Critical Roles of Threonine 187 Phosphorylation in Cellular Stress-induced Rapid and Transient Activation of Transforming Growth Factor-β-activated Kinase 1 (TAK1) in a Signaling Complex Containing TAK1-binding Protein TAB1 and TAB2*

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Transforming growth factor-β-activated kinase 1 (TAK1) mitogen-activated protein kinase kinase kinase (MAP3K) has been shown to be activated by cellular stresses including tumor necrosis factor-α (TNF-α). Here, we characterized the molecular mechanisms of cellular stress-induced TAK1 activation, focusing mainly on the phosphorylation of TAK1 at Thr-187 and Ser-192 in the activation loop. Thr-187 and Ser-192 are conserved among species from Caenorhabditis elegans to human, and their replacement with Ala resulted in inactivation of TAK1. Immunoblotting with a novel phospho-TAK1 antibody revealed that TNF-α significantly induced the phosphorylation of endogenous TAK1 at Thr-187, and subsequently the phosphorylated forms of TAK1 rapidly disappeared. Intermolecular autophosphorylation of Thr-187 was essential for TAK1 activation. RNA interference and overexpression experiments demonstrated that TAK1-binding protein TAB1 and TAB2 were involved in the phosphorylation of TAK1, but they regulated TAK1 phosphorylation differentially. Furthermore, SB203580 and p38α small interfering RNA enhanced TNF-α-induced Thr-187 phosphorylation as well as TAK1 kinase activity, indicating that the phosphorylation is affected by p38α/TAB1/TAB2-mediated feedback control of TAK1. These results indicate critical roles of Thr-187 phosphorylation in the stress-induced rapid and transient activation of TAK1 in a signaling complex containing TAB1 and TAB2.

Cellular stresses stimulate several intracellular signaling pathways leading to the activation of transcription factors AP-11 and NF-κB. The transcriptional activity of AP-1 is regulated by the stress-activated protein kinases (SAPKs)/mitogen-activated protein kinases (MAPKs) cascades, including the c-Jun NH2-terminal kinase (JNK) and p38 pathways (1–5). The JNK and p38 pathways are activated by corresponding upstream MAP2Ks, such as MKK4/7 and MKK3/6, respectively (6–10). In contrast to the simple combinations of MAPKs and MAP2Ks, more than 10 MAP3Ks that control the JNK and p38 pathways have been identified to date, and it has not been clear which MAP3K contributes mainly to the stress-induced MAPKs pathway (11–14). On the other hand, the transcriptional activity of NF-κB is regulated by IkB kinase (IKK)-mediated degradation of IκBs and subsequent nuclear translocation of the NF-κB complex (15). Several MAP3Ks have also been shown to regulate the NF-κB pathway by controlling the IKK complex (16–18). However, the precise functions of MAP3Ks in the regulation of IKK are still controversial, mainly because of the poor characterization of the regulatory mechanisms of MAP3K activation under physiological conditions.

Transforming growth factor-β-activated kinase 1 (TAK1) is one of the most characterized MAP3K family members and is activated by various cellular stress, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), lipopolysaccharide, and osmotic stress (5, 7, 17–27). It has recently been shown that TAK1 participates in diverse cellular functions, including Wnt signaling (28, 29), Epstein-Barr virus latent membrane protein 1 signaling (30), CD3/CD28 signaling in T lymphocytes (31), and double strand RNA/Toll-like receptor 3 signaling (32). In addition, TAK1 is essential for antibacterial innate immunity in Drosophila (33, 34). In these signaling pathways, TAK1 functions as an upstream stimulatory molecule of the JNK, p38, and IKK signaling pathways. Therefore, TAK1 has been recognized as a potential candidate that regulates the cellular stress-induced activation of both AP-1 and NF-κB. Other MAP3Ks such as MAPK/extracellular signal-regulated kinase kinase 1 (MEKK1), MEKK3, and mixed lineage kinases (MLKs) have been also reported to activate both the AP-1 and NF-κB pathways (16, 35, 36). In contrast, apoptosis signal-regulated kinase 1 (ASK1) has been characterized as a MAP3K specifically activating JNK/p38 pathways (11).

