

TAK1-dependent Signaling Requires Functional Interaction with TAB2/TAB3*

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Transforming growth factor β -activated kinase 1 (TAK1), a member of the MAPKKK family, was initially described to play an essential role in the transforming growth factor β -signaling pathway, but recent evidence has emerged implicating TAK1 in the interleukin (IL)-1 and tumor necrosis factor (TNF) pathways. Notably, two homologous proteins, TAB2 and TAB3, have been identified as adaptors linking TAK1 to the upstream adaptors TRAFs. However, it remains unclear whether the interaction between TAB2/TAB3 and TAK1 is necessary for its kinase activation and subsequent activation of the IKK and MAPK pathways. Here, we characterized the TAB2/TAB3-binding domain in TAK1 and further examined the requirement of this interaction for IL-1, TNF, and RANKL signaling. Through deletion mapping experiments, we demonstrated that the binding motif for TAB2/TAB3 is a non-contiguous region located within the last C-terminal 100 residues of TAK1. However, residues 479–553 of TAK1 appear to be necessary and sufficient for TAB2/TAB3 interaction. Conversely, residues 574–693 of TAB2 were shown to interact with TAK1. A green fluorescent protein fusion protein containing the last 100 residues of TAK1 (TAK1-C100) abolished the interaction of endogenous TAB2/TAB3 with TAK1, the phosphorylation of TAK1, and prevented the activation of IKK and MAPK induced by IL-1, TNF, and RANKL. Furthermore, TAK1-C100 blocked RANKL-induced nuclear accumulation of NFATc1 and consequently osteoclast differentiation consistent with the ability of a catalytically inactive TAK1 to block RANKL-mediated signaling. Significantly, our study provides evidence that the TAB2/TAB3 interaction with TAK1 is crucial for the activation of signaling cascades mediated by IL-1, TNF, and RANKL.

The pro-inflammatory cytokines interleukin-1 (IL-1)² and tumor necrosis factor (TNF) elicit a critical function in the

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² The abbreviations used are: IL-1, interleukin-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; TNF, tumor necrosis factor; TAB1, -2, and -3, TAK1-binding protein 1, 2, and 3; GST, glutathione S-transferase; GFP, green fluorescent protein; TRAF, TNF receptor-associated factor;

innate immune response. Following receptor activation, these cytokines induce a cascade of signaling events leading to the activation of transcription factors such as NF- κ B and AP1 through upstream kinases including I κ B kinase (IKK) and the mitogen-activated protein kinases (MAPKs; JNK, p38, and ERK). These events culminate in the expression and regulation of numerous pro-inflammatory genes (1, 2).

Transforming growth factor- β -activated kinase 1 (TAK1), a member of the MAP3K family, was initially identified as a kinase in transforming growth factor β signaling (3). Recent evidence has emerged indicating that TAK1 is involved in various signaling pathways, including IL-1, IL-18, TNF, and receptor activator of NF- κ B ligand (RANKL) (4–7). Because TAK1 has the capacity to be activated by various cytokines, upstream adaptor molecules are critical in providing the selectivity of initiating distinct signaling events upon receptor activation.

Members of the TNF receptor-associated factor (TRAF) family of adaptor proteins are involved in coupling activated TNF and IL-1 receptors to NF- κ B and MAPKs pathways (8–11). Although TRAF proteins have no known enzymatic activity, recent evidence indicates that they act as E3s through their N-terminal really interesting new gene (RING) domain (12–14). RING-type E3s contain a series of Cys and His residues distinctly separated to constitute a novel zinc-binding domain. TRAF6 together with the heterodimeric E2 complex (Ubc13/Uev1A) facilitates the synthesis of unique Lys⁶³-linked polyubiquitin chains on itself, rather than the conventional Lys⁴⁸-linked polyubiquitin chains that target proteins for degradation. This unique Lys⁶³-linked polyubiquitin chain likely provides a scaffold to recruit downstream effector molecules to activate various signaling components.

TAK1-binding protein 2 (TAB2), or its homologue TAB3, appears to be the adaptor molecule linking these TRAF molecules to activate TAK1 (15–19). Both TAB2 and TAB3 contain a highly conserved C-terminal novel zinc finger domain, which binds preferentially to Lys⁶³-linked polyubiquitin chains (20).³ Mutation of this zinc finger domain abolishes the ability of TAB2 and TAB3 to bind to polyubiquitin chains, as well as their

TAK1, transforming growth factor β -activated kinase 1; IKK, I κ B kinase; RANK, receptor activator of NF- κ B; WT, wild type; HEK, human embryonic kidney; NF- κ B, nuclear factor- κ B; ERK, extracellular signal-regulated kinase; NFAT, nuclear factor of differentiation factor; RANKL, RANK ligand; TRAP, tartrate-resistant acid phosphatase; MAP3K, MAPKK kinase; LPS, lipopolysaccharide; MKK, mitogen-activated protein kinase; RING, really interesting new gene; FL, full-length.

³ B. G. Darnay, unpublished observations.

ability to activate TAK1 that appears to be a ubiquitin-dependent kinase of MKK and IKK (21). However, the mechanism by which the polyubiquitination events induce TAK1 activation has yet to be determined.

Following IL-1, TNF, or LPS stimulation, two possible TAK1 complexes exist, one which consists of TAB2-TAK1-TAB1 and another that consists of TAB3-TAK1-TAB1, which causes the activation of MAPKs and NF- κ B pathways (18, 21). These data suggest that TAB2 and TAB3 may play redundant roles in TAK1 activation (18). Consistent with this hypothesis, activation of NF- κ B and MAPKs pathways by IL-1 and TNF is intact in TAB2-deficient mouse embryonic fibroblasts (22).⁴ Additionally, knockdown of both TAB2/TAB3 by small interfering RNA results in complete loss of TAK1 activation (19).

Whereas it appears that TAK1 activation requires polyubiquitination of TRAF6 and RIP1 in the IL-1 and TNF pathways, respectively, the precise role of TAB2/TAB3 in TAK1 activation has not been clearly investigated. Furthermore, the dissection of the respective roles of TAB2 and TAB3 in regulating TAK1 signaling is prevented due to the compensatory nature of these proteins. The development of a construct that could ablate the functional interaction of both TAB2/TAB3 with TAK1 could be an alternative strategy to examine the functional role of TAB2/TAB3 in regulating TAK1-dependent signaling. In this study, we demonstrated that the binding motif for TAB2/TAB3 on TAK1 is a non-contiguous region within the last C-terminal 100 residues of TAK1 (TAK1-C100), and more specifically encompassing residues 479–553 of TAK1. Furthermore, TAK1-C100 prevented endogenous interaction of TAB2 and TAK1, and blocked IL-1 and TNF signaling. Additionally, we show the requirement of TAK1 kinase activity in RANKL signaling and osteoclast differentiation. Notably, we demonstrate that TAK1-C100 abolishes RANKL-mediated activation of IKK and JNK, nuclear accumulation of NFATc1, and terminal osteoclast differentiation. Our results reveal the critical function of the TAB2/TAB3 and TAK1 interaction for eliciting TAK1-dependent signals generated by the cytokines IL-1, TNF, and RANKL.

EXPERIMENTAL PROCEDURES

Cell Lines, Reagents, and Antibodies—Human embryonic kidney 293 cells (HEK293), mouse macrophage cell line RAW264.7 (RAW), and mouse fibroblastic cell line L929 were purchased from the American Type Cell Culture Collection (Manassas, VA) and cultured as previously described (23–25). Retroviral packaging cell line GP2-293 was purchased from Clontech (Palo Alto, CA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Recombinant TNF and IL-1 were purchased from PeproTech (Rocky Hill, NJ). Monoclonal antibody to phospho-I κ B α and rabbit polyclonal antibodies to phospho-c-Jun, phospho-p38, and phospho-TAK1 (Thr^{184/187}) were purchased from Cell Signaling Technology (Beverly, MA); rabbit polyclonal antibodies to JNK1, NEMO, TAB2, and monoclonal antibody to NFATc1 was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody for α -tubulin was pur-

chased from Calbiochem, and rabbit polyclonal antibody for β -actin from Cytoskeleton (Denver, CO). Goat anti-rabbit IgG-conjugated horseradish peroxidase antibody was purchased from Bio-Rad and goat anti-mouse IgG-conjugated horseradish peroxidase from BD Pharmingen. Monoclonal anti-FLAG antibody, glutathione-agarose, and tartrate resistance acid phosphatase (TRAP) kit were purchased from Sigma. Protein A/G-agarose beads were purchased from Pierce.

Plasmids—Mouse TAK1-K63W cDNA was a generous gift from Dr. Tan (Baylor College of Medicine, Houston, TX). The cDNA encoding full-length TAK1-K63W was amplified by PCR using gene-specific primers containing EcoRI (5') and BamHI (3') sites that was subsequently digested and cloned into p3XFLAG-CMV-14 (Sigma). To obtain wild-type TAK1 (TAK1-WT), the K63W mutation was reversed using the QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). The various C-terminal truncated forms of TAK1 Δ 1– Δ 5 were generated by PCR using gene-specific primers containing EcoRI (5') and BamHI (3') sites that was subsequently digested and cloned into p3XFLAG-CMV-14. To generate the fragments encoding residues 479–579 (TAK1- Δ 6) and 479–513 (TAK1- Δ 7), we performed PCR with gene-specific primers containing ScaI (5') and BamHI (3') sites and after digesting with the appropriate enzymes, the fragments were fused to the sequence encoding residues 1–300 of TAK1 and inserted into p3XFLAG-CMV-14. Using full-length TAK1 coding sequence, TAK1- Δ 8, - Δ 9, and - Δ 10 constructs were generated using the QuikChange Site-directed Mutagenesis kit. Retroviral vectors expressing FLAG-tagged TAK1-WT and TAK1-K63W were generated by PCR and inserted into a modified pMX vector (26, 27) containing an IRES-GFP and a puromycin selectable marker. The GFP-TAK1-C100, GFP-TAK1-(479–520), GFP-TAK1-(505–536), GFP-TAK1-(505–547), GFP-TAK1-(505–553), and GFP-TAK1-(479–553) fusion protein constructs were generated by PCR using gene-specific primers corresponding to residues 479–579, 479–520, 505–536, 505–547, 505–553, and 479–553 of TAK1 with SalI (5') and BamHI (3') sites and inserted in-frame with GFP in the pLEGF-C1 retroviral vector (Clontech). The entire GFP-TAK1-C100 fragment was subsequently cloned into pMX-puro. The entire TAB2 cDNA was subcloned (EcoRI/XbaI) by PCR into a modified pCR3 mammalian expression vector (Invitrogen) containing two tandem FLAG sequences at the N terminus. Human TAB3 cDNA was purchased from ATCC and initially cloned into a pCR3-T7 vector using BamHI (5') and NotI (3') sites. We subcloned it from pCR3-T7-TAB3 into pGEX-KG between BamHI (5') and a blunted SalI (3') site. Expression vectors encoding FLAG-RANK, FLAG-TRAF6, GST-I κ B α , and GST-c-Jun have been previously described (28, 29). All constructs were verified by automated DNA sequencing.

Recombinant Proteins—Purification of all GST fusion proteins has been previously described (28, 29). Recombinant RANKL has been previously described (24, 25).

Isothermal Titration Calorimetry and Data Analysis—Prior to analysis, purified GST fusion proteins consisting of TAK1-(479–579) and TAB2-(574–693) in which the GST portion was cleaved with thrombin were further purified by gel filtration (Superdex 200). Following overnight dialysis with identical

⁴ A. Besse, unpublished observations.

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buffer (50 mM NaH_2PO_4 , pH 7.4, 1 mM tris(2-carboxyethyl)phosphine hydrochloride), the protein was concentrated in Amicon Ultra-4 tubes (Millipore) with a 5-kDa molecular mass cutoff. Isothermal titration calorimetry measurements were performed at 25 °C using a VP-ITC machine that was connected to a computer with ORIGIN software (Microcal Inc., Northampton, MA). Prior to titration, protein samples were centrifuged at $10,000 \times g$ at 4 °C for 10 min and degassed by vacuum aspiration for ~10 min. The calorimeter cell and titration syringe were extensively rinsed with dialysis buffer (50 mM NaH_2PO_4 , pH 7.4, 1 mM tris(2-carboxyethyl)phosphine hydrochloride). The calorimetric titrations were carried out with 20–45 injections of 5–10 μl of TAK1 residues 479–579 (0.5–0.8 μM), spaced 180 s apart, into the sample cell containing a solution of 1.334 ml of TAB2 residues 574–693 (20–80 μM). After subtracting the heat of dilution, the association constant (K_A), enthalpy change (ΔH), and the stoichiometry (N) were obtained by fitting the thermograms to a one-set binding site model using ORIGIN software. The remaining thermodynamic parameters, the dissociation constant (K_D), free energy change (ΔG), and the entropy change (ΔS) were calculated from the relationships: $K_A^{-1} = K_D$ and $-RT \ln K_A = \Delta G = \Delta H - T\Delta S$.

Transfection and Retroviral Infection—Transfection of HEK293 cells was performed using the Calcium Phosphate Transfection kit (Invitrogen) according to the manufacturer's instructions. For production of retroviral supernatants, GP2-293 cells were co-transfected with the indicated retroviral vector and pVSV-G (Clontech) using FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Twelve hours after transfection, the medium was replaced with fresh medium. Viral supernatants were collected after 48–72 h and used to infect HEK293, RAW264.7, or L929 cells. After 2 days, selection was initiated with G418 (500 $\mu\text{g}/\text{ml}$) or puromycin (4 $\mu\text{g}/\text{ml}$). Stable pools of cells or clones were maintained in G418 (300 $\mu\text{g}/\text{ml}$) or puromycin (2 $\mu\text{g}/\text{ml}$).

Reporter Gene Assays—HEK293 cells were plated into 6-well plates and transfected as above together with an NF- κ B-driven luciferase reporter construct (0.5 $\mu\text{g}/\text{well}$). The total amount of DNA was adjusted to 2.5 $\mu\text{g}/\text{well}$ with empty vector. Thirty-six hours after transfection, cells were harvested and lysates were analyzed for luciferase activity using the Dual Luciferase Reporter Assay System from Promega (Madison, WI).

Protein Extraction, Immunoprecipitation, and Western Blotting—Cells were harvested, washed two times with ice-cold phosphate-buffered saline, and then lysed in buffer containing 20 mM Tris, pH 7.4, 250 mM NaCl, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 2 mM EDTA, 1% Triton X-100, 2 mg/ml leupeptin, and 2 mg/ml aprotinin for 20 min on ice. Cellular debris was removed by centrifugation at $13,000 \times g$ for 15 min. Protein was estimated by using the Bio-Rad Protein Assay dye. For immunoprecipitation, equivalent amounts of cell lysate were incubated with various antibodies for 2 h at 4 °C under rotation. Then 15 μl of Protein A/G-agarose beads were added and incubated for an additional hour at 4 °C under rotation. The immune complex was washed 3 times with lysis buffer and one time with low-salt buffer (20 mM Tris, pH 7.4, 25 mM NaCl, 1 mM dithiothreitol). For GST pull-down assays, glu-

tathione-agarose beads coated with the indicated GST fusion proteins were mixed with equivalent amounts of cell lysate for 2 h at 4 °C under rotation. The protein complexes were washed 3 times with lysis buffer and one time with low-salt buffer. For immunoblotting, the immunoprecipitates, GST pulled-down protein complexes, or cell lysates were resolved on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad). The membranes were immunoblotted with the indicated antibodies followed by the appropriate secondary antibody and then visualized by using the Enhanced Chemiluminescence (ECL) Western blotting system (Amersham Biosciences).

In Vitro Kinase Assays—JNK and NEMO activities were analyzed using GST-Jun and GST-I κ B α as substrates as previously described (23–25). Briefly, cells were plated in 6-well or 10-cm plates and the next day they were starved overnight. The following day, cells were treated with the indicated cytokine and washed three times in ice-cold phosphate-buffered saline. Cells were lysed for 20 min on ice in lysis buffer and cellular debris was removed by centrifugation at $13,000 \times g$ for 15 min. Equivalent amounts of proteins were immunoprecipitated with anti-JNK1 or anti-NEMO antibodies as described above. The kinase assay was performed in a 2 times reaction mixture containing 50 mM HEPES, pH 7.4, 20 mM MgCl_2 , 2 mM dithiothreitol, 2.5 μg of GST-Jun or GST-I κ B α , and 10 μCi of [γ - ^{32}P]ATP at 30 °C for 20 min. Reactions were stopped by addition of SDS sample buffer and subjected to 10% SDS-PAGE. Phosphorylation of GST-Jun or GST-I κ B α was detected using autoradiography. The incorporation of [γ - ^{32}P]ATP into the substrate was quantitated with a PhosphorImager using ImageQuant software (GE Healthcare).

Osteoclast Differentiation—Stable RAW cell lines were plated in 24-well plates at 1×10^4 cells/well in triplicate, and the next day treated with 100 ng/ml with RANKL. After 5 days, cells were fixed and stained for TRAP after which pictures were taken of representative fields. Osteoclast formation was assessed by counting the number of TRAP-positive multinucleated cells per well. In some cases prior to fixing the cells, pictures were taken with a $\times 10$ objective lens under bright or fluorescent light.

RESULTS

Binding of TAB2 Occurs in a Non-contiguous Region within the C-terminal Tail of TAK1—Whereas previous studies have shown that TAB2 could interact with the C terminus of TAK1, the precise domain in TAK1 that is required for TAB2 binding has not been mapped. To further map the region of TAK1 that is required to bind TAB2, we took two alternative approaches. In the first approach, we created a series of C-terminal deletion mutants of FLAG-tagged TAK1 (Fig. 1A) and co-transfected full-length TAK1 (TAK1-FL) or the indicated TAK1 deletion mutants with FLAG-TAB2 in HEK293 cells. The cell lysates were either immunoprecipitated with anti-TAB2 and immunoblotted with anti-FLAG or immunoblotted with anti-FLAG. TAB2 co-precipitated with only TAK1-FL, TAK1- $\Delta 5$, and TAK1- $\Delta 6$ (Fig. 1B). Analysis of these TAK1 deletion mutants indicated that TAB2 appears to interact with residues 479–547 of TAK1, because TAK1- $\Delta 5$ and TAK1- $\Delta 6$ both interacted

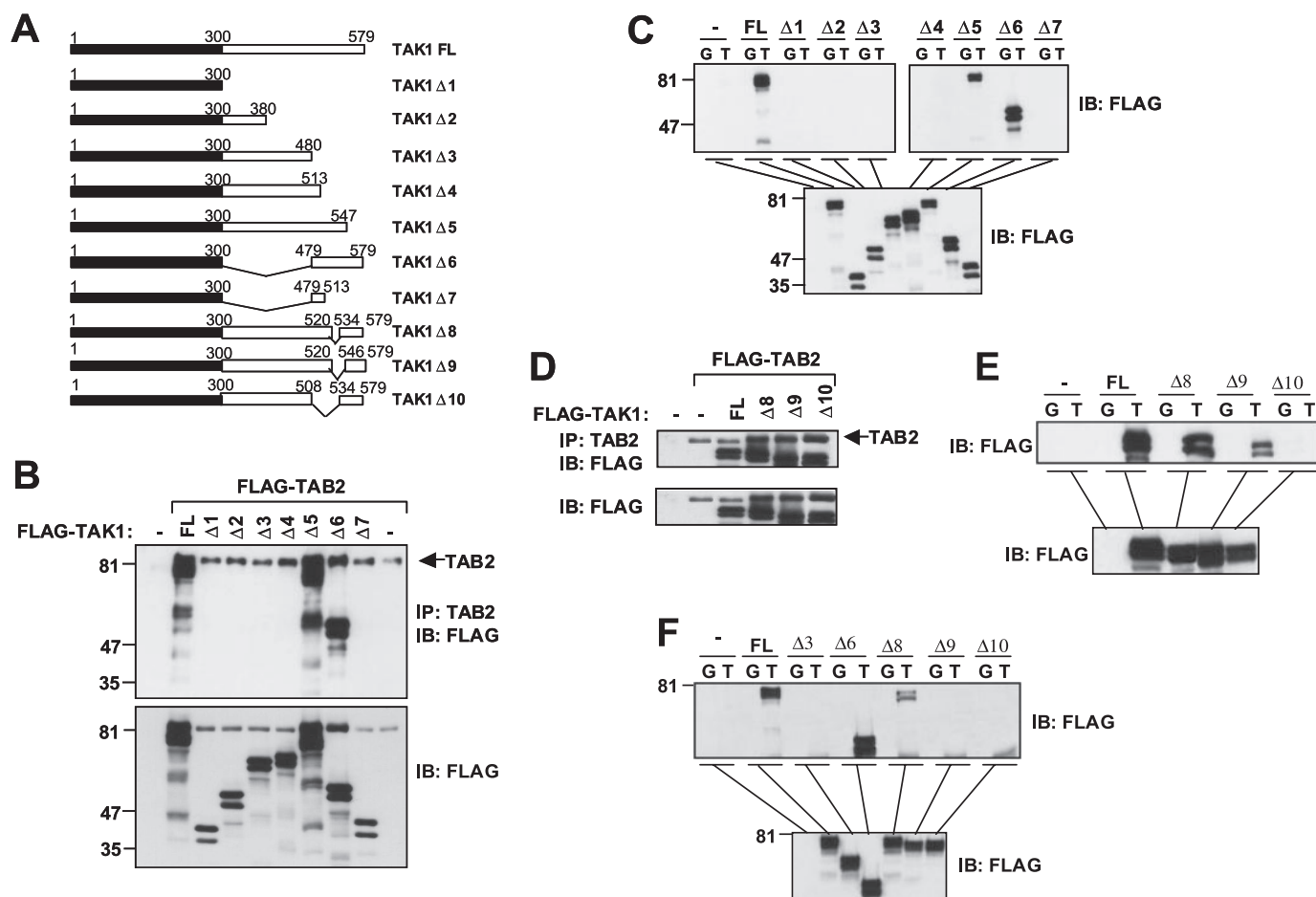


FIGURE 1. Defining the region of TAK1 that interacts with TAB2 and TAB3. *A*, schematic diagram of TAK1 constructs used in this study. The N-terminal region of TAK1 containing the kinase domain is highlighted in *black*. The C-terminal region is in *white*. The numbers for mouse TAK1 represent the N- and C-terminal residues of the respective construct. *B*, the region between residues 479 and 547 of TAK1 is required to bind TAB2. HEK293 cells were transiently transfected with empty vector (–) or co-transfected with FLAG-TAB2 in the absence or presence of the indicated FLAG-TAK1 constructs. After 36 h, cells were harvested and lysates were immunoprecipitated (IP) with anti-TAB2 and immunoblotted (IB) with anti-FLAG (top). Expression of the proteins in the cell lysates was determined by immunoblotting with anti-FLAG (bottom). *C*, TAB2 binds a motif between residues 479 and 547 within the C-terminal region of TAK1. HEK293 cells were transiently transfected with empty vector (–) or with the indicated FLAG-TAK1 constructs. After 36 h, cells were harvested and lysates were mixed with glutathione-agarose beads coated with either GST (G) or GST-TAB2 (T). Bound proteins were determined by immunoblotting with anti-FLAG (top) and expression of the proteins in the lysates was determined by immunoblotting with anti-FLAG (bottom). *D–E*, residues 521–545 are not essential for TAB2 interaction. HEK293 cells were transiently transfected with empty vector (–) or co-transfected with FLAG-TAB2 in the absence or presence of the indicated FLAG-TAK1 constructs and immunoprecipitation with anti-TAB2 (*D*) or a GST pull down (*E*) was performed essentially as described above. *F*, TAB3 binds to a similar region on TAK1. HEK293 cells were transiently transfected with empty vector (–) or with the indicated FLAG-TAK1 constructs. After 36 h, cells were harvested and lysates were mixed with glutathione-agarose beads coated with either GST (G) or GST-TAB3 (T). Bound proteins were determined by immunoblotting with anti-FLAG (top) and expression of the proteins in the lysates was determined by immunoblotting with anti-FLAG (bottom).

with TAB2, but not TAK1- $\Delta 1$ – $\Delta 4$ or TAK1- $\Delta 7$. In confirmation of these results, we performed GST fusion pull-down experiments with either GST or GST-TAB2 and lysates from transiently transfected HEK293 cells programmed to express various TAK1 deletion mutants. Similar to the co-immunoprecipitation experiments, only TAK1-FL, TAK1- $\Delta 5$, and TAK1- $\Delta 6$ interacted with GST-TAB2 (Fig. 1C). These results demonstrate that the region of TAK1 comprised between the residues 479 and 547 interacts with TAB2.

In the alternative approach, we compared the primary amino acid sequence within the last 100 residues of TAK1 from multiple species and designed three internal TAK1 deletion mutants that lacked residues between 508 and 547 (Fig. 1A, $\Delta 8$ – $\Delta 10$). We analyzed the ability of these TAK1 mutants to interact with co-transfected TAB2 in HEK293 cells. TAB2 coprecipitated with TAK1-FL and TAK1- $\Delta 8$, but weakly with

TAK1- $\Delta 9$ and not at all with TAK1- $\Delta 10$ (Fig. 1D). Similarly, GST-TAB2 also interacted with TAK1-FL and TAK1- $\Delta 8$, but weakly with TAK1- $\Delta 9$ and not at all with TAK1- $\Delta 10$ (Fig. 1E), suggesting that residues 521–545 are not essential for the interaction but residues 509–520 are important.

TAB3, a Homologue of TAB2, Binds to the Same Region of TAK1—TAB3 has recently been described as a functional homologue of TAB2 (17, 18). To determine whether the TAB2-binding region in TAK1 interacts also with TAB3, we transiently transfected HEK293 cells with various TAK1 deletion mutants and examined their ability to precipitate with GST-TAB3. Similar to TAB2, GST-TAB3 interacted only with TAK1-FL, TAK1- $\Delta 6$, and TAK1- $\Delta 8$ (Fig. 1F). Notably, TAK1- $\Delta 9$ interacted weakly with GST-TAB2, but not at all with GST-TAB3 suggesting a slight variation in the binding motif.

Functional Interaction of TAB2/TAB3 with TAK1

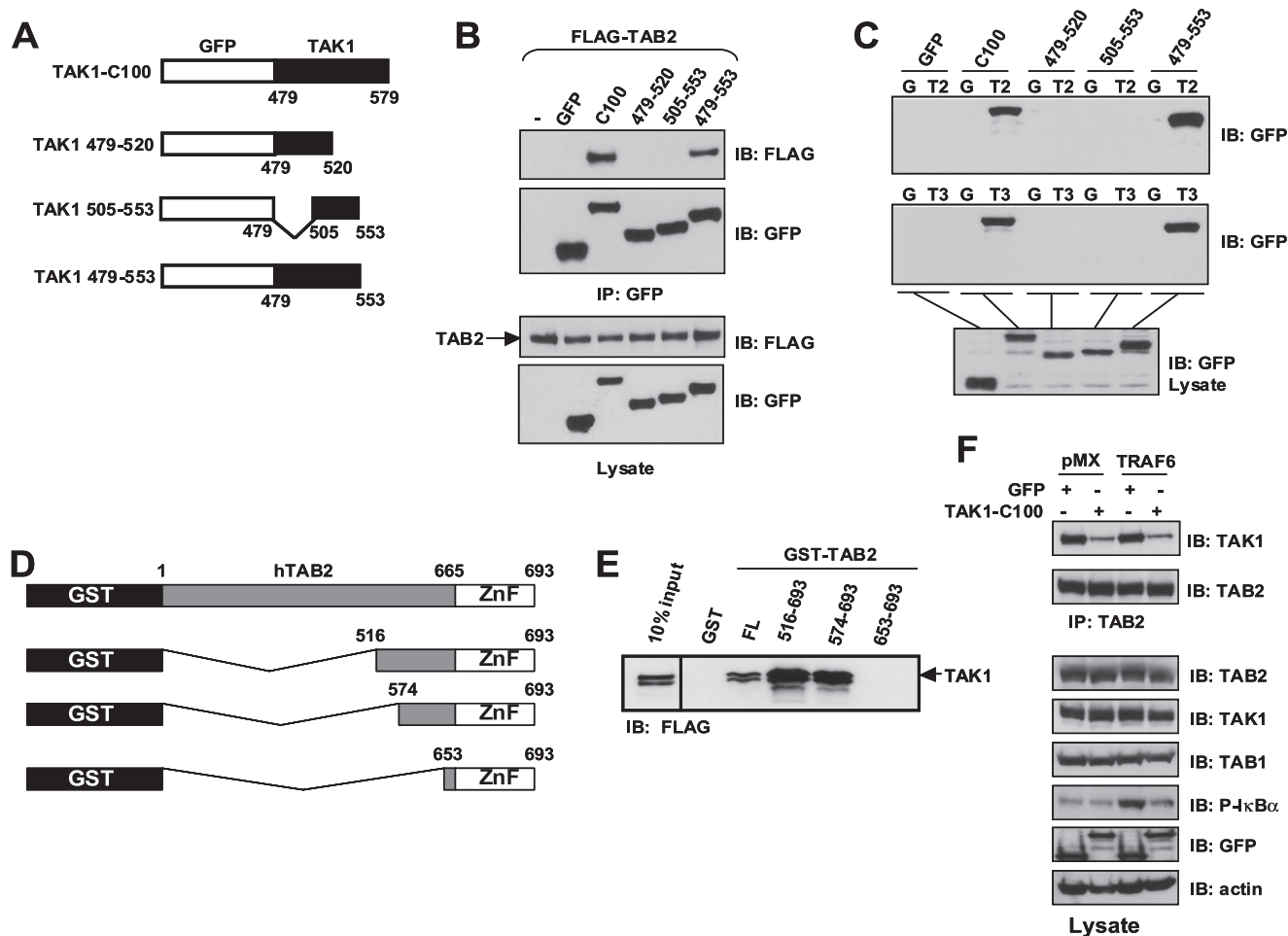


FIGURE 2. TAK1-C100 interacts with both TAB2 and TAB3 and prevents TRAF6-mediated phosphorylation of I κ B α . *A*, schematic diagram of the GFP fusion proteins TAK1-C100, TAK1-(479–520), TAK1-(505–553), and TAK1-(479–553). The indicated residues of TAK1 were fused to the C terminus of GFP in the retroviral vector pLEGFP-C1. *B*, co-immunoprecipitation of TAK1-C100 and -(479–553) with TAB2. HEK293 cells were transiently co-transfected with the indicated constructs. After 36 h, cells were harvested and immunoprecipitated with anti-GFP and bound proteins were visualized by immunoblotting with anti-FLAG. The membrane was stripped and probed with anti-GFP. Expression of the proteins in the lysates was determined by immunoblotting (IB) with anti-GFP or anti-FLAG. *C*, TAK1-C100 and -(479–553) bind TAB2 and TAB3. HEK293 cells were transiently transfected with the indicated fusion proteins. After 36 h, cells were harvested and lysates were mixed with glutathione-agarose beads coated with GST (G), GST-TAB2 (T2), or GST-TAB3 (T3). Bound proteins were visualized by immunoblotting with anti-GFP (top) and expression of the proteins in the lysates was determined by immunoblotting with anti-GFP (bottom). *D*, schematic diagram of the GST-TAB2 fusion proteins encompassing the C-terminal domain of TAB2. The indicated GST fusion proteins were constructed with human TAB2. ZnF, zinc finger domain. *E*, residues 574–653 of TAB2 are sufficient to bind to TAK1. HEK293 cells were transfected with an expression plasmid encoding FLAG-tagged full-length TAK1. Equal amounts of cell lysate were incubated with GST or the different GST-TAB2 mutants immobilized on glutathione-agarose beads. After incubation for 2 h at 4 °C, the beads were washed, and bound proteins were separated by SDS-PAGE and the bound TAK1 was detected by immunoblotting with an anti-FLAG. Ten percent of the extract used for the pull-down assay is shown on the left. *F*, TAK1-C100 significantly impairs the interaction between endogenous TAB2 and TAK1 resulting in loss of TRAF6-mediated IKK activation. HEK293 cells stably expressing empty vector (pMX) or TRAF6 were subsequently infected with retrovirus expressing either GFP or TAK1-C100. After neomycin selection, cells were harvested and lysates were immunoprecipitated with anti-TAB2 and then immunoblotted with the indicated antibodies (top two panels). Expression of the indicated proteins in the lysates was visualized with their respective antibody (bottom 6 panels).

A GFP Fusion Protein Containing the C-terminal 100 Residues of TAK1 Binds Both TAB2 and TAB3—The data presented thus far indicate that residues 509–520 of TAK1 are important for binding both TAB2 and TAB3. To determine whether this region is sufficient to interact with TAB2/TAB3, we created GFP fusion proteins containing residues 505–536 or 505–547 of TAK1 and examined their ability to bind TAB2; however, both of these constructs failed to interact with TAB2 and TAB3 (data not shown). Because both TAB2 and TAB3 interacted with TAK1- Δ 6, which contains the C-terminal 100 residues of TAK1, we generated additional GFP fusion proteins consisting of residues 479–579 (TAK1-C100), 479–520 (TAK1-(479–520)), 505–553 (TAK1-(505–553)), and 479–553 (TAK1-

(479–553)) of TAK1 (Fig. 2*A*). The GFP fusion protein containing TAK1-C100 and TAK1-(479–553) interacted with both TAB2 and TAB3 by co-immunoprecipitation and GST fusion pull-down experiments, whereas GFP fusion proteins containing TAK1-(479–520) or TAK1-(505–553) failed to interact with TAB2 and TAB3 (Fig. 2, *B* and *C*). Collectively, these data indicate that the interaction of TAB2/TAB3 with TAK1 resides in a non-contiguous region of TAK1, and suggests that residues 509–520 of TAK1 are important for this interaction, but not sufficient for binding TAB2 and TAB3 (see “Discussion”).

The C-terminal 100 Residues of TAK1 Bind to Residues 574–693 of TAB2—Because we mapped a small region of TAK1 that is necessary for interaction with TAB2/TAB3, we also deter-

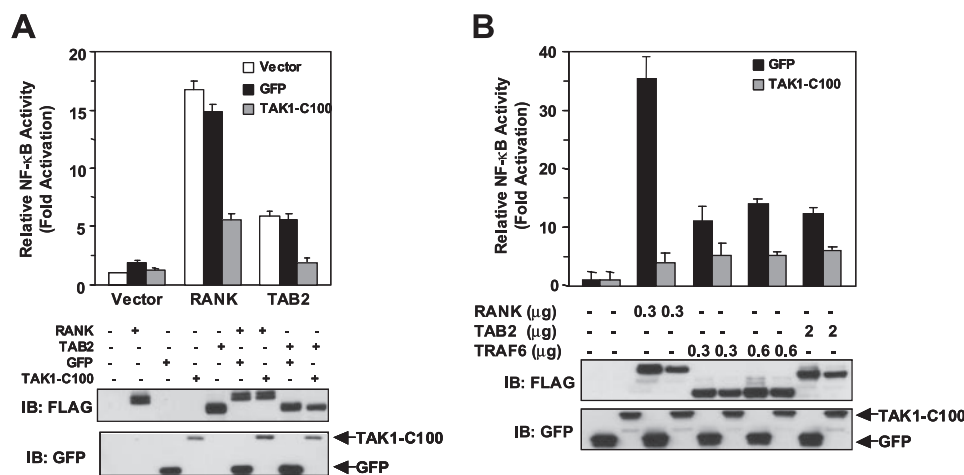


FIGURE 3. TAK1-C100 blocks RANK-, TRAF6-, and TAB2-induced NF- κ B activation. A, RANK- and TAB2-induced NF- κ B activation is blocked by TAK1-C100. HEK293 cells were transiently transfected with the indicated constructs together with an NF- κ B luciferase reporter vector. After 36 h, cells were harvested and luciferase activity was measured as described under "Experimental Procedures." Expression of the indicated proteins was detected by immunoblotting (IB) the lysates with anti-FLAG (lower panel). B, RANK, TRAF6, and TAB2-induced NF- κ B activation is dependent on the interaction between TAB2/TAB3 and TAK1. HEK293 cells stably expressing GFP or TAK1-C100 were transfected with the indicated constructs together with an NF- κ B luciferase reporter vector. Cells were processed as described in A.

mined the region of TAB2 that interacts with TAK1. We constructed a series of GST fusion proteins containing the C-terminal region of TAB2 (Fig. 2D) and similar to previous experiments, we examined their ability to interact with TAK1. HEK293 cells were transfected with FLAG-tagged TAK1 and cell lysates were used in GST pull-down experiments. TAK1 interacted with TAB2 mutants containing residues 516–693 or 574–693, but not with residues containing primarily the C-terminal zinc finger domain of TAB2-(653–693) (Fig. 2E). These data suggest that a region between residues 574 and 653 of TAB2 is necessary to interact with TAK1. Consistent with these observations, mutation of the zinc finger domain (C670A/C673A) of TAB2 did not prevent TAK1 from interacting with TAB2 (data not shown). In confirmation of our findings, we examined quantitatively the interaction between recombinant proteins consisting of the C termini of both TAK1-(479–579) and TAB2-(574–693) by isothermal titration calorimetry. Measurements from this experiment indicated a stoichiometric binding ratio of 1:1 between TAK1 and TAB2 with a calculated K_D of 65 μ M (data not shown). Taken together, these results indicate that the C-terminal 100 residues of TAK1 are sufficient to interact with the C terminus of TAB2.

TAK1-C100 Significantly Impairs TAB2-TAK1 Interaction and Inhibits TRAF6-induced Activation of IKK—We hypothesized that because TAB2/TAB3 both interacted with the C-terminal 100 residues of TAK1, expression of only the C-terminal tail of TAK1 may function as a dominant negative mutant and block subsequent TAK1 activation and downstream signaling. To explore this possibility, we created a retroviral vector expressing a GFP fusion protein consisting of the C-terminal 100 residues of TAK1 (TAK1-C100) (Fig. 2A). Whereas a somewhat smaller region was sufficient to interact with TAB2/TAB3, we opted to use TAK1-C100 to ensure correct folding and increase the ability to interact with both TAB2 and TAB3.

The activation of IKK by TRAF6 has been reported to involve the Lys⁶³-linked autoubiquitination of TRAF6, which serves as

a scaffold to recruit TAB2 by its ability to selectively bind Lys⁶³-linked polyubiquitin chains (12, 21, 30, 31).³ The formation of a TRAF6-TAB2-TAK1 complex may cause conformational changes in the catalytic domain of TAK1 thereby inducing its kinase activity. HEK293 cells stably expressing TRAF6 or empty vector (pMX) were infected with retrovirus expressing either GFP or TAK1-C100 and selected with neomycin for 2 weeks. After selection, the cells were harvested and lysates were examined for the interaction between TAB2 and TAK1 by immunoprecipitation with anti-TAB2 and immunoblotting with anti-TAK1. The interaction between the TAB2-TAK1 complex was significantly impaired in cells expressing TAK1-C100 compared with the

cells expressing only GFP (Fig. 2F). We observed equivalent expression of all of the exogenous proteins as well as TAB1 indicating that the loss of the TAB2-TAK1 complex was not due to down-regulation of any of these proteins (Fig. 2F). Furthermore, expression of TRAF6 caused activation of IKK as judged by immunoblotting with anti-phospho-I κ B α , which was significantly reduced in TRAF6-expressing cells that also expressed TAK1-C100 (Fig. 2F). These data reveal the essential requirement of the TAB2-TAK1 interaction for TAK1-dependent activation of the NF- κ B pathway.

TAK1-C100 Blocks NF- κ B Activation by RANK, TRAF6, and TAB2—Because TAK1 lies downstream of both RANK and TAB2, we next determined whether TAK1-C100, through its ability to sequester both TAB2/TAB3 could inhibit NF- κ B activation induced by RANK and TAB2. HEK293 cells were transiently co-transfected with RANK and TAB2 in combination with GFP or TAK1-C100 together with a NF- κ B luciferase reporter vector. Whereas expression of GFP had no effect, TAK1-C100 significantly inhibited RANK- and TAB2-induced NF- κ B activation (Fig. 3A). Furthermore, we generated HEK293 cells stably expressing either GFP or TAK1-C100 and then examined the ability of RANK, TRAF6, and TAB2 to activate an NF- κ B luciferase reporter in these cell lines. As previously observed, the RANK-, TRAF6-, and TAB2-induced NF- κ B activation was significantly impaired in cells expressing TAK1-C100 compared with GFP alone (Fig. 3B). Taken together, these results demonstrate that the interaction between TAB2/TAB3 and TAK1 is critical for TAK1 activation and subsequent NF- κ B activation.

TAK1-C100 Inhibits Signaling by IL-1 and TNF—The essential role of TAK1 in activation of NF- κ B by IL-1 and TNF has been reported in embryonic fibroblasts lacking TAK1 (32, 33). To determine whether TAK1-C100 inhibits IL-1 and TNF signaling, we generated L929 (mouse fibroblast) cell lines stably expressing either GFP or TAK1-C100. Non-infected L929 cells were used as autofluorescence staining control (Fig. 4A). Next,

Functional Interaction of TAB2/TAB3 with TAK1

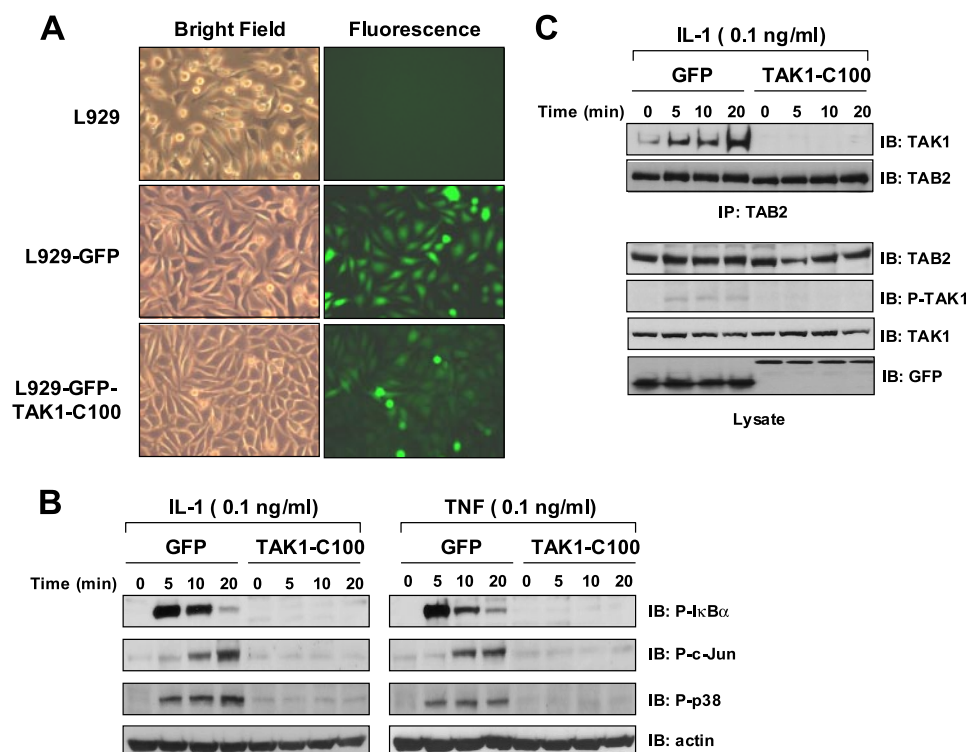


FIGURE 4. Interaction between TAB2 and TAK1 is required for IL-1- and TNF-induced signaling. *A*, stable expression of GFP or TAK1-C100 in L929 cells. L929 murine fibroblasts were infected with retrovirus expressing either GFP (pMX-GFP) or TAK1-C100 (pMX-GFP-TAK1-C100). After puromycin selection, pictures were taken with a $\times 10$ objective lens under bright light or fluorescence. Non-infected L929 cells were used as autofluorescence staining control. *B*, TAK1-C100 inhibits the phosphorylation of I κ B α , c-Jun, and p38 induced by IL-1 and TNF. The indicated cell lines were plated in 10-cm dishes and serum starved overnight. The next day, the cells were stimulated with either IL-1 (*left panels*) or TNF (*right panels*) for the indicated times. The cells were harvested and lysates were subjected to SDS-PAGE and immunoblotted (*IB*) with the indicated antibodies. The membrane was stripped and reprobed with anti-actin. *C*, TAK1-C100 blocks the interaction between TAB2 and TAK1 and inhibits the phosphorylation of TAK1. The indicated cell lines were treated as in *A* and the lysates immunoprecipitated with anti-TAB2 and then immunoblotted with anti-TAK1 and anti-TAB2 (*upper panels*). The lysates were subjected to SDS-PAGE and immunoblotted with the indicated antibodies (*bottom panels*).

serum-starved cells were stimulated with either IL-1 or TNF for the indicated times and cell lysates prepared. Stable expression of TAK1-C100, but not GFP, inhibited both IL-1- and TNF-induced phosphorylation of I κ B α , c-Jun, and p38 (Fig. 4*B*), which suggests that TAK1-C100 function as a dominant negative inhibitor by blocking the interaction between TAB2/TAB3 and TAK1. To further confirm this hypothesis, we stimulated the two different cell lines with IL-1 and examined whether TAK1 was present in the TAB2 complex by immunoprecipitating with anti-TAB2 antibody. After IL-1 stimulation, L929 cells expressing GFP showed increased amounts of TAK1 co-precipitating with TAB2, but TAK1 was absent in the anti-TAB2 complex from cells expressing TAK1-C100 (Fig. 4*C*), although equivalent amounts of TAB2 were immunoprecipitated. Furthermore, using a phospho-specific antibody directed against the active form of TAK1 (15, 34), the phosphorylation of TAK1 induced by IL-1 stimulation was also inhibited in cells expressing TAK1-C100 (Fig. 4*C*). These data reveal an essential role of the TAB2-TAK1 complex for both IL-1 and TNF signaling.

TAK1 Is Required for RANKL-induced Osteoclast Differentiation—From the data presented above, TAK1-C100 blocked NF- κ B activation induced by RANK and its essential modulator TRAF6 (Fig. 3), which suggests that TAK1 may function in the RANKL-RANK pathway. As a prelude to investigating the

TAB2-TAK1 complex in the RANK pathway, we first sought to determine whether TAK1 is required for osteoclast differentiation *in vitro*. The mouse macrophage cell line RAW264.7 (RAW) was infected with a control retrovirus (pMX) or expressing either FLAG-tagged TAK1 (TAK1-WT) or a catalytically inactive mutant of TAK1 (TAK1-K63W) followed by selection with puromycin. After selection, cells were examined for their ability to differentiate into TRAP-positive osteoclast following RANKL treatment for 5 days. Results from this experiment revealed that only control cells or cells expressing TAK1-WT have the ability to form osteoclasts, whereas cells expressing TAK1-K63W failed to form osteoclasts (Fig. 5, *A* and *B*). These data demonstrate that the kinase activity of TAK1 is crucial for *in vitro* RANKL-stimulated osteoclast differentiation.

Catalytically Inactive TAK1 Inhibits RANKL-induced IKK and JNK Activation—Loss of osteoclast differentiation by RANKL in cells expressing TAK1-K63W may reflect the absence of IKK and/or JNK activation by RANKL. To test this hypothesis, we examined the activation of IKK

and JNK after RANKL stimulation in RAW cells stably expressing empty vector, TAK1-WT, or TAK1-K63W. The RANKL-mediated activation of both IKK and JNK was significantly reduced in cells expressing TAK1-K63W as compared with either cells expressing empty vector or TAK1-WT (Fig. 5*C*). These data demonstrate that TAK1-K63W functions as a dominant negative regulator of RANKL-mediated osteoclast differentiation most likely by blocking both IKK and JNK activation.

