A Novel Zinc Finger Protein That Inhibits Osteoclastogenesis and the Function of Tumor Necrosis Factor Receptor-associated Factor 6*

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A variety of surface receptors eliciting diverse cellular responses have been shown to recruit tumor necrosis factor receptor-associated factor (TRAF) adaptor molecules. However, a few TRAF-interacting intracellular proteins that serve as downstream targets or regulators of TRAF function have been identified. In search of new intracellular molecules that bind TRAF6, we carried out a yeast two-hybrid cDNA library screening with an N-terminal segment of TRAF6 as the bait. A novel human C2H2-type zinc finger family protein was identified, which when coexpressed with TRAF6 led to a suppression of TRAF6-induced activation of NF-κB and c-Jun N-terminal kinase. This novel protein was designated TIZ (for TRAF6-inhibitory zinc finger protein). TIZ expression also inhibited the signaling of RANK (receptor activator of NF-κB), which together with TRAF6 has been shown to be essential for osteoclastogenesis. Furthermore, the expression level of TIZ appeared to be regulated during the differentiation of human peripheral blood monocytes into osteoclasts. More significantly, transfection of TIZ into the monocytic/macrophage cell line Raw264.7 reduced the RANK ligand-induced osteoclastogenesis of this cell line. Our findings suggest that the novel zinc finger protein TIZ may play a role during osteoclast differentiation by modulating TRAF6 signaling activity.

The tumor necrosis factor receptor-associated factor (TRAF) family proteins have initially been identified as adaptor proteins that bind to the cytoplasmic domain of tumor necrosis factor receptor 2 (TNFR2) (1). To date, six mammalian TRAF genes and three Drosophila homologs have been described (2–4). All TRAF proteins, except TRAF1, are composed of an N-terminal RING finger domain, several repeats of the zinc finger motif, the TRAF-N domain with a coiled-coil structure, and the TRAF-C domain (2, 3). TRAF1 lacks the RING finger and contains only one zinc finger (3). TRAF4 is also distinct from other TRAFs in that it possesses nuclear localization signals (5).

The TRAF domain mediates the homo- and heteroassociation between TRAF proteins and the interaction of TRAF proteins with the cytoplasmic domain of TNFR family members such as TNFR2, CD40, CD30, and receptor activator of NF-κB (RANK). The interaction of a TRAF and a receptor appears to be trimeric in nature, since crystal structure studies revealed a homotrimer of tumor necrosis factor (6) that induces receptor cross-linking and trimerization (7) and a mushroom-shaped trimer of the TRAF domain of TRAF2 (8, 9). TRAF proteins are also recruited to cell surface receptors indirectly through other intracellular proteins. TRAF2 binds TNFR-associated death domain protein, which interacts with TNFR1 (10), and TRAF6 interacts with interleukin-1 receptor (IL-1R)-associated kinase, which is recruited to the Toll/IL-1R family receptor complexes through MyD88 (11, 12). Other intracellular adaptor proteins have also been shown to interact with TRAF proteins and regulate the function of TRAF proteins. Among these adaptor proteins are TANK/TRAF, c-IAPs, TRIP, and A20 (3). The cytokine-induced zinc finger protein A20 has been shown to bind the TRAF domain of TRAF1, TRAF2, and TRAF6 and to inhibit the NF-κB activation by TRAF2 and TRAF6 (13, 14).

The N-terminal portion of TRAF proteins seems to have effector functions. The regulation of NF-κB and c-Jun N-terminal kinase (JNK) is the most extensively studied function of TRAF proteins. Overexpression of TRAF2, TRAF5, or TRAF6 has been shown to induce the activation of NF-κB and JNK (15), and oligomerization of only the N-terminal portion of TRAF2 and TRAF6 has been demonstrated to be sufficient to induce the response (16). The TRAF-induced NF-κB activation is believed to occur by sequential processes of the activation of NF-κB-inducing kinase, which induces activation of IκB kinase, phosphorylation and subsequent degradation of IκB, and nuclear translocation of liberated active NF-κB (17). For the activation of JNK, other serine/threonine kinases (apoptosis-inducing kinase, mitogen-activated protein kinase/extracellular-signal-regulated kinase 1 and 2), which are recruited to the cell surface, are activated by TRAFs (18).}

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‡ The abbreviations used are: TRAF, tumor necrosis factor receptor-associated factor; TNFR, tumor necrosis factor receptor; RANKL, receptor activator of nuclear factor-κB ligand; IL, interleukin; IL-1R, interleukin-1 receptor; JNK, c-Jun N-terminal kinase; PBMC, peripheral blood mononuclear cell(s); OCL, osteoclast-like cell; M-CSF, macrophage colony-stimulating factor; TRAP, tartrate-resistant acid phosphatase; KRAB, Krüppel-associated box; RACE, rapid amplification of cDNA ends; FBS, fetal bovine serum; GST, glutathione S-transferase; MEM, minimum essential medium.
lar signal-regulated kinase kinase, and transforming growth factor-β-activating kinase 1) have been reported to channel the TRAF signal to SEK1 and MKK7, the upstream activating kinases of JNK (3).

The in vivo function of some TRAF proteins has been demonstrated by knock-out mouse studies. TRAF6-deficient mice generated independently by two research groups displayed severe osteopetrosis caused by impaired activity of osteoclasts, cells primarily involved in bone resorption (18, 19). Naito et al., demonstrated that the differentiation of osteoclasts by the well known osteoclastogenesis factor RANK ligand (RANKL; also called ODF, OPG, and TRANCE) is blocked in cells derived from TRAF6−/− mice, underlining the essential role of TRAF6 for RANK signaling in vivo (18). In support of this result, several in vitro studies showed binding of TRAF6 to RANK and involvement of TRAF6 in the RANK-induced activation of NF-κB and JNK (20–22). The importance of TRAF6 in signaling of IL-1, CD40, and lipopolysaccharide was also demonstrated with TRAF6−/− cells (19).

Considering the importance of TRAF6 in signaling of RANK and other TNFR and IL-1R/Toll family proteins, we sought to find new molecules that bind TRAF6 and possibly work as new downstream targets or regulators of TRAF6. Since the N-terminal region of TRAF6, which does not contain any catalytic activity, is sufficient for its effector functions (16), we reasoned that this part might mediate protein-protein interactions with potential downstream targets and regulators of TRAF6 function. Therefore, we used the N-terminal part of human TRAF6 as a bait for the yeast two-hybrid screening. A novel protein containing several repeats of a zinc finger motif was found to bind TRAF6. Transfection of the gene encoding this protein suppressed the NF-κB and JNK activation by TRAF6 and the RANK-dependent signaling, leading to our nomenclature TIZ, for TRAF6-inhibitory zinc finger protein. TIZ expression was found to change during differentiation of osteoclasts from peripheral blood monocytes, and transfection of TIZ into the macrophage/macrophage cell line Raw264.7 resulted in a decrease in formation of multinuclear osteoclast-like cells in response to RANKL.

**EXPERIMENTAL PROCEDURES**

Yeast Two-hybrid Assays—The yeast two-hybrid screening was performed using the MATCHMAKER GAL4 Two-Hybrid System (CLONTECH, Palo Alto, CA). To construct the bait vector, DNA fragments encoding amino acids 1–274 of human TRAF6 were amplified by PCR with the forward primer 5′-CGCGATATCGTGTCTAAACTGTG-GAA-3′ (EcoRI site underlined) and the reverse primer 5′-CGCGATCCGGCCCAACATCTGTCG-3′ (BamHI site underlined) and cloned into pGK19 using EcoRI and BamHI restriction sites. The bait vector and human HeLa MATCHMAKER cDNA library DNAs were sequentially transformed into Saccharomyces cerevisiae yeast reporter strain Y190 and cultured on SD/Leu plates (8–10×10^5) adding control vector DNAs. 20 μl of purified GST fusion proteins were precipitated with 2 μl of anti-TRAF6 and 20 μl of protein A-Sepharose beads and incubated for 1 h at 4 °C. The beads were washed five times in the lysis buffer and subjected to Western blotting.

**Confocal Microscopy**—The full-length hTIZ and TRAF6 were subcloned into the pDsRed1-C1 and pEGFP-C2 vectors (CLONTECH), respectively. COS-7 cells were transfected with pDsRed1-C1-TIZ and pEGFP-C2-TRAF6 using Superfect (Qiagen) following the manufacturer’s instruction. 24 h after transfection, confocal microscopy was performed with a Carl Zeiss Axiovert 135 M microscope and LSM410 at Korea Basic Science Institute (Daejeon, Korea).

**JNK Activity Assays**—293-EBNA cells were plated into six-well plates (8 × 10^5/well) the next day. Cells were transfected with 0.5 μg of pEG-JNK and 2 μg of other indicated DNAs plus 8 μg of SuperFect reagent (Qiagen). The total amounts of DNA were kept constant by adding control vector DNAs. 20–36 h after transfection, cell lysates were prepared as described above. 300 μg of cleared lysates were incubated with glutathione-Sepharose beads for 2 h at 4 °C. The precipitated beads were extensively washed and subjected to kinase reactions as previously described (23) except omitting radiolabeled ATP and detecting the phosphorylated substrates by Western blotting with an anti-phospho-c-Jun (Cell Signaling, Beverly, MA).

**Electrophoretic Mobility Shift Assays**—Cells were lysed in a hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and scraped into a microcentrifuge tube. Nonidet P-40 was added to 0.1% and cells were extensively washed with the lysis buffer and then microcentrifuged for 20 min at 10,000 × g. Protein concentrations of the supernatants were determined by using the DC Protein Assay Kit (Bio-Rad), and 10–20 μg of cellular proteins were resolved by SDS-PAGE and then transferred to Immobilon membranes (Millipore, Watford, England). The membranes were blocked with 5% nonfat dry milk in TBS-T for 1 h and then incubated for 1 h with primary antibodies. The membranes were washed extensively, then incubated with a 1:1000 dilution of anti-FLAG (Eastman Kodak Co.) or anti-TRAF6 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). For immunoprecipitation, cell extracts were five times ultracentrifuged in and out, sonicated for 10 min with a sonicator (Pomadex, Madison, WI), and 20 μl of lysates were used for detection of luciferase activity with a luminometer (EG&G Berthold, Wilbad, Germany).

**GST Pull-down Assays**—The partial cDNA of TIZ (74–212) was subcloned into pGEX4T-1 (Amersham Biosciences, Inc.) for bacterial GST fusion protein expression. The transformed Escherichia coli culture was stimulated with 1 μg/mL isoprison-β-thiogalactoside for 2 h, and the whole cell lysate was obtained by sonication. The GST proteins were purified using glutathione-Sepharose beads. 2 μg of purified GST fusion proteins were incubated with lysates of transfected 293-EBNA cells and 20 μl of glutathione-Sepharose beads for 1 h at 4 °C. The precipitates were extensively washed in the lysis buffer and subjected to Western blotting as described above.

**Western Blotting and Immunoprecipitation**—Western blotting and immunoprecipitation analyses were performed as described (21). Briefly, cells were incubated in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors for 30 min on ice and scraped into microcentrifuge tubes. The whole cell extracts were vigorously pipetted in and out, sonicated for 5 min with a setting of 2–3 pulse and 7–8 rest at 50% amplification (Vibra Cell, Sonics & Materials, Danbury, CT), and then microcentrifuged through 100 μl of a 1:1 mixture of 105–g of purified GST fusion proteins were precipitated with 2 μl of anti-TRAF6 and 20 μl of protein A-Sepharose beads and incubated for 1 h at 4 °C. The beads were washed five times in the lysis buffer and subjected to Western blotting.

For immunoprecipitations, lysates were mixed with 2 μl of anti-TRAF6 and 20 μl of protein A-Sepharose beads and incubated for 1 h at 4 °C. The beads were washed five times in the lysis buffer and subjected to Western blotting.

**RT-PCR Analyses**—100 ng of total RNA were reverse-transcribed with SuperScriptII reverse transcriptase (Invitrogen) and amplified by PCR. For TIZ expression analyses of samples from various human cells and tissues, a forward primer (5′-GTGAGTTAGACCCCCCAGTAAGTG-3′) and a reverse primer (5′-TTTTAGTTAGCAGCTCAAG-3′) were used. The expected size was 207 bp. The forward and reverse primers for β-actin were 5′-CAAGAGATGGCCAGCGGCTG-3′ and 5′-TCCCTTC- GCATCTCTGTGCGGCA-3′, respectively. The PCR products were separated on agarose gels and stained with ethidium bromide.
4000 rpm for 15 min, the pellet was lysed in 15 μl of a high salt buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 25% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) for 20 min on ice. 75 μl of storage buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) was added, agitated for 10 s by vortexing, and then microcentrifuged at 14,000 rpm for 20 min. Protein concentrations of the supernatants were measured with the DC Protein Assay Kit (Bio-Rad). The NF-κB binding site oligomer 5′-AGTT-GAGGGGACTTCCACGGC-3′ (Santa Cruz Biotechnology) was random labeled with [α-32P]ATP and Klenow enzyme. 10 μg of nuclear extracts were incubated with ~20,000 cpm of probe in 20 μl of the reaction buffer containing 10 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 5% glycerol, 2 mM dithiothreitol, and 1 μg of poly(dI-dC) for 30 min at 20 °C. The DNA-bound NF-κB proteins were subjected to 4–5% polyacrylamide gel electrophoreses followed by autoradiography.

**DIFFERENTIATION OF PBMC AND RAW264.7 CELLS TO OSTEOCLAST-LIKE CELLS**—Differentiation of human peripheral blood mononuclear cells (PBMC) was carried out as previously described (24, 25). Human peripheral blood was obtained from healthy volunteers and mononuclear cell (PBMC) was carried out as previously described (24, 25). Human peripheral blood mononuclear cells were plated at 1 × 10^6 cells/well in 48-well plates for the observation of cell morphology and at 7 × 10^5 cells/well in six-well plates for RNA preparation and were incubated for 16 h at 37 °C in a humidified atmosphere of 5% CO2. The next day, incubation with 50 ng/ml RANKL (Peptropatch EC, London, UK) and 100 ng/ml M-CSF (Chemicon, Temecula, CA) was started. The RANKL/M-CSF treatment was continuous for 14 days, with half of the medium being changed every 3–4 days. Cells at 0, 5, 10, and 14 days of treatment were photographed or harvested for RT-PCR analyses.

**RESULTS**

**Identification of TIZ**—To find a new molecule that binds TRAF6, the yeast two-hybrid screening was carried out. Since the N-terminal region of TRAF6 has been suggested to be sufficient for the effector functions of TRAF6 (16), we reasoned that this part might interact with potential downstream targets and regulators of TRAF6 function. Therefore, we used the N-terminal 274 amino acid residues, encompassing the Ring and zinc finger domains, of human TRAF6 as the bait. When a HeLa cell cDNA library was screened, 14 positive clones were obtained. One clone (74-2; encodes amino acids 95–213 of TIZ) showed the strongest binding on filter and liquid β-galactosidase assays. The yeast two-hybrid interaction of the 74-2 clone with TRAF6 was specific, since this clone did not interact with TRAF2, TRAF3, and a non-TRAF family protein, and at 7 days of treatment were photographed or harvested for RT-PCR analyses.
A Novel Zinc Finger Protein Inhibitory to TRAF6 Function

TIZ interacts with TRAF6 in mammalian cells. A, 293-EBNA cells were transfected with a TRAF6 expression plasmid or the control vector. The lysates were prepared and incubated with 2 μg of either GST or GST fusion protein containing the part of TIZ that bound TRAF6 in the yeast two-hybrid screening. The GST proteins were precipitated with glutathione beads and subjected to Western blotting with anti-TRAF6. B, 293-EBNA cells were transfected with 200 ng of pM-TRAF6 and/or pVP16-TIZ plasmids along with 25 ng of GAL4/Tx-luc reporter vector. 24 h after transfection, the luciferase activity was measured with cell lysates. Data are presented as means ± S.D. of triplicate samples. C, 293-EBNA cells were transfected with the indicated plasmids. Cell lysates were prepared, and immunoprecipitations were carried out with anti-TRAF6. The precipitated proteins were resolved by SDS-PAGE and subjected to Western blotting with anti-FLAG (top panel). The same membrane was stripped and reprobed with anti-TRAF6 (middle panel). Aliquots of whole cell extracts were subjected to anti-FLAG Western blotting (bottom panel). D, COS-7 cells were cotransfected with pDsRed1-TIZ and pEGFP-TRAF6 plasmids. 24 h after transfection, cells were fixed, and confocal microscopy was performed. The light microscopic image (i), the TIZ expression pattern (ii), and the TRAF6 expression pattern (iii) of a single cell and the combined picture of the three images (iv) are shown.

identical to this gene. This novel gene was named TIZ, and its sequence information was deposited in GenBank™ (accession number AY044432). Cloning of a partial cDNA for mouse TIZ, which corresponds nucleotides 879–1732 of human TIZ, and shows 99.6% identity to human TIZ (data not shown), further suggests that TIZ is a new gene. Amino acid sequence analyses revealed a region homologous to the (Krüppel-associated box) (KRAB) domain at the N terminus (amino acids 4–75) and 14 repeats of the C_{3H2} type zinc finger motif at the C-terminal region (amino acids 174–558) (Fig. 1B). Among the 14 zinc finger repeats, 10 have authentic C_{3H} sequences, whereas four had variations in the histidine residues. The amino acid sequences of TIZ showed the highest homology to human zinc finger protein 85 (82% homology by the ClustalW program). The relative closeness of TIZ to other zinc finger proteins analyzed using the ClustalW algorithm is shown in Fig. 1C. TIZ showed very low sequence identity (less than 15%) to the zinc finger proteins A20, Cezanne, and TRABID, which were previously reported to bind TRAF proteins. RT-PCR analyses showed expression of TIZ in various human cell types and tissues (Fig. 1D). No apparent induction of TIZ expression contingent to tumorigenesis was detected in an examination of three cancers and their control normal tissues (Fig. 1D, lane 6 versus lane 7, lane 9 versus lane 10, and lane 11 versus lane 12).

Interaction of TIZ with TRAF6 in Mammalian Cells—The result of yeast-two-hybrid assay (Fig. 1A) suggested specific interaction of TIZ with TRAF6 in yeast. To directly assess the binding capability of TIZ to TRAF6, the TIZ partial cDNA from the yeast two-hybrid positive clone 74-2 was subcloned into a GST fusion vector. When the GST-TIZ and control GST proteins were incubated with lysates from 293-EBNA cells transfected with TRAF6 (Fig. 2A, lane 2) or the control (lane 1) expression plasmids, GST-TIZ specifically bound both the endogenous (lane 4) and transfected (lane 6) TRAF6 protein, while GST alone did not interact with TRAF6 (lanes 3 and 5). To confirm the interaction in mammalian cells, we performed the two-hybrid assay in 293-EBNA cells. Cotransfection of pM-TRAF6 encoding the full-length TRAF6 fused to the Gal4 DNA binding domain and pVP16-TIZ encoding the conjugate of VP16 transactivation domain and TIZ greatly induced transcription of the reporter gene under the control of the Gal4-responsive element (Fig. 2B). Coimmunoprecipitation of TIZ and TRAF6 also supported the interaction of these proteins in mammalian cells (Fig. 2C). We next examined the intracellular distribution of TIZ and TRAF6. TIZ and TRAF6 were expressed as the red fluorescent protein and green fluorescent protein fusion proteins, respectively, in COS-7 cells, and the confocal microscopy was performed. As shown in Fig. 2D, both TIZ and TRAF6 were detected in perinuclear cytoplasmic regions as dotlike structures. Weak signals of TIZ were also observed in the nucleus (Fig. 2D, ii). When transfected into 293 cells, the nuclear signal was stronger (data not shown). The superimposition of the red and green channels revealed yellow signals in many, but not in all, locations, indicating colocalization of TIZ and TRAF6 in discrete intracellular regions (Fig. 2D, iv).

Effects of TIZ on TRAF6 Signaling Pathways—Two well-characterized effects of TRAF6 signaling are the activation of NF-κB transcription factor and c-Jun N-terminal kinase (JNK). We investigated whether TIZ has effects on the signaling functions of TRAF6. Overexpression of TRAF6 in 293 cells has been reported to result in NF-κB activation (15). Confirming previous reports, transient transfection of TRAF6 into 293-EBNA
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A, 293-EBNA cells were transfected with 50 ng of pNF-κB-Luc and 500 ng of indicated TRAF plasmid with or without 500 ng of TIZ DNA. 36 h after transfection, the luciferase assay was performed. Results of duplicate samples are shown (mean ± S.D.). B, cells were transfected with 50 ng of pNF-κB-Luc, 500 ng TRAF6, and the indicated amounts of TIZ plasmids. 24 h after transfection, cells were harvested, nuclear extracts were prepared, and the electrophoretic mobility shift assay was performed with an NF-κB binding site oligomer as described under "Experimental Procedures." D, 293-EBNA cells plated in a six-well plate were transfected with 2 μg of indicated plasmids. 24 h after transfection, cells were harvested, nuclear extracts were prepared, and the electrophoretic mobility shift assay was performed with an NF-κB binding site oligomer as described under "Experimental Procedures." E, 293-EBNA cells were transfected with 50 ng of pAP-1-Luc, 500 ng of TRAF6, and 500 ng of TIZ plasmids. The luciferase assay was performed 24 h after transfection. The means ± S.D. of triplicate samples are shown.

cells led to a great increase in the reporter luciferase activity under the control of NF-κB (Fig. 3A). Cotransfection of TIZ reduced the TRAF6-induced luciferase activity in a dose-dependent manner (Fig. 3B). The inhibitory effect of TIZ was specific to TRAF6 as the NF-κB activation induced by TRAF2 and TRAF5 was not affected (Fig. 3A). Whether the effect of TIZ on NF-κB activation was due to a reduced DNA binding of the transcription factor was assessed by the electrophoretic mobility shift assay. The transfection of TRAF6 caused a retarded electrophoretic mobility of NF-κB binding element oligonucleotides in the electrophoretic mobility shift assay (Fig. 3D, lane 2), which was suppressed upon cotransfection of TIZ (Fig. 3D, lane 4). This inhibition was not due to a reduction in TRAF6 expression, since comparable levels of TRAF6 were detected in Western blotting analyses (data not shown). We next examined whether TIZ can also regulate JNK activation by TRAF6. The kinase activity of JNK was stimulated by TRAF6 transfection (Fig. 3D, lane 2). This TRAF6-induced JNK activation was attenuated by cotransfection of TIZ (Fig. 3D, lane 4). JNK can phosphorylate and activate c-Jun that forms with other Jun and Fos family protein AP-1 transcription complexes (26). Therefore, we examined the effect of TIZ on AP-1 activation by the luciferase reporter assay. TRAF6 transfection into 293-EBNA cells increased the AP-1-responsive luciferase activity, and cotransfection of TIZ abrogated the TRAF6-induced AP-1 activation (Fig. 3E). The inhibitory effects of TIZ on the TRAF6-induced activation of NF-κB, JNK, and AP-1 suggest that TIZ may play a role as a negative regulator of TRAF6.

Effects of TIZ on NF-κB Activation by Tumor Necrosis Factor Family Cytokines—TRAF6 has been shown to be an important signaling component for RANK, a tumor necrosis factor receptor family protein essential for osteoclast differentiation (20, 27, 28). Since TIZ suppressed the TRAF6-induced NF-κB activation, we investigated whether TIZ interferes with RANK signaling to NF-κB. Transient transfection of RANK into 293-EBNA cells led to the activation of NF-κB, reflected as an increase in the reporter luciferase activity (Fig. 4A, lane 2). The NF-κB activation by RANK overexpression was abrogated when TIZ was cotransfected (Fig. 4A, lane 2). Stimulation of RANK-transfected 293-EBNA cells with its ligand RANKL further increased the NF-κB activation (Fig. 4A, lane 5), which was abolished by TIZ expression (Fig. 4A, lane 7). We next tested whether this effect of TIZ can be observed with endogenous levels of RANK. The macrophage/monocyte lineage cell line Raw264.7 has been shown to differentiate into multinucleated osteoclast-like cells (OCLs) upon incubation with RANKL (29). The expression of RANK protein on these cells was detected by immunohistochemistry. When Raw264.7 cells were transfected with an NF-κB-responsive luciferase plasmid and stimulated with RANKL, ~4-fold increase in the luciferase activity was observed (Fig. 4B, lane 3). Transfection of TIZ attenuated the RANKL-induced NF-κB activation to 1.9-fold

Fig. 3. TIZ inhibits TRAF6 signaling functions. A, 293-EBNA cells were transfected with 50 ng of pNF-κB-Luc and 500 ng of indicated TRAF plasmid. The luciferase assay was performed. Results of triplicate samples are shown (mean ± S.D.). B, cells were transfected with 50 ng of pNF-κB-Luc, 500 ng TRAF6, and the indicated amounts of TIZ plasmids. After 24 h, the luciferase assay was performed. Results are means ± S.D. of triplicate samples. C, 293-EBNA cells plated in a six-well plate were transfected with 2 μg of indicated plasmids. 24 h after transfection, cells were harvested, nuclear extracts were prepared, and the electrophoretic mobility shift assay was performed with an NF-κB binding site oligomer as described under "Experimental Procedures." D, 293-EBNA cells plated in a six-well plate were transfected with 2 μg of indicated DNA. Cells were harvested 24 h after transfection, and JNK assays were performed as described under "Experimental Procedures." E, 293-EBNA cells were transfected with 50 ng of pAP-1-Luc, 500 ng of TRAF6, and 500 ng of TIZ plasmids. The luciferase assay was performed 24 h after transfection. The means ± S.D. of triplicate samples are shown.

Fig. 4. TIZ suppresses NF-κB activation by RANKL and IL-1 but not by TNF-α. A, 293-EBNA cells were transfected with 50 ng of pNF-κB-Luc, 500 ng of RANK, and 500 ng of TIZ plasmids as indicated. After 24 h, cells were stimulated with 50 ng/ml RANKL or the control vehicle for 16 h. Cells were lysed and subjected to luciferase assays. Relative luciferase activities are shown as means ± S.D. of triplicate samples. B, Raw264.7 cells were transfected with 1 μg of TIZ or the control vector together with 200 ng of pNF-κB-Luc. 24 h after transfection, cells were stimulated with 100 ng/ml RANKL for 16 h, and luciferase activities were measured. Data from triplicate samples are presented as means ± S.D. C, 293-EBNA cells were transfected with 50 ng of pNF-κB-Luc plasmid and 500 ng of TIZ DNA. 16 h after transfection, cells were stimulated with 20 ng/ml IL-1β for 4 h and subjected to luciferase assays. The relative luciferase activities of triplicate samples are shown (means ± S.D.). D, 293-EBNA cells were transfected with 500 ng of TIZ or the control vector along with 50 ng of pNF-κB-Luc DNA. 16 h cells were stimulated with 20 ng/ml TNF-α for 4 h, and the luciferase assay was performed. Results of triplicate samples are presented as means ± S.D.

References

2 J. Y. Lee, H.-H. Kim, and Z. H. Lee, unpublished observation.
A Novel Zinc Finger Protein Inhibitory to TRAF6 Function

Expression Levels of TIZ mRNA during Osteoclastogenesis—Since TRAF6 has been shown to be essential for RANKL-induced differentiation and activation of osteoclasts (18, 19), it was intriguing to know whether the expression of TIZ is regulated during osteoclastogenesis. Human PBMC were induced to differentiate into osteoclasts with M-CSF and RANKL for 14 days as previously reported (24, 25). The fused cells started to appear at day 5, and multinuclear OCLs were clearly detected at day 10 (Fig. 5B). The extent of differentiation was further increased by day 14 (Fig. 5B). The expression levels of TIZ during the osteoclastogenesis of PBMC were assessed by RT-PCR (Fig. 5A). TIZ mRNA was detected in PBMC before M-CSF/RANKL treatment, but the level was reduced by day 5 of the treatment. By day 10, the expression level of TIZ mRNA was recovered to near the level at the start and further increased by day 14 (Fig. 5A). These results may indicate that TIZ expression is negatively regulated during the early stage of osteoclastogenesis, and the negative regulation is released once the progenitor cells are differentiated.

Inhibition of Osteoclast Differentiation by TIZ—Since TIZ inhibits the signaling function of TRAF6 (Fig. 3) and TIZ expression is decreased during the early phase of osteoclastogenesis (Fig. 5), we next examined the effect of untimely TIZ expression on osteoclast differentiation. Raw264.7 cells that can differentiate into OCLs by 4–5 days of incubation of RANKL were transfected with a TIZ expression plasmid or the control vector DNA before initiation of RANKL stimulation. The efficiency of the transfection conditions employed was more than 90% when tested with a green fluorescent protein vector (data not shown). Transfected cells were cultured in the differentiation medium containing 50 ng/ml RANKL for 4 days to induce osteoclastogenesis and then stained for TRAP, a marker of osteoclasts. Transfection of TIZ significantly reduced the number of multinuclear TRAP-positive OCLs compared with the control vector transfection (Fig. 6A). The mock transfection had little effect compared with the untransfected control cells, suggesting that the transfection method used did not affect the differentiation of Raw264.7 cells under the experimental conditions (Fig. 6A). Quantitation of TRAP-positive OCLs revealed about 40% reduction in the number of OCLs containing more than 10 nuclei upon TIZ transfection (Fig. 6B). These results suggest that the proper regulation of TIZ expression is important for osteoclast differentiation.

DISCUSSION

The signaling adaptor molecule TRAF6 has been suggested to mediate the NF-κB activation by CD40, IL-1 receptor, RANK, two lipopolysaccharide receptors (TLR2 (Toll-like receptor 2) and TLR4), and the neurotrophin receptor p75NTR based on results from in vitro transfection studies (11, 20, 30–33). Some of the results were confirmed by studies with cells derived from TRAF6 knock-out mice; the NF-κB activation induced by CD40 ligation, IL-1, lipopolysaccharide, and RANKL was impaired in the TRAF6-deficient cells (18, 19, 34). The IL-1, lipopolysaccharide, and RANKL-induced activation of JNK and p38 mitogen-activated protein kinases is also impaired in the TRAF6-null cells (19, 34). Despite the various functions of TRAF6 in response to many cell surface receptors, not many intracellular proteins that interact with TRAF6 and serve downstream targets or modulators of its effector functions have been identified. In search of new TRAF6-interacting molecules by the yeast two-hybrid approach, we identified a zinc finger protein that appeared to negatively regulate TRAF6 signaling function. This protein was named TIZ (TRAF6-inhibitory zinc finger protein). The gene expression of TIZ was detected in various cell types, including monocytes and macrophages, but not in every cell type (Fig. 1). The TRAF6/TIZ interaction was confirmed in mammalian cells by mammalian two-hybrid, GST pull-down, communoprecipitation, and confocal microscopic analyses (Fig. 2). Ectopic overexpression of TIZ suppressed the TRAF6-, RANKL-, and IL-1-induced activation of NF-κB and JNK (Figs. 3 and 4). The functional significance of TIZ was inferred by the regulated expression pattern of this gene during the RANKL-driven differentiation of PBMC into osteoclast-like cells (Fig. 5). Furthermore, enforced
expression of this gene suppressed the RANKL-induced OCL formation from Raw264.7 cells (Fig. 6).

The amino acid sequences of TIZ predict that this protein belongs to the family of KRAB zinc finger proteins (35). The KRAB domain consists of about 75 amino acids and is generally located in the N-terminal to zinc finger repeats. KRAB zinc finger proteins are predicted to be involved in hematopoietic cell development and differentiation (36). Interestingly, this family of zinc finger proteins appears to be specifically expressed in vertebrates (36). These supposed features are in agreement with the proposed role of TIZ in osteoclastogenesis in our study (Figs. 5 and 6) in that the only known physiological function of osteoclasts is to resorb bones during skeletal development and bone remodeling and that major portions of osteoclasts are believed to be derived from hematopoietic progenitor cells. Many KRAB zinc finger proteins have been suggested to function as transcription repressors through the KRAB domain. However, only a few target sequences and one target gene (GADD45 for ZBRK1) have been identified (36, 37). One potential scheme for KRAB-mediated repression is sequence-specific binding of the zinc finger repeats to target DNA and recruitment of a corepressor through the KRAB domain (37, 38).