1 The abbreviations used are: AP-1, activator protein-1; ASK1, apoptosis signal-regulated kinase 1; EGFP, enhanced green fluorescence protein; GST, glutathione S-transferase; HA, hemagglutinin; HEK, human embryonic kidney; His6, hexahistidine; IKK, IκB kinase; IL-1, interleukin 1; JNK, c-Jun NH2-terminal kinase; kd, kinase domain; luc, luciferase; MAPK, mitogen-activated protein kinase; MAP2K (MKK), MAPK kinase; MAP3K, MAPKK kinase; MEKK, MAPK/extracellular signal-related kinase kinase kinase; MLK, mixed lineage kinase; NF-κB, nuclear factor-κB; PCNA, proliferating cell nuclear antigen; PP, protein phosphatase; RNAi, RNA interference; siRNA, small interfering RNA; TAB, TAK1-binding protein; TAK1, transforming growth factor-β-activated kinase 1; TNF, tumor necrosis factor; TRAF, TNF receptor associated factor; MKK, MAPK kinase.
TAK1 is a unique MAP3K whose kinase activity is controlled by specific TAK1-binding proteins (37). Two TAK1-binding proteins, TAB1 and TAB2, have been well characterized in the IL-1 signaling pathway (20, 24, 39). TAB1 binds to the NH2-terminal catalytic domain of TAK1 and is essential for TAK1 kinase activity (37). The COOH-terminal 68 amino acids of TAB1 are sufficient for full activation of TAK1 (37, 40–42). It has been shown that the NH2-terminal region of TAB1 shares a putative protein phosphatase 2C domain and a p38α binding domain (43, 44). Recently, Cheung et al. (26) reported that the association of TAB1 with p38α negatively regulates TAK1 kinase activity by phosphorylating TAB1 at Ser-423 and Thr-431. On the other hand, TAB2 interacts with the COOH-terminal region of TAK1, which functions as an adaptor protein to recruit TAK1 to TNFα receptor-associated factor (TRAF). Family members TRAF2 and TRAF6 in the TNF-α and IL-1 signaling pathways, respectively (20, 25, 27). The translocation of TAB2 from the membrane to the cytosol on stimulation of IL-1 facilitates the interaction between TAK1 and TRAF6 (20). Furthermore, IL-1 signaling leads to the ubiquitination of TAB2 through ubiquitin E3 ligase activity of TRAF6 (23, 27).

The TAK1 kinase activity is regulated restrictedly in TNF-α and IL-1 signaling pathways. We demonstrated previously that TNF-α induces the kinase activity within a few min after stimulation, with a rapid turnover within 10 min (18). However, the molecular mechanisms of the rapid and transient activation of TAK1 are still unclear. TAK1 has been suggested to be phosphorylated in the stress signaling pathway. Mutational experiments defined Thr-187 and Ser-192 in the activation loop as target residues to trigger TAK1 kinase activity (41, 42, 45). However, there is no evidence of the phosphorylation of endogenous TAK1 under physiological conditions. In this study, we generated a novel phospho-TAK1 antibody specifically recognizing the Thr-187 phosphorylated form. The present study using the antibody demonstrated that phosphorylation of TAK1 at Thr-187 is correlated with endogenous TAK1 kinase activity.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**—Expression vectors for TAK1, TAB1, and IKKβ were reported previously (18, 19, 41, 42). Point mutations were made by using a QuickChange site-directed mutagenesis kit (Stratagene), and all of the mutations were verified by DNA sequencing analysis. The expression vector for TAB2 was kindly provided by Dr. Kunihiro Matsumoto (Nagoya University, Japan).

**Antibodies and Reagents**—A novel antiphospho-TAK1 (Thr-187) antibody was generated by immunizing rabbits with the synthetic phosphopeptide corresponding amino acids 180–194 of human TAK1. The sequence of peptide antigen is as follows, NH2-CDIQTHM[pT]NNKG-7360.

**Cell Lines and Transfection**—HELa and HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO2. HEK293 and HELa cells were transduced with siRNAