Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis

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Signals emanating from the receptor for interleukin-1 (IL-1), lipopolysaccharide (LPS) or osteoclast differentiation factor/receptor activator of NFκB ligand (ODF/RANKL) stimulate transcription factors AP-1 through mitogen-activated protein kinase (MAPK) activation and NFκB through IκB kinase (IKK) activation. These kinases are thought to be activated by tumor necrosis factor receptor-associated factor 6 (TRAF6). However, molecular mechanisms by which TRAF6 activates various downstream kinases remain to be elucidated. We identified functional domains of TRAF6 under physiological conditions established by appropriate expression of TRAF6 mutants in TRAF6-deficient cells. In IL-1 and LPS signaling pathways, the RING finger and first zinc finger domains are not required for NFκB activation but are required for full activation of MAPK. However, IL-1 and LPS signals utilize distinct regions within the zinc finger domains of TRAF6 to activate NFκB. Furthermore, the RING finger domain is not required for differentiation of splenocytes to multinuclear osteoclasts, but is essential for osteoclast maturation. Thus, TRAF6 plays essential roles in both the differentiation and maturation of osteoclasts by activating various kinases via its multiple domains.
Keywords: MAPK/NFκB/osteoclastogenesis/RANK/TRAF

Introduction

The tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family proteins are cytoplasmic adapter proteins that mediate cytokine signaling (Arch *et al*., 1998). Among the receptors that recruit TRAF proteins are members of the TNFR superfamily and the Toll/interleukin-1 receptor (IL-1R) family. To date, six members of this family have been described. The TRAF domain is located in the C-terminal region of all members of the TRAF family and is composed of two subregions (Cheng *et al*., 1995): TRAF-C, which is the C-terminal half of the TRAF domain, is highly conserved among all members, and TRAF-N, which is the N-terminal half of the TRAF domain, is predicted to form a coiled-coil structure. Crystallographic studies of the TRAF domain of TRAF2 suggest that this domain forms a mushroom-shaped trimer, which is likely to interact with the trimer form of CD40 or TNFRII in response to ligand stimulation (McWhirter *et al*., 1999; Park *et al*., 1999). With the exception of TRAF1, all TRAFs contain an N-terminal RING finger domain and a stretch of predicted zinc fingers. Whereas TRAF2, TRAF5 and TRAF6 activate transcription factors NFκB through IκB kinase (IKK) activation and AP-1 through activation of mitogen-activated protein kinases (MAPKs) including Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), neither TRAF3 nor TRAF4 activates these pathways (Arch *et al*., 1998). Recently, signal-dependent interaction of MAP kinase kinase kinase (MAP3K) with TRAFs was demonstrated. TRAF6 interacts with transforming growth factor β (TGFβ)-activated kinase 1 (TAK1) via TAK1 binding protein 2 (TAB2) to activate NFκB-inducing kinase (NIK) and JNK (Ninomiya *et al*., 1999; Takaesu *et al*., 2000). TRAF2 interacts with apoptosis signal-regulating kinase-1 (ASK1) (Nishitoh *et al*., 1998) and MEKK1 (Baud *et al*., 1999). Thus, TRAF2, TRAF6 and probably TRAF5 exert their function by activating downstream MAP3Ks or MAP4Ks.

TRAF6 has the most divergent TRAF-C and is the only TRAF that is involved in the signal from members of the Toll/IL-1R family by interacting with the IL-1R-associated kinase (IRAK) (Cao *et al*., 1996; Ishida *et al*., 1996a) and that from p75 neutrophin receptor (Khursigara *et al*., 1999). Furthermore, TRAF2, TRAF3 and TRAF5 bind to the membrane-distal domain in the cytoplasmic tail of CD40 and receptor activator of NFκB (RANK), whereas TRAF6 interacts with the membrane-proximal domain (Galibert *et al*., 1998; Wong *et al*., 1998; Tsukamoto *et al*., 1999). We have previously shown that TRAF6-deficient (TRAF6$^{-/-}$) mice exhibit severe osteopetrosis and are defective in osteoclast formation due to defective signaling from RANK upon binding of osteoclast differentiation factor/RANK ligand (ODF/RANKL) (also known as OPGL and TRANCE) (Naito *et al*., 1999). Furthermore, TRAF6$^{-/-}$ mice are defective in normal B-cell differentiation, lymph node organogenesis and IL-1 signaling. Thus, TRAF6 plays pivotal roles in immune and inflammatory systems *in vivo*. Although TRAF6-mediated signal transduction is indispensable, the molecular mechanism by which TRAF6 exerts its biological effects remains unknown. Moreover, results of earlier studies designed to

investigate the role of TRAF zinc-binding regions, which are required for TRAF signal transduction, were often contradictory, most likely because TRAF proteins were grossly overexpressed in cell lines that endogenously express TRAF (Cao *et al*., 1996; Takeuchi *et al*., 1996; Dadgostar and Cheng, 1998; Baud *et al*., 1999). In this study, we sought to identify critical regions within TRAF6 that are involved in activation of NFκB and MAPK pathways, and to define the essential roles of TRAF6 in osteoclastogenesis by expressing mutant TRAF6 transgenes at endogenous wild-type levels in TRAF6$^{-/-}$ cells.

Results

TRAF6 is essential for activation of NFκB, JNK and p38 by IL-1 and lipopolysaccharide (LPS) signaling

TRAF6$^{-/-}$ mice display osteopetrosis with defects in bone remodeling due to impaired ODF/RANKL–RANK signaling (Lomaga *et al*., 1999; Naito *et al*., 1999). However, we scarcely observed tartrate-resistant acid phosphatase-positive (TRAP$^+$) osteoclast-like cells (OCLs) in bone tissue, whereas normal numbers of TRAP$^+$ OCLs that lacked contact with bone surfaces were observed by Lomaga *et al*. (1999). Furthermore, our *in vitro* culture experiments revealed that osteoclast progenitors derived from TRAF6$^{-/-}$ mice are unable to differentiate into OCLs in response to ODF/RANKL. This discrepancy could be due to differences in targeting constructs. However, other unexpected genetic defects besides TRAF6 deficiency are possible. Thus, whether TRAF6 is essential for NFκB and MAPK activation via IL-1 and LPS signaling was first tested. Two independent mouse embryonic fibroblast (MEF) cell lines were generated from wild-type and TRAF6$^{-/-}$ mice (Figure 1B). MEF cells were stimulated with either IL-1 or LPS, and activation of NFκB, JNK and p38 was analyzed. Activation of NFκB, JNK and p38 in response to IL-1 and LPS was abrogated in TRAF6$^{-/-}$ MEF cells (Figures 2 and 3). In contrast, activation of NFκB and MAPK pathways by tumor necrosis factor α (TNFα) or sorbitol in TRAF6$^{-/-}$ MEF cells was similar to that in wild-type MEF cells (data not shown). To confirm that failure of MEF cells to respond to IL-1 and LPS was due solely to lack of TRAF6 expression, TRAF6 cDNA was introduced into TRAF6$^{-/-}$ MEF cells by a retrovirus vector carrying a puromycin resistance gene. The amount of TRAF6 expressed from the introduced cDNA was comparable to that of endogenous TRAF6 in wild-type MEF cells by incubating TRAF6$^{-/-}$ MEF cells with diluted virus solution to avoid multiple infection of virus (Figure 1B). Complementation of TRAF6$^{-/-}$ MEF cells with TRAF6 restored activation of NFκB, JNK and p38 in response to IL-1 and LPS treatment (Figures 2 and 3). These results indicate that TRAF6 is essential for IL-1 and LPS signaling linked to NFκB and MAPK activation.

TRAF6 does not associate with TRAF2 or TRAF5 in response to IL-1

Since both TRAF2 and TRAF5 activate NFκB and MAPK (Rothe *et al*., 1994; Ishida *et al*., 1996b; Nakano *et al*., 1996), and since the TRAF family of proteins can associate to form homomeric or heteromeric complexes (Arch *et al*., 1998), TRAF6 is also likely to form complexes with TRAF2 or TRAF5. Thus, complex
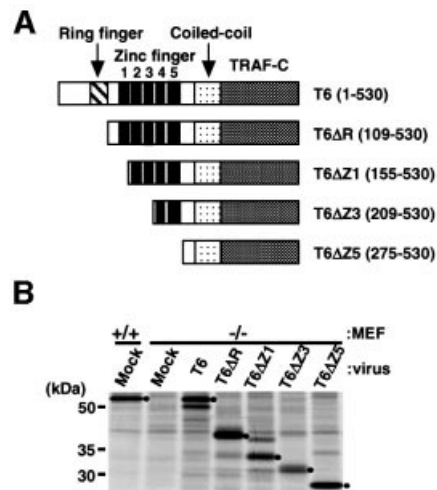

Fig. 1. Establishment of TRAF6$^{-/-}$ embryo-derived fibroblasts stably expressing physiological levels of exogenous wild-type or mutant TRAF6. (**A**) Structure of wild-type and N-terminally truncated TRAF6 mutants. Numbers in parentheses indicate amino acids that comprise wild-type and mutant TRAF6. (**B**) Expression level of wild-type and mutant TRAF6 in MEF cells. Cells were metabolically labeled with [^{35}S]methionine, and wild-type and mutant TRAF6 were immuno-precipitated with anti-TRAF6 antibody. The number of methionine residues in each protein: T6 (18), T6ΔR (15), T6ΔZ1 (14), T6ΔZ3 (13) and T6ΔZ5 (10). Dots denote the positions of protein bands that correspond to the indicated form of TRAF6 (shown above each lane).

formation was analyzed under two different conditions where TRAF6 activates NFκB and MAPK pathways. TRAF6 did not associate with TRAF2 or TRAF5 when they were overexpressed in 293T cells, although TRAF6 forms a homomeric complex (Figure 4A). Furthermore, endogenous TRAF6 did not co-precipitate with TRAF2 or TRAF5 irrespective of IL-1 stimulation of MEF cells (Figure 4B). In contrast, stimulation-dependent association of TRAF6 with IRAK was reproduced (Cao *et al*., 1996). Thus, wild-type and mutant TRAF6 described below are not likely to associate with TRAF2 or TRAF5 in MEF cells, even upon stimulation.

IL-1 and LPS signals utilize distinct regions within the zinc finger domain of TRAF6 to activate NFκB pathways

Because IL-1- and LPS-dependent activation of NFκB and MAPK were restored by complementation of TRAF6$^{-/-}$ MEF cells with TRAF6, we attempted to delineate the domains in TRAF6 required for NFκB and MAPK activation. Previous studies designed to determine functional domains of TRAF6 were performed by over-expression of TRAF6 mutants programmed by transient transfection in cell lines having endogenous TRAF6 (Cao *et al*., 1996; Baud *et al*., 1999). Thus, the ability of TRAF6 mutants to transmit a physiological signal has never been appropriately addressed. Since the TRAF domain is required for binding to upstream molecules such as CD40 or IRAK (Cao *et al*., 1996; Ishida *et al*., 1996a), a series of N-terminal deletion mutants (Figure 1A) was generated, and their cDNAs were inserted into retrovirus vector. To avoid multiple integration of the mutant TRAF6 transgene, TRAF6$^{-/-}$ MEF cells were incubated with adequately diluted solutions of recombinant virus and
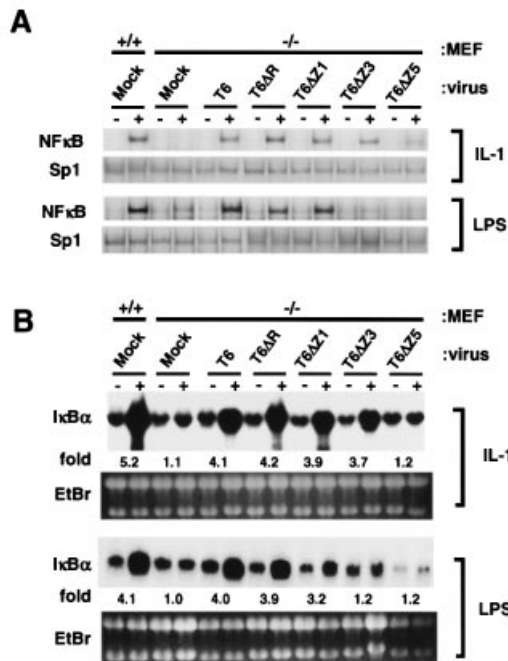
Fig. 2. The RING finger and first zinc finger of TRAF6 are not required for NFκB activation in response to IL-1 and LPS in MEF cells. MEF cells were derived from wild-type (+/+) or TRAF6$^{-/-}$ (–/–) mice and infected with recombinant retrovirus harboring either wild-type or mutant TRAF6 transgenes. (**A**) DNA binding activity of nuclear NFκB. Cells were either unstimulated (–) or stimulated (+) with IL-1 (20 ng/ml) for 30 min or LPS (200 ng/ml) for 60 min. Nuclear extracts were prepared and EMSAs were performed. The quantity of nuclear extracts used was confirmed by the DNA binding activity of Sp1. (**B**) NFκB-induced expression of endogenous IκBα gene. Cells were either unstimulated (–) or stimulated (+) with IL-1 (20 ng/ml) for 60 min or LPS (200 ng/ml) for 90 min. Expression of endogenous IκBα gene was determined by northern blotting. The fold increase in IκBα mRNA in stimulated cells compared with unstimulated cells was determined by phosphoimaging and normalized to the level of total RNA loaded into each lane as determined by ethidium bromide (EtBr) staining.

Fig. 3. Both the RING finger and the zinc finger domain are required for full activation of JNK and p38 in MEF cells. MEF cells were derived from wild-type (+/+) or TRAF6$^{-/-}$ (–/–) mice. (**A**) Activation of JNK. Cells were either unstimulated (–) or stimulated (+) with IL-1 (20 ng/ml) for 15 min or LPS (500 ng/ml) for 90 min. After stimulation, cells were assayed for endogenous JNK activity by immunocomplex kinase assay with GST–c-Jun (1–89) as a substrate (GST–c-Jun). The amount of JNK in the immunocomplex is also shown (JNK). The fold increase in endogenous JNK activity in stimulated cells compared with untreated cells was determined by phosphoimaging and normalized to the level of JNK in the immunocomplex, which was determined by immunoblotting using ImageQuant (Molecular Dynamics). (**B**) Activation of p38 MAPK. After stimulation as described in (A), cells were lysed with sample buffer. p38 MAPK (p38) and phosphorylated p38 (p-p38) were visualized by immunoblotting with anti-p38 and anti-p-p38 antibodies. The fold increase in the amount of p-p38 in stimulated cells compared with unstimulated cells was determined and normalized to the amount of p38 as measured by ImageQuant.

subsequently selected for puromycin resistance. Expression levels of exogenously introduced TRAF6 mutants were comparable to that of endogenous TRAF6 in wild-type MEF cells (Figure 1B). The ability of TRAF6 mutants to activate NFκB in response to IL-1 was first determined by electrophoretic mobility shift assays (EMSAs) (Figure 2A). T6ΔR, T6ΔZ1 and T6ΔZ3 were capable of activating the NFκB pathway to a level almost comparable to that observed with wild-type TRAF6. Thus, these results indicate that the RING finger and first three zinc fingers were not required for NFκB activation in response to IL-1. T6ΔZ5, which does not have any zinc-binding region, still had residual yet detectable activity. These results were supported by the results of another set of experiments in which IL-1-induced expression of the IκBα gene was analyzed (Figure 2B). The IκBα gene is under the transcriptional control of NFκB (Sun *et al*., 1993). Consistent with the EMSA results, full induction of the IκBα gene was observed in MEF cells expressing wild-type TRAF6, T6ΔR, T6ΔZ1 and T6ΔZ3. However, expression of T6ΔZ5 did not result in IκBα gene induction.
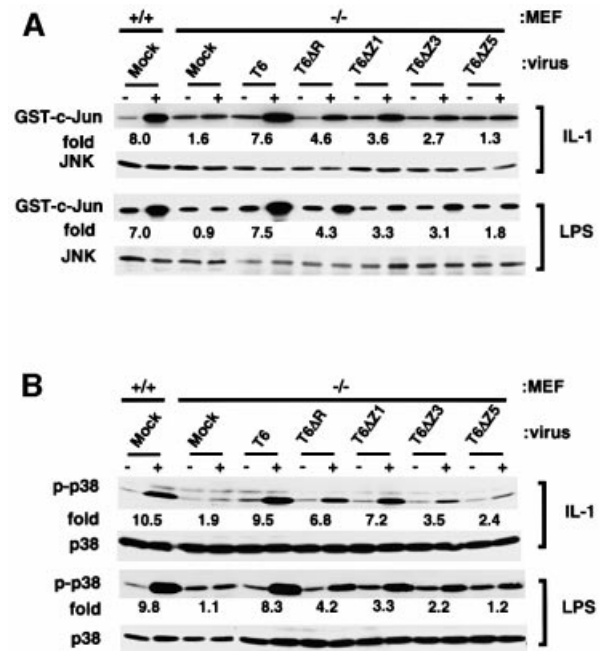
LPS signal transduction was also analyzed (Figure 2A and B). Both EMSAs and IκBα northern blot analysis revealed that NFκB was fully activated in cells expressing T6ΔZ1. Thus, the RING finger and the first zinc finger are not required for LPS-induced NFκB activation. Interestingly, unlike IL-1 signaling, further deletion of the second and third zinc fingers completely abolished the ability of TRAF6 to mediate the NFκB signaling pathway. Taken together, results indicate that the RING finger and first zinc finger of TRAF6 are not required for full activation of the NFκB pathway by both IL-1 and LPS. Interestingly, the second and third zinc fingers were essential for NFκB activation by LPS but not required for that by IL-1. In contrast, NFκB activation by IL-1 required the fourth and fifth zinc fingers.

RING finger and zinc finger domains of TRAF6 are required for full activation of MAPKs

Domains of TRAF6 required for JNK or p38 activation were mapped. Wild-type MEF cells or TRAF6$^{-/-}$ MEF cells expressing full-length TRAF6 or one of the TRAF6 mutants described above were stimulated by either IL-1 or
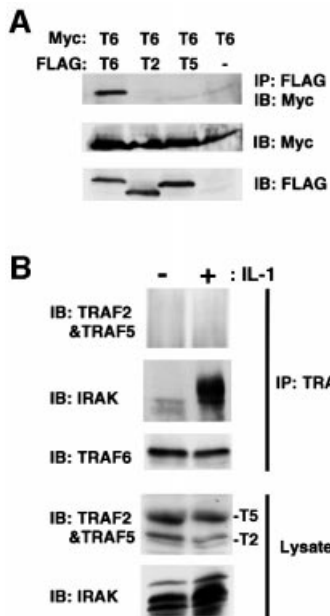
Fig. 4. TRAF6 does not associate with TRAF2 or TRAF5 in MEF cells. (**A**) TRAF6 does not heterodimerize with TRAF2 or TRAF5 when overexpressed in 293T cells. Myc-TRAF6 expression plasmid was co-transfected with control expression plasmid or expression plasmid encoding Flag-TRAF2, Flag-TRAF5 or Flag-TRAF6 into 293T cells. After 36 h, cell lysates were prepared and incubated with anti-FLAG monoclonal antibody. Co-precipitating Myc-TRAF6 was detected by immunoblot analysis with anti-Myc monoclonal antibody (top). Amounts of Myc-TRAF6 in total lysates (middle) and FLAG-TRAFs in immunocomplexes (bottom) are shown. (**B**) Endogenous TRAF6 does not associate with TRAF2 or TRAF5 upon IL-1 stimulation. MEF cells derived from wild-type mice were either unstimulated (–) or stimulated with IL-1 (20 ng/ml) for 15 min (+). Cell lysates were prepared and incubated with anti-TRAF6 antibody. Amounts of TRAF2, TRAF5, TRAF6 and IRAK in the immuno-precipitates were determined by immunoblot analysis with antibodies recognizing each protein (upper three panels). Amounts of endogenous TRAF2, TRAF5 and IRAK in the lysates were determined by immunoblotting (lower two panels).

LPS. JNK activity was determined by *in vitro* kinase assay (Figure 3A), and p38 activation was estimated by immunoblotting phosphorylated p38 (Figure 3B). Deletion of the RING finger resulted in ~40% reduction of IL-1-induced JNK and p38 activation. Further deletion of zinc fingers led to the gradual reduction of JNK and p38 activation, and the entire deletion of all zinc-binding domains abolished the ability of TRAF6 to activate JNK and p38 almost completely. Almost identical results were obtained when JNK and p38 activation were induced by LPS. Taken together, these results indicate that the zinc-binding motifs including the RING finger as well as all of the zinc fingers are required for full activation of JNK and p38. Thus, the RING finger and first zinc finger of TRAF6 are solely required for full activation of MAPK but not essential for activation of NFκB.

The RING finger domain of TRAF6 is essential for IL-1-induced activation of TAK1

TAK1, a member of MAP3K, is activated by association with TRAF6 via TAB2 in an IL-1 signal-dependent manner, and TAK1 in turn activates NIK, which is likely to activate the IKK complex (Ninomiya *et al.*, 1999;
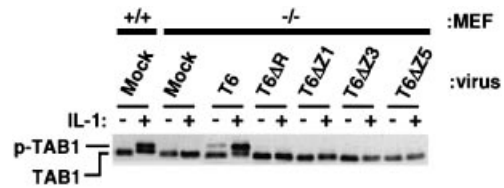

Fig. 5. The RING finger of TRAF6 is essential for TAK1 activation in response to IL-1. Various MEF cells were either unstimulated (–) or stimulated (+) with IL-1 (20 ng/ml) for 15 min. Cell lysates were analyzed by immunoblotting with anti-TAB1 antibody. Phosphorylated TAB1 (p-TAB1) shows less mobility than TAB1.

Takaesu *et al.*, 2000). TAK1 is capable of activating NFκB and JNK in the presence of TAB1. Thus, experiments designed to identify the domain of TRAF6 required for TAK1 activation by IL-1 were carried out. Activation of TAK1 coincides with phosphorylation of TAK1 and TAB1, which results in the mobility shift of these two proteins on SDS–polyacrylamide gels (Ninomiya *et al.*, 1999). MEF cells were treated with IL-1, and lysates were prepared and assayed for TAB1 mobility shift (Figure 5). No activation of TAK1 was observed in TRAF6⁻/⁻ MEF cells treated with IL-1, whereas TAK1 activation was observed in IL-1-treated TRAF6⁻/⁻ MEF cells comple-mented with TRAF6. Thus, TRAF6 is essential for IL-1-induced activation of TAK1. Interestingly, in TRAF6⁻/⁻ MEF cells expressing T6ΔR, no activation of TAK1 by IL-1 was detected, whereas IL-1 activated JNK by 60% in these cells (Figure 3A). These results indicate that TRAF6 activates other JNK-activating kinases via its zinc finger domain. Furthermore, since the RING finger is not required for NFκB activation, the contribution of TAK1 to NFκB activation via an IL-1 pathway is negligible in MEF cells. Therefore, involvement of TAK1 in NFκB activation is likely to be cell-type specific.

The RING finger domain of TRAF6 is essential for osteoclast maturation but is not required for osteoclast differentiation

Formation of functional osteoclasts requires differenti-ation of monocyte–macrophage lineage precursor cells (colony-forming unit monocyte–macrophage, CFU-M) to TRAP⁺ multinuclear cells and subsequent maturation, where cells polarize and form actin rings and ruffled border membranes for bone resorption (Teitelbaum, 2000). We have previously shown that TRAF6⁻/⁻ mice exhibit severe osteopetrosis due to the lack of osteoclast formation (Naito *et al.*, 1999). Furthermore, *in vitro* culture experiments revealed that osteoclast precursor cells derived from TRAF6⁻/⁻ mice are unable to differen-tiate into osteoclasts in response to ODF/RANKL and macrophage colony-stimulating factor (M-CSF). However, Lomaga *et al.* (1999) demonstrated that the TRAF6⁻/⁻ mice are osteopetrotic and have abundant dysfunctional osteoclasts. These observations prompted us to think that the RANK–TRAF6 pathway could be required for both differentiation and maturation of osteoclasts. Since the numbers of osteoclast progenitor cells in the spleens of wild-type and TRAF6⁻/⁻ mice are comparable (Naito *et al.*, 1999), we first checked various cytokine-signaling pathways in splenocytes pre-cultured for 3 days in the presence of M-CSF. Degradation of IκBα

and phosphorylation of JNK and p38 in response to IL-1 or soluble ODF/RANKL (sODF/RANKL) were observed only in wild-type progenitor cells but not in TRAF6$^{-/-}$
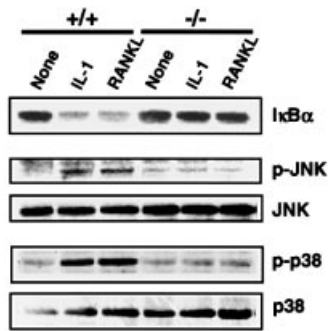

Fig. 6. TRAF6 is essential for ODF/RANKL-induced activation of NFκB and MAPK in osteoclast progenitors. Spleen cells derived from wild-type (+/+) or TRAF6$^{-/-}$ (–/–) mice were cultured for 3 days in the presence of M-CSF. After serum starvation for 30 min, cells were either unstimulated (None) or stimulated with IL-1 (10 ng/ml) or sODF/RANKL (100 ng/ml) for 20 min. Cell lysates were prepared and immunoblot analysis was performed with antibodies reactive with IκBα, JNK, p38, phosphorylated JNK (p-JNK) or phosphorylated p38 (p-p38).

progenitors (Figure 6). These results indicate that ODF/RANKL-induced RANK signaling is impaired in TRAF6$^{-/-}$ progenitor cells.

To address the role of TRAF6 in the differentiation and maturation of osteoclasts, splenocytes from wild-type or TRAF6$^{-/-}$ mice were either mock infected or infected with retrovirus carrying wild-type TRAF6 or TRAF6 mutant in the presence of M-CSF. The infected cells were further cultured in the presence of both M-CSF and sODF/RANKL, and the number of OCLs identified as multi-nuclear TRAP$^+$ cells was counted (Figure 7). Under this condition, splenocytes from wild-type mice efficiently differentiated into OCLs, ~90% of which formed apparent actin rings. Non-infected or mock-infected splenocytes did not differentiate into OCLs. Expression of wild-type TRAF6 in TRAF6$^{-/-}$ splenocytes resulted in the appearance of OCLs, ~50% of which formed actin rings (Figure 7A, c and d). Complementation of TRAF6$^{-/-}$ splenocytes with T6ΔR or T6ΔZ1 resulted in the appearance of significant numbers of OCLs, whereas complementation of these cells with T6ΔZ3 or T6ΔZ5 did not lead to formation of OCLs (Figure 7C). Interestingly, none of the OCLs generated by the expression of either T6ΔR or T6ΔZ1 was able to form the actin ring (Figure 7A, e and f
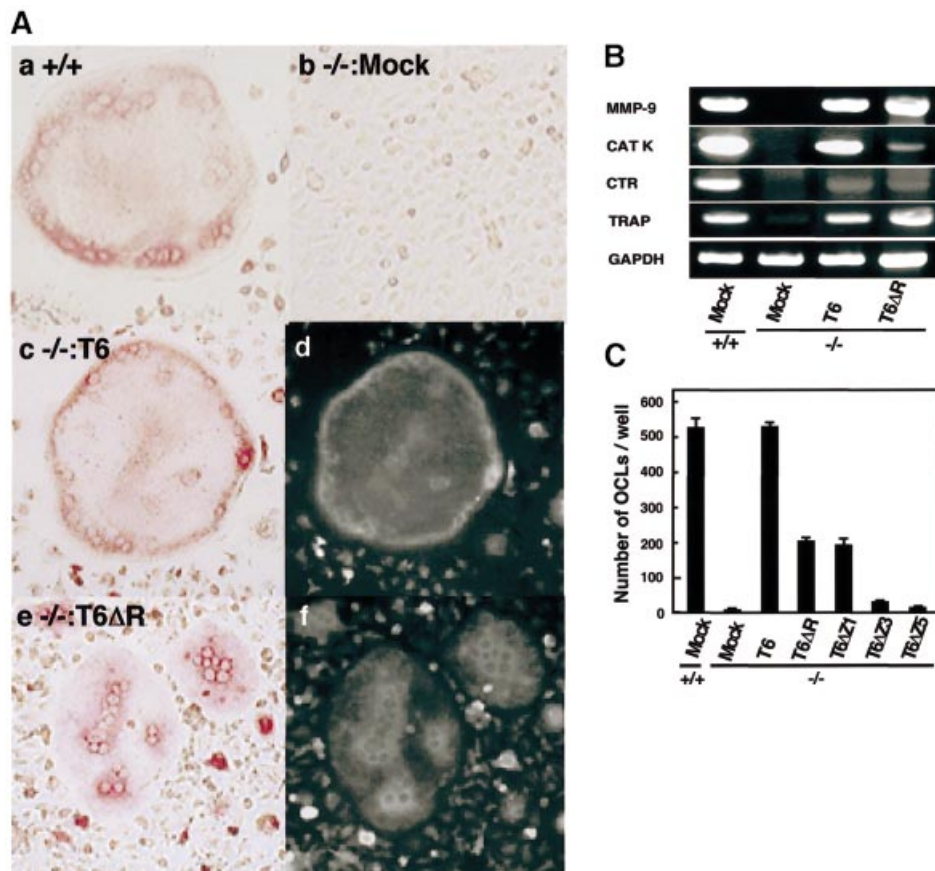

Fig. 7. The RING finger and first zinc finger of TRAF6 are not required for the formation of multinuclear TRAP$^+$ cells. (**A**) Microscopic observation of TRAP$^+$ cells and actin ring formation. Spleen cells from wild-type mice (a) and TRAF6$^{-/-}$ mice (b–f). TRAF6$^{-/-}$ cells were mock-infected (b) or infected with retrovirus encoding wild-type TRAF6 (c and d), or T6ΔR (e and f). After infection, cells were cultured in the presence of M-CSF (10 ng/ml) and sODF/RANKL (100 ng/ml). Then, cells were fixed and stained for TRAP (a, b, c and e) and F-actin (d and f) to visualize actin rings. (**B**) Expression of osteoclast marker genes. MMP-9 (nucleotides 111–864), cathepsin K (CATK) (nucleotides 1–505), calcitonin receptor (CTR) (nucleotides 1311–1765), TRAP (nucleotides 586–1082) and GAPDH (nucleotides 319–1047) were amplified by RT–PCR. (**C**) Quantitative analysis of the formation of OCLs. TRAP$^+$ cells containing more than three nuclei were counted as OCLs.
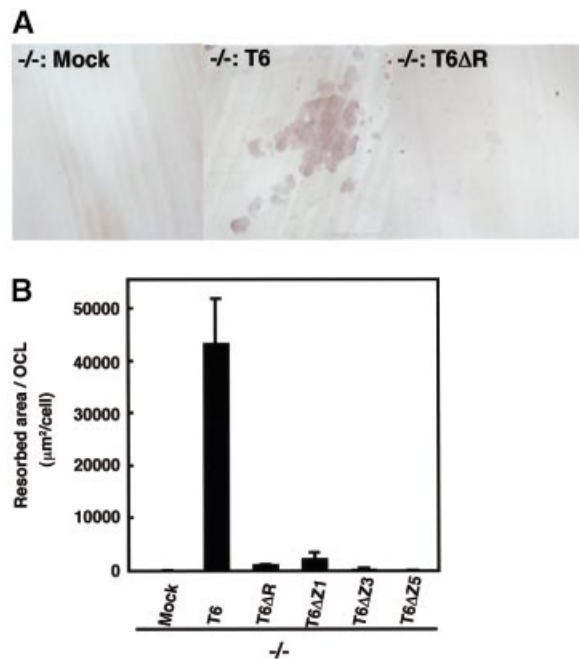
Fig. 8. The RING finger of TRAF6 is essential for osteoclast maturation. (**A**) Microscopic view of resorption pits. Cells were cultured with normal osteoblasts on dentine slices in the presence of prostaglandin E2 (1 μM) and 1α,25-dihydroxyvitamin D3 (10 nM). Spleen cells from TRAF6⁻/⁻ mice were mock infected (left) or infected with retrovirus encoding wild-type TRAF6 (center) and T6ΔR (right). (**B**) Quantitative analysis of the resorbed area. Resorption pits on dentine slices were visualized by staining with 0.5% toluidine blue, and the total pit area on dentine slices was measured. OCLs formed under identical conditions were counted, and the resorption area per single OCL was calculated.

and data not shown), although these OCLs expressed typical marker genes required for osteoclasts (Figure 7B). These results indicate that the RING finger domain is not required for differentiation of progenitor cells to TRAP⁺ OCLs, but is required for formation of the actin ring.

We next analyzed the bone-resorbing activity of these OCLs. Splenocytes from TRAF6⁻/⁻ mice infected with retrovirus harboring wild-type or mutant TRAF6 transgene were co-cultured with osteoblasts from wild-type mice in the presence of 1α,25-dihydroxyvitamin D3 and prostaglandin E2 for 7 days. The number of OCLs was counted, and the area of resorption pits was measured. Formation of OCLs was comparable to the results shown in Figure 7C, where recombinant M-CSF and sODF/RANKL were used instead of osteoblasts (data not shown). Complementation of TRAF6⁻/⁻ splenocytes with wild-type TRAF6 restored resorption activity, as was shown by the generation of OCLs, which formed a number of resorption pits (Figure 8A). However, complementation of TRAF6⁻/⁻ splenocytes with T6ΔR did not restore OCL resorption activity. Quantitation of resorption, expressed as the resorbed area per single OCL, revealed that OCLs derived from TRAF6⁻/⁻ splenocytes expressing T6ΔR or T6ΔZ1 have impaired bone resorbing ability (Figure 8B). Therefore, the RING finger of TRAF6 is essential for the maturation of osteoclasts, but is not required for generating multinuclear TRAP⁺ OCLs. Because OCLs were absent from cultures of ODF/RANKL-treated TRAF6⁻/⁻

splenocytes (Naito *et al*., 1999), it is clear that TRAF6 is required for both differentiation and maturation of osteoclasts.

Discussion

Identification of functional domains of TRAF6 under physiological conditions

In this paper, we have identified the signal-transmitting domains of TRAF6 by measuring the activity of N-terminally truncated mutants of TRAF6 under conditions where the two critical problems in previous reports can be overcome, as follows. First, the activity of mutants was analyzed in the absence of endogenous TRAF6 using TRAF6⁻/⁻ MEF cells. Therefore, possible interaction of TRAF6 mutants and endogenous TRAF6 can be ruled out. Moreover, interaction of TRAF6 with TRAF2 or TRAF5, which also activates MAPK and NFκB, was not detected in MEF cells irrespective of the extracellular stimulation or the overexpression experiments in 293T cells (Figure 4). Both TRAF2- and TRAF5-deficient mice are likely to have intact ODF/RANKL signal transduction pathways, because they do not have osteopetrosis (Yeh *et al*., 1997; Nakano *et al*., 1999). Therefore, it is unlikely that NFκB activation exerted by expression of mutants T6ΔR, T6ΔZ1 and T6ΔZ3 was due to association of these mutants with an undetectable amount of TRAF2 or TRAF5 in the TRAF6⁻/⁻ MEF cells. Secondly, the expression level of the TRAF6 mutants was adjusted to that of endogenous TRAF6 in wild-type MEF cells (Figure 1B). Thus, the system presented here is likely to be the most appropriate so far to identify functional domains of TRAF6.

In our assay system, neither IL-1 nor LPS activates NFκB or JNK in the absence of TRAF6, and complementation of TRAF6⁻/⁻ MEF with wild-type TRAF6 protein restores all of these pathways (Figures 2 and 3). These results clearly indicate that TRAF6 is an essential component of both IL-1 and LPS signal transduction pathways linked to NFκB and MAPK activation.

The zinc-binding domain of TRAF6 consists of subdomains that interact with molecules linked to the distinct downstream pathways

Functional domains within the zinc-binding domain of TRAF6 identified in this paper are summarized in Figure 9. The NFκB activation domains are located in a relatively small segment within the zinc finger domain, while JNK activation domains were scattered throughout the zinc-binding domain. These results revealed that TRAF6 activates various downstream kinases, probably MAP3Ks or MAP4Ks, through its distinct subdomains present in the zinc-binding domain. In fact, the RING finger is essential for activating TAK1 (Figure 5), which is capable of activating both NFκB and MAPK pathways by its overexpression with TAB1 (Ninomiya *et al*., 1999). However, the T6ΔR mutant, which lacks the RING finger domain, still activates NFκB and MAPK significantly. These results clearly indicate that other unidentified kinases or adapter proteins are likely to be activated by the zinc finger domain of TRAF6. One candidate is MEKK1, which is activated by TNFα and associates with TRAF2 (Baud *et al*., 1999). Evolutionarily conserved signaling intermediate in Toll/IL-1 pathways (ECSIT),
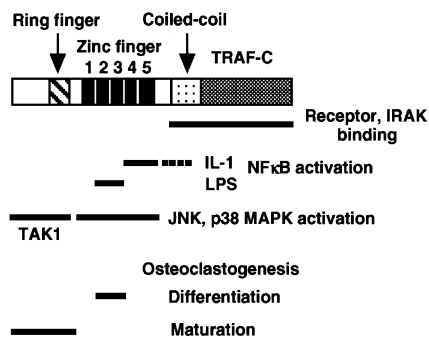
Fig. 9. Functional domains of TRAF6. Functional domains in the zinc-binding region of TRAF6 identified in this study are summarized. Domains within the primary structure of TRAF6 (long rectangular box, top) are represented as smaller rectangular boxes having different patterns and are labeled accordingly. The underlined domains are required for the functions indicated to the right. A dotted line indicates weak NFκB activation domain in response to IL-1.

which bridges TRAF6 to MEKK1 and enhances MEKK1 processing, could also be a candidate, since ECSIT has been shown to bind to the TRAF domain of TRAF6 (Kopp *et al.*, 1999). Interestingly, residual yet detectable NFκB activation was induced without a zinc-binding region in response to IL-1 (Figure 2A). Although this activation did not lead to IκBα induction (Figure 2B), there could be physiological target genes. Taken together, our results strongly suggest that there are subdomains in the zinc-binding domain of TRAF6 that lead to distinct post-receptor pathways.

TLR4, a receptor for LPS (Poltorak *et al.*, 1998), and IL-1R are thought to share the same intermediates, including MyD88, IRAK and TRAF6, for transmitting signals (Adachi *et al.*, 1998; Kawai *et al.*, 1999). Interestingly, distinct regions of TRAF6 are responsible for NFκB activation by IL-1 and LPS: IL-1 requires the fourth and fifth zinc fingers, whereas LPS requires the second and third (Figures 2 and 9). Although the molecular mechanisms underlying this discrepancy are not clear, results suggest that the downstream signaling components of TRAF6 for IL-1 signal transduction are different from those for LPS signaling. It is also possible that the upstream molecules that mediate TRAF6 activation by IL-1R occupation may be different from those of the LPS signaling pathways, even though these different pathways may share common molecules such as IRAK. This possibility is supported by studies showing that LPS is able to induce NFκB activation in cells derived from MyD88-deficient mice (Kawai *et al.*, 1999), while IL-1- and IL-18-mediated NFκB activation are abrogated in these mice (Adachi *et al.*, 1998).