TAK1-C100 Inhibits RANKL-mediated Osteoclast Differentiation and Signaling—The data presented thus far have demonstrated that the interaction between TAB2 and TAK1 is essential for activation of NF- κ B and MAPK induced by IL-1 and TNF or by transient expression of RANK, TAB2, and TRAF6. We next addressed whether the interaction of TAK1 with TAB2 is required for RANKL-mediated signaling and osteoclast differentiation. We generated RAW cells stably expressing GFP or TAK1-C100 and selected individual clones expressing each protein for further analysis. First, we examined RANKL-mediated osteoclast differentiation in these clones expressing either GFP or TAK1-C100. Expression of TAK1-C100, but not GFP alone, prevented RANKL-dependent osteoclast differentiation as visualized by TRAP-positive staining (Fig. 6*A*). As shown graphically, TAK1-C100 significantly

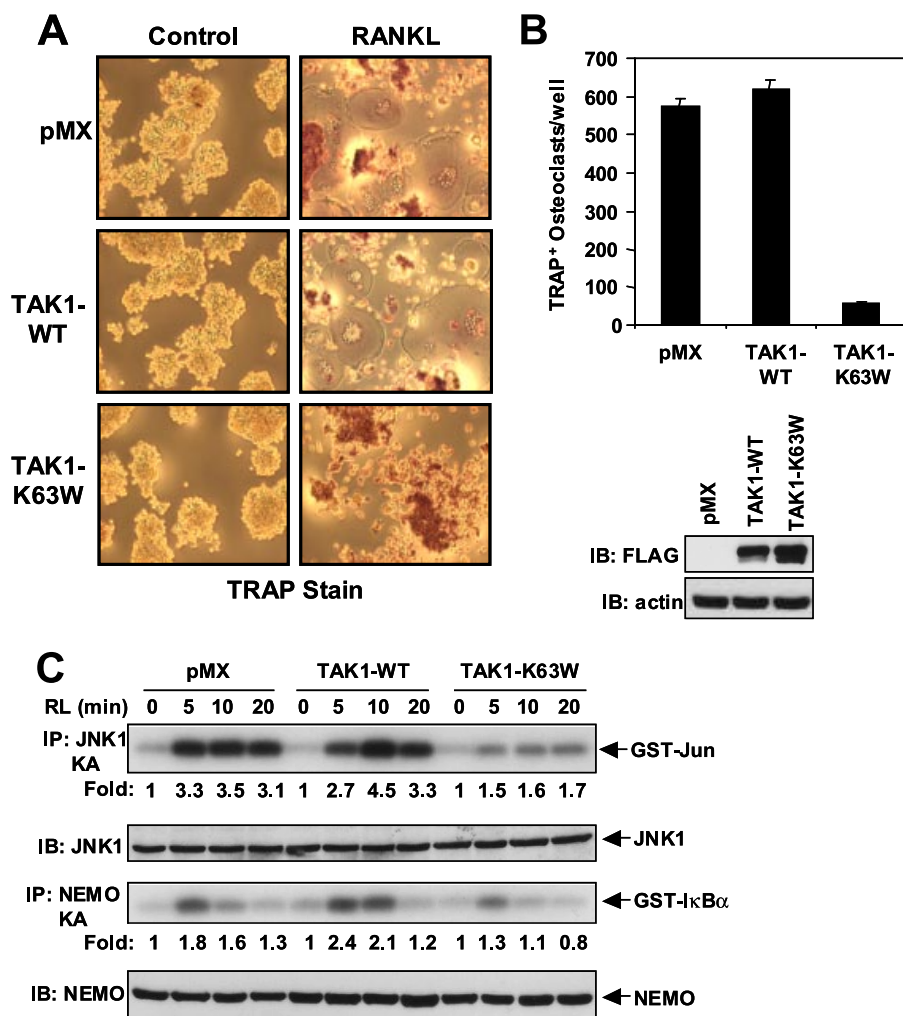


FIGURE 5. TAK1 is required for osteoclast differentiation. *A* and *B*, catalytically inactive TAK1 inhibits RANKL-mediated osteoclast differentiation. RAW cells stably expressing empty vector (*pMX*), FLAG-TAK1 (*TAK1-WT*), or catalytically inactive TAK1 (*TAK1-K63W*) were plated under osteoclastic conditions and treated the next day with RANKL (100 ng/ml). After 5 days, cells were fixed and stained for TRAP. Pictures of representative wells were taken with a $\times 10$ objective lens (*A*) and the number of TRAP-positive multinucleated osteoclasts per well were counted and represented as the average per well (in triplicate) from two independent experiments (*B*). The expression of TAK1-WT and TAK1-K63W was detected by immunoblotting (*IB*) with anti-FLAG antibody. The membrane was stripped and reprobed with anti-actin. *C*, TAK1-K63W inhibits RANKL-induced JNK and IKK activation. The indicated RAW cell lines were treated with RANKL (100 ng/ml) for the indicated times and the cells harvested. Lysates were subjected to immunoprecipitation with either JNK1 or NEMO antibodies and *in vitro* kinase assays performed using GST-Jun or GST-I κ B α , respectively, as substrates. The incorporation of 32 P into the substrates was quantitated with a PhosphorImager and ImageQuant software, and represented as -fold activation compared with time 0. Lysates were also immunoblotted with JNK1 and NEMO antibodies.

reduced the number of TRAP-positive osteoclasts induced by RANKL (Fig. 6*B*). These results demonstrate that the interaction between TAB2 and TAK1 is necessary for RANKL-induced osteoclast differentiation.

We next examined the activation of IKK and JNK upon RANKL stimulation in RAW cells expressing GFP or TAK1-C100. The RANKL-induced activation of both JNK and IKK was notably impaired in cells expressing TAK1-C100 as compared with cells expressing only GFP (Fig. 6*C*). Furthermore, the transcription factor NFATc1 plays an important role in terminal differentiation of osteoclast progenitors (35–39). We examined for expression of NFATc1 in nuclear extracts following RANKL stimulation for 0, 12, 24, and 48 h in cells expressing either GFP or TAK1-C100. RAW cells expressing GFP exhib-

ited nuclear accumulation of NFATc1 after RANKL stimulation, which was completely abolished in cells expressing TAK1-C100 (Fig. 6*D*). Taken together these results demonstrate that the interaction between TAB2/TAB3 and TAK1 is required to obtain full activation of TAK1, which in turn activates both the IKK complex and MAPKs pathways. Moreover, interfering with the interaction between TAB2 and TAK1 also results in the loss of induction of NFATc1 by RANKL.

DISCUSSION

In the IL-1 and TNF pathways, genetic ablation of TAK1 in mice has implicated this MAP3K in the activation of IKK and JNK, which results in the induction of NF- κ B and AP1 transcription factors, respectively (32, 33). However, IL-1 and TNF activate these pathways in the absence of either TAB1 or TAB2; the later of which is likely compensated by its homologue TAB3. The functional role of TAB1 in this process remains unclear at present. Previous studies have demonstrated that TRAF6 interacts with TAB2 in an IL-1-dependent manner, resulting in the formation of a TRAF6-TAB2-TAK1 complex that is required for activation of NF- κ B and JNK (15). In this model, TAB2 acts as a bridge linking TRAF6 to TAK1 and thereby mediating the activation of TAK1 in the IL-1 pathway. Similarly, because TRAF6 is essential for RANKL-mediated signaling (11, 40–44), engagement of the RANK pathway may use the identical TRAF6-TAB2-TAK1 complex

for its signaling leading to osteoclast differentiation. In contrast, stimulation by TNF causes the site-specific Lys⁶³ ubiquitination of RIP1 in the TNFR1 complex by an unidentified E3 ligase (possibly TRAF2 or cIAP1), which serves to recruit TAB2 and NEMO resulting in the receptor recruitment of the TAK1 (through the novel zinc finger ubiquitin binding domain of TAB2/3) and IKK complexes (45). Whereas this model demonstrates an essential requirement of TAK1 in activating these signaling cascades, the functional interaction between TAB2/TAB3 and TAK1 remains elusive due to a suitable cell system lacking both TAB2 and TAB3.

We hypothesized that disruption of the constitutive interaction between TAB2/TAB3 may interfere with TAK1 activation because previous data indicated that silencing both TAB2 and

Functional Interaction of TAB2/TAB3 with TAK1

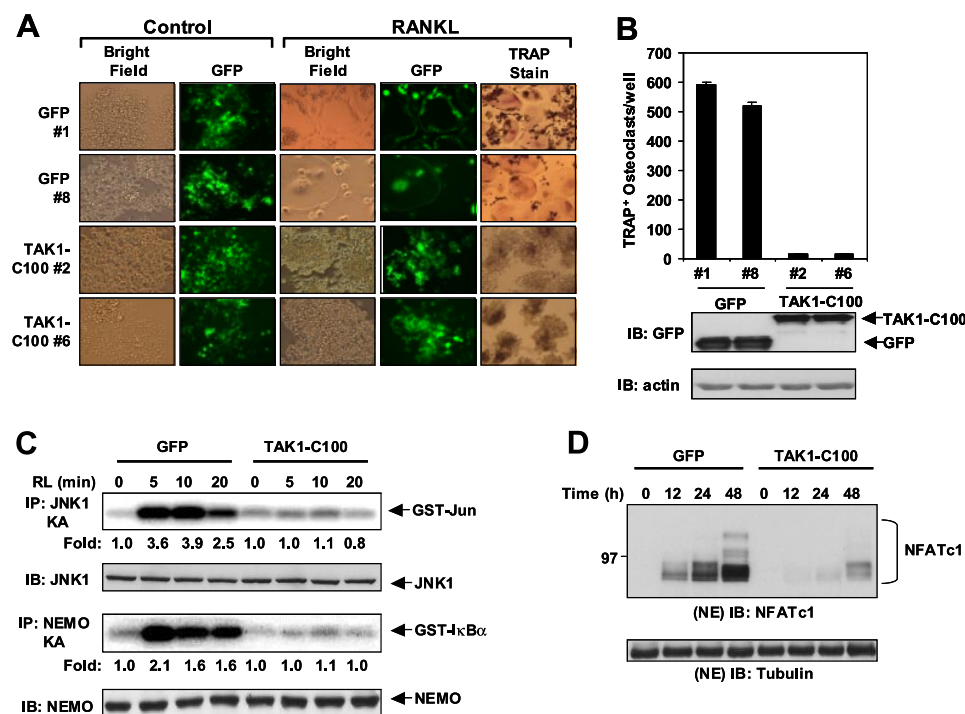


FIGURE 6. RANKL-mediated osteoclast differentiation and signaling is dependent on the interaction between TAB2/TAB3 and TAK1. *A* and *B*, blocking the interaction between TAB2/TAB3 and TAK1 inhibits osteoclast differentiation. RAW clones stably expressing GFP or TAK1-C100 were plated under osteoclastic conditions and the next day treated with RANKL (100 ng/ml). After 5 days, the cells were fixed, TRAP stained, and pictures were taken using a $\times 10$ objective lens as indicated (*A*). The number of TRAP-positive multinucleated osteoclasts per well were counted and represented as the average per well (in triplicate) from two independent experiments (*B*). The expression of GFP and TAK1-C100 was detected by immunoblotting (IB) with anti-GFP antibody. The membrane was stripped and reprobed with anti-actin. *C*, TAK1-C100 inhibits RANKL-induced JNK and IKK activation. RAW cells stably expressing GFP or TAK1-C100 were treated with RANKL (100 ng/ml) for the indicated times. Cells were harvested and lysates were processed as described in the legend to Fig. 5C. *D*, TAK1-C100 abolishes RANKL-induced NFATc1 accumulation in the nucleus. RAW cells stably expressing GFP or TAK1-C100 were plated at 5×10^4 cells/well in 6-wells plates and the next day stimulated with RANKL (100 ng/ml) for the indicated times. Cells were harvested and nuclear extracts were subjected to SDS-PAGE and immunoblotted with anti-NFATc1 (upper panels). The membrane was stripped and reprobed with anti-tubulin (lower panels). IP, immunoprecipitated.

TAB3 by small interfering RNA knockdown abolished IL-1 and TNF signaling (19). We sought an alternative strategy "to neutralize" both TAB2 and TAB3 by developing a construct that could interact with both TAB2/TAB3 and block their interaction with TAK1. To test our hypothesis, we first characterized the region of TAK1 that is necessary to interact with TAB2 and TAB3. Through deletion mapping, co-immunoprecipitation, and GST pull-down experiments, we demonstrated that residues 509–520 of TAK1 appear to be important for binding both TAB2 and TAB3, but not sufficient. However, a larger region encompassing residues 479–553 of TAK1 was sufficient for interacting with TAB2 and TAB3.

Further analysis utilizing programs to predict secondary structure (46) of the C-terminal 100 residues of TAK1 indicated an α -helical structure (Fig. 7). In brief, the last 100 residues of TAK1 are predicted to form four α -helices (helix 1, 490–505; helix 2, 512–526; helix 3, 536–549; helix 4, 553–573). Because TAK1- $\Delta 5$, which terminated with residue 547, and GFP-TAK1-(479–553) interacted with TAB2/TAB3, helix 4 probably does not contribute to this interaction as summarized in Fig. 7. We also generated GFP fusion constructs encompassing helix 1 and part of helix 2 (TAK1-(479–520)), helix 2 (TAK1-(505–536)), both helix 2 and 3 (TAK1-(505–547) and -(505–

553)), or helix 1, 2, and 3 (TAK1-(479–553)), but all of these constructs except TAK1-(479–553) failed to interact with TAB2/TAB3. These results suggest that the C-terminal 100 residues of TAK1, with the exception of helix 4, forms a tertiary conformation and that eliminating any one or more of these predicted helices may interfere with the intermolecular contacts required for the proper folding of this domain. Nonetheless, the C-terminal region of TAK1 appears as a functional domain that is required for interacting with TAB2/TAB3.

Because TAK1-C100 was able to bind both TAB2 and TAB3, we next investigated whether this region could functionally block the interaction of endogenous TAB2/TAB3 and TAK1. Using a retroviral delivery system, we presented evidence that a GFP fusion protein consisting of TAK1-C100 was able to significantly impair the constitutive interaction of TAB2/TAB3 with TAK1. Furthermore, using L929 mouse fibroblasts stably expressing TAK1-C100 we clearly demonstrated that interfering with the TAB2/TAB3-TAK1 interaction blocked NF- κ B and MAPKs signaling upon IL-1 and TNF stimulation through the inhibition of TAK1 phosphorylation.

Moreover, expression of TAK1-C100 in HEK293 cells abolished the ability of RANK, TRAF6, and TAB2 to activate an NF- κ B luciferase reporter. Collectively, these data support a critical role of the interaction of either TAB2 or TAB3 with TAK1 for its activation and therefore its ability to stimulate the IKK and JNK pathways in response to IL-1 and TNF stimulation.

Analogous to the IL-1 signal transduction pathway, RANKL requires engagement of the TRAF6 machinery for its signaling because TRAF6 deficiency results in complete loss of RANKL signaling and osteoclast differentiation (11, 40). Notably, the ubiquitin ligase activity of TRAF6 and its autoubiquitination are essential for NF- κ B and JNK activation in osteoclast progenitors.⁵ Ectopic expression of a catalytically inactive TAK1 abolished RANKL-mediated IKK and JNK activation and consequently the differentiation of these cells into TRAP-positive osteoclasts, suggesting an essential role of TAK1 in this process. In support of this hypothesis, RAW cells stably expressing TAK1-C100 showed dramatic loss of RANKL-mediated IKK and JNK

⁵ B. Lamothe and B. G. Darnay, unpublished observations.

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