, there are no reports that IL-1 can itself replace RANK-L in the differentiation of osteoclast precursors. Although earlier studies suggested that TNFα, acting directly on precursors, could act independently of RANK-L to induce osteoclast formation (36, 37), recent efforts reveal that cells need to be primed by preexposure to RANK-L before TNFα alone can be osteoclastogenic (38). In summary, we provide the first data that RANK-L activates the NF-κB signaling pathway in murine osteoclast precursors. As neither IL-1 nor TNFα, both of which activate the same NF-κB signals as does RANK-L, can replace the latter molecule in the osteoclastogenic process, we conclude that NF-κB activation is necessary, but not sufficient, for osteoclastogenesis. However, the fact that RANK-L and these inflammatory, bone-resorptive cytokines prompt NF-κB activation postures this transcription complex as a potential antiosteoclastogenic therapeutic target.

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