Several groups reported functional domains within the zinc-binding region of TRAFs, which were identified by overexpression of TRAF mutants programmed by transient transfection. In domain mapping studies of TRAF2 and TRAF5, several discrepancies in the identification of various functional domains are apparent. However, TRAF2 and TRAF5 always required the RING finger domain for NFκB activation (Takeuchi *et al.*, 1996; Dadgostar and Cheng, 1998; Baud *et al.*, 1999). It was reported that NFκB activation by overexpression of

TRAF6 lacking the RING finger was ~60% of that by wild-type TRAF6, and that further deletion of the first two zinc fingers of TRAF6 resulted in the complete abrogation of NFκB activation (Cao *et al.*, 1996). Although this suggests that TRAF6 could transmit signals in a different way from TRAF2 and TRAF5, the RING finger of all these TRAFs plays an important role in NFκB activation. However, our results show that deletion of the N-terminal region up to the first zinc finger scarcely affects the ability of TRAF6 to mediate IL-1- or LPS-induced NFκB activation. Furthermore, T6ΔZ3, which further lacks the second and third zinc fingers, is still capable of mediating IL-1-dependent NFκB activation (Figures 2 and 9). To explain discrepancies between our findings and those of others, we propose a model in which signal-dependent structural change or oligomerization of TRAF6 for signaling could require stabilization by both its zinc-binding region and an unidentified stabilizer protein. T6ΔR, when expressed at physiological levels, is likely to form putative, stabilizer-mediated oligomers that are sufficient for NFκB activation; however, these putative T6ΔR oligomers may be unstable or structurally insufficient for activation of molecules linked to MAPK, such as TAK1, due to lack of the RING finger. Overexpression of TRAF6 or T6ΔR could titrate out the stabilizer. However, the majority of TRAF6 could form stable oligomers without the stabilizer via its RING finger to activate both NFκB and MAPK, since the concentration of TRAF6 is significantly higher than the physiological condition. T6ΔR oligomers, which could form when overexpressed, are likely to be unstable because they lack the RING finger domain; thus, these molecules may only partially activate NFκB. The stabilizer is likely to be IRAK or the cytoplasmic tail of membrane receptors, including CD40, RANK and p75 NGFR. IL-1-induced TAK1 activation requires TAB2-mediated linkage of TAK1 to TRAF6 (Takaesu *et al.*, 2000). Since the interaction of TAB2 with TRAF6 requires the entire structure of TRAF6, TAB2 is not likely to interact with unstable TRAF oligomers. It has been reported recently that the RING finger of TRAF6 is required when TRAF6 functions as an E3 ubiquitin protein ligase together with TRAF6-regulated IKK activator 1 (TRIKA1) to activate NFκB pathways (Deng *et al.*, 2000). The discrepancy between this report and our results remains to be elucidated. However, there could be ubiquitylation-dependent and -independent IKK activation pathways downstream of TRAF6.

Role of TRAF6 in osteoclastogenesis: identification of the TRAF6 RING finger domain as a critical motif for osteoclast maturation

We have previously shown severe osteopetrosis in TRAF6$^{-/-}$ mice due to blockade of the ODF/RANKL–RANK pathway (Naito *et al.*, 1999). The cytoplasmic tail of RANK, a receptor of ODF/RANKL, was shown to bind to TRAF6 through its membrane-proximal TRAF-binding domain (Galibert *et al.*, 1998; Wong *et al.*, 1998). Consistent with these findings, both ODF/RANKL- and RANK-deficient mice were characterized by profound osteopetrosis resulting from a block in osteoclast differentiation (Dougall *et al.*, 1999; Kong *et al.*, 1999). Here we show that expression of wild-type TRAF6 in TRAF6$^{-/-}$ osteoclast progenitor cells resulted in the

appearance of mature osteoclasts, which were able to resorb bone (Figures 7 and 8). Results confirmed that TRAF6 is essential for differentiation of osteoclasts and ruled out other possible genetic defects that affect osteoclast differentiation in our TRAF6⁻/⁻ cells. With this system, we provide evidence indicating that the RING finger of TRAF6 is responsible for the maturation of osteoclasts, whereas the second and third zinc fingers play essential roles in the differentiation of OCLs (Figure 9). Thus, we are the first to show that TRAF6 plays essential roles in both the differentiation and maturation of osteoclasts. Our findings strongly suggest that linkage between RANK and TRAF6 is also required for both steps of osteoclastogenesis.

Since the RING finger is required for the full activation of JNK and p38 but is not required for NFκB activation in MEF cells (Figures 2 and 3), the level of MAPK activation is likely to be critical for maturation of osteoclasts. Thus, the requirement of the RING finger for TAK1 activation suggests that TAK1-mediated JNK or p38 activation is responsible for osteoclast maturation. Osteoclasts in mice lacking c-fos are unable to differentiate (Grigoriadis *et al.*, 1994). Moreover, expression of the Fra-1 transgene in c-fos⁻/⁻ mice relieves osteopetrosis, and treatment of CFU-M with ODF/RANKL induces Fra-1 expression in a c-Fos-dependent manner (Matsuo *et al.*, 2000). Thus, ODF/RANKL-induced AP-1 activation is likely to be linked to Fra-1 induction, which may be required for both differentiation and maturation of osteoclasts. On this basis, the partial activation of AP-1, which is a consequence of reduced JNK and p38 activation by T6ΔR and T6ΔZ1, is likely to induce differentiation but not maturation of osteoclasts. Therefore, the level of AP-1 activation required for osteoclast differentiation is likely to be lower than that for osteoclast maturation. NFκB is also essential for osteoclastogenesis because mice lacking NFκB subunits p50 and p52 do not form OCLs from CFU-M and consequently have osteopetrosis (Franzoso *et al.*, 1997; Iotsova *et al.*, 1997). Thus, the extent of AP-1 and NFκB activation mediated by the RANK–TRAF6 linkage is likely to control osteoclastogenesis.

The β3 integrin subunit and c-Src are two signal transduction molecules required for maturation of osteoclasts; mice lacking one of them have dysfunctional osteoclasts, leading to osteopetrosis (Lowe *et al.*, 1993; McHugh *et al.*, 2000). β3 integrin is a component of the αvβ3 integrin complex, which is a major functional adhesion receptor on osteoclasts. αvβ3 integrin is thought to transmit signals that are linked to the formation of actin rings and ruffled border membranes via Src-dependent tyrosine phosphorylation of PYK2 and c-Cbl (Tanaka *et al.*, 1996; Duong *et al.*, 1998). Interestingly, TRAF6 was recently shown to associate with and activate c-Src upon stimulation of RANK (Wong *et al.*, 1999). Although the TRAF domain of TRAF6 was demonstrated to be sufficient for interaction with c-Src, the RING finger domain may be required for linkage of c-Src with downstream intermediates such as PYK2 and c-Cbl. However, expression of kinase-deficient Src mutant rescues osteopetrosis of c-src⁻/⁻ mice almost completely (Schwartzberg *et al.*, 1997). The relationship between TRAF6-mediated signal transduction and this kinase-independent function of c-Src remains to be elucidated. Further investigation is needed to broaden our understanding of the regulation of osteoclastogenesis by TRAF6-mediated signaling pathways.