Transcription repressor activity of KRAB proteins presupposes their nuclear localization. The confocal microscopic analysis of cells cotransfected with TIZ and TRAF6 revealed mostly perinuclear localization of TIZ, while some nuclear expression is also extant (Fig. 2D). Although many KRAB domain-containing zinc finger proteins were shown to be expressed in the nucleus, extranuclear detection of this family protein has also been reported. The zinc finger protein NRIF that interacts with the neurotrophin receptor p75NTR showed both cytoplasmic and nuclear expression when the two proteins were coexpressed (39). This was in contrast to the exclusively nuclear detection when NRIF was expressed alone (39). The differential expression pattern of NRIF led to the proposition that NRIF may become released from p75NTR and translocate to the nucleus upon receptor activation. This mode of signaling has been demonstrated using the SMAD transcription factor and the transforming growth factor-β receptor (40). An analogous mode may operate for TIZ; the activation of TRAF6-recruiting receptors such as RANK and IL-1R may render the TIZ protein released from TRAF6 and translocated to the nucleus to regulate gene transcription. An alternative mechanism by which TIZ may play its role is sequestration of TRAF6 in discrete intracellular locations, preventing recruitment of TRAF6 to the cell surface receptors. This possibility may be supported by our observation that the TIZ protein was difficult to obtain as detergent-soluble fractions to carry out the in vitro binding and coimmunoprecipitation assays under general cell lysis conditions. Also, our preliminary result of subcellular fractionation experiments indicated that the majority of TIZ protein was recovered in fractions of cytoskeletal and nuclear matrix components.

Several TRAF-binding molecules that interfere with NF-κB activation have been reported. The zinc finger protein A20 and an A20-like protein Cezanne were shown to bind TRAF6 and suppress TRAF6-induced NF-κB activation (14, 41). A20 and Cezanne contain seven and one, respectively, repeats of a zinc finger motif with the consensus sequences of C\textsubscript{X}C\textsubscript{X}\textsubscript{2}CX\textsubscript{1}C\textsubscript{X}C\textsubscript{X}C\textsubscript{X}C, which is distinct from the zinc finger repeats present in TIZ. This opens the possibility that there might be other types of zinc finger proteins that bind TRAF proteins and modulate TRAF signaling function. The NF-κB-inhibitory effect of A20 has been suggested to be mediated by the A20-binding proteins ABIN and ABIN2 at a level upstream of the IkB kinase complex (42, 43). Similarly, TIZ may recruit yet unidentified adaptor molecules to block the IkB kinase complex activation. Other modes of inhibition of the TRAF6-induced NF-κB activation have been demonstrated. The MAPKKK family of protein apoptosis-inducing kinase was shown to bind TRAF proteins and suppress NF-κB activation by destroying the TRAF6-transforming growth factor-β-activating kinase 1 complexes (44, 45). Also interfering with the Ras pathway with a dominant negative mutant led to inhibition of the TRAF6- but not MyD88-induced NF-κB activation (46). Diverse mechanisms may have been developed during evolution to selectively modulate the level of NF-κB activity in response to various external and internal changes that cells encounter.

In our study, TIZ appears to play a role in osteoclast differentiation potentially by down-regulating the RANK-TRAF6 signaling. The pivotal role of RANK-TRAF6 signaling for the generation of functional osteoclasts has been evidenced by knock-out mouse studies in which TRAF6 deficiency led to the osteopetrotic phenotype (18, 19). The defect in bone modeling is probably due to impaired TRAF6-mediated signal transduction for the RANKL/RANK interaction that initiates either osteoclast activation (19) or osteoclast differentiation (18). Consistently, both RANKL and RANK knock-out mice also developed osteopetrosis (28, 47). The roles of each domain of TRAF6 in osteoclastogenesis were dissected by reintroducing various deletion constructs of TRAF6 into TRAF6-deficient mouse embryonic fibroblasts (34). In the study, it was proposed that the level of MAPK and NF-κB activation induced by RANK-TRAF6 signaling might govern the course of functional osteoclast formation (34). Thus, during the osteoclast differentiation process, the strength of TRAF6 signaling activity may need to be fine tuned. One way of achieving such goal would be the temporal regulation of expression of positive and negative modulators of TRAF6. TIZ may be one of the negative regulators of TRAF6 function. Therefore, whether TIZ expression changes during development of bone would be an intriguing question. For in situ analyses of TIZ expression, we are currently undergoing identification of the mouse form of the gene. The ultimate evidence for the real physiological function of this novel TRAF6 regulator will come from an approach that manipulates the gene in the in vivo level, such as a knock-out mouse study.

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A Novel Zinc Finger Protein That Inhibits Osteoclastogenesis and the Function of Tumor Necrosis Factor Receptor-associated Factor 6
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