Materials and methods

Plasmids and antibodies
To construct retrovirus vectors carrying a cDNA for wild-type or mutant TRAF6, each cDNA was inserted into pMX-puro vector (Kitamura, 1998). N-terminally truncated mutants of TRAF6 were generated by introducing an initiation methionine codon into the appropriate positions of TRAF6 cDNA by site-directed mutagenesis. The following mutants were used: T6ΔR (amino acids 109–530), T6ΔZ1 (amino acids 155–530), T6ΔZ3 (amino acids 209–530) and T6ΔZ5 (amino acids 275–530). Anti-TRAF6 (C-20), anti-TRAF2, anti-TRAF5, anti-Myc, anti-JNK and anti-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-FLAG antibody from Sigma Chemicals (St Louis, MO), anti-IRAK and anti-IκBα antibodies from Transduction Laboratories (Lexington, KY), and anti-phospho-p38 and anti-phospho-JNK from NEB, Inc. (Beverly, MA).

Introduction of wild type and TRAF6 mutants into MEF cells by retrovirus vector
Two MEF cell lines each from 14.5-day-old TRAF6⁻/⁻ and wild-type embryos were established. Similar results were obtained from both cell lines. Packaging cell line BOSC23 (2×10^6) was transfected with 2 μg of the retrovirus vector plasmid. Virus stocks were prepared by collecting the media from the cultures 48 h after transfection. MEF cells (3×10^5) were incubated with 2 ml of virus stock for 4 h in the presence of polybrene (1 μg/ml). Forty-eight hours after infection, the medium was changed to Dulbecco's modified Eagle's medium (DMEM)/10% fetal calf serum (FCS) containing 0.5 μg/ml puromycin. Puromycin-resistant cell pools were used for further experiments.

EMSAs
A double-stranded oligonucleotide containing a κB site from the mouse κ-light chain enhancer was used for NFκB and that containing a GC box was used for Sp1. Equal amounts of extract (5 μg protein) were incubated with ^{32}P-labeled probe and the DNA–protein complex was analyzed on a 4% polyacrylamide gel.

Immunoprecipitation and western blotting
MEF cells were either left untreated or treated with IL-1 (20 ng/ml) or LPS (200 ng/ml for NFκB and 500 ng/ml for JNK and p38) for the period indicated. Cells were lysed in 500 μl of TNE buffer (50 mM Tris–HCl pH 8.0, 1% NP-40, 1 mM EDTA, 150 mM NaCl, 0.5 mM dithiothreitol) and centrifuged to remove cellular debris. The resulting supernatant was subjected to immunoprecipitation by adding 1 μg of the appropriate antibody and 20 μl of protein G–Sepharose (Amersham Pharmacia, UK). Mouse spleen cells (5×10^6 cells) from wild-type or TRAF6⁻/⁻ mice were cultured in α-MEM/10% FCS for 3 days on 35 mm dishes in the presence of M-CSF (10 ng/ml). After serum starvation for 30 min, cells were stimulated with IL-1 (10 ng/ml) or sODF/RANKL (100 ng/ml) for 20 min, and whole-cell lysates were prepared. The immunoprecipitates or whole-cell lysates were separated by SDS–PAGE and transferred to PVDF membrane (Millipore Corp., Bedford, MA). The membranes were immunoblotted with specific antibodies, visualized with the appropriate horseradish peroxidase-conjugated secondary antibody using the ECL western blotting system (Amersham Pharmacia). For metabolic labeling of MEF cells, 2×10^6 cells were first incubated in methionine-free DMEM for 2 h and the medium was then changed to 5 ml of fresh methionine-free DMEM containing 250 μCi of [^{35}S]methionine. After 20 h of labeling, cells were washed twice and lysed in TNE buffer. Wild-type and mutant TRAF6 were immunoprecipitated with anti-TRAF6 antibody that recognizes all of the TRAF6 mutants.

In vitro kinase assay of JNK
Endogenous JNK was immunoprecipitated with anti-JNK antibody. Immunoprecipitates were incubated with 2 μg of glutathione S-transferase (GST)–c-JUN (1–89) fusion protein (New England Biolabs Inc.) in 20 μl of kinase buffer [20 mM HEPES pH 7.5, 20 mM MgCl₂, 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol)] at 30°C for 30 min.

Northern analysis
Twenty micrograms of total RNA were electrophoresed in 1% agarose gel containing formaldehyde and transferred to nylon membrane (NEN Inc.,

Boston, MA). The filter was incubated with ^{32}P-labeled DNA probe overnight at 42°C, and then washed in 0.5× SSC/0.2% (w/v) SDS for 30 min at 65°C. The entire coding region of IκBα cDNA was used as a probe.

In vitro differentiation and activation of osteoclasts

Spleen cells (1 × 10^6 cells) were cultured in α-MEM/10% FCS with M-CSF (10 ng/ml) overnight in 24-well plates. Cells were subsequently cultured in virus stock medium containing polybrene (4 µg/ml) for 4 h at 37°C. Infected cells were cultured for 2 days in the presence of M-CSF (10 ng/ml), and for an additional 4 days in the presence of both M-CSF (10 ng/ml) and sODF/RANKL (100 ng/ml). Adherent cells were then fixed with 10% formaldehyde, treated with ethanol–acetone (50:50), and stained for TRAP (Naito *et al.*, 1999). TRAP$^+$ multinucleated cells containing more than three nuclei were counted as osteoclasts. For visualizing actin rings, fixed cells were stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR). For gene expression analysis, total RNA was isolated and subjected to RT–PCR using a One Step RNA PCR Kit (TAKARA, Tokyo) to amplify the DNA fragments of MMP-9, cathepsin K (CATK), calcitonin receptor (CTR), TRAP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. For pit formation assays, osteoblasts from calvariae of wild-type newborn mice were cultured on dentine slices in α-MEM/10% FCS overnight in 48-well plates (two slices/well). Spleen cells were then co-cultured with these osteoblasts in the presence of 10 nM 1α,25-dihydroxyvitamin D3 and 10 µM prostaglandin E2 overnight prior to infection. Cells were then subjected to retroviral infection overnight. After the incubation for an additional week, cells were removed from dentine slices by adding 1 M NH$_4$OH and resorption pits were visualized by 0.5% toluidine blue staining. The total pit areas were measured using an image analysis system (System Supply, Nagano, Japan). TRAP$^+$ cells were counted in an identical set of cultures.

Acknowledgements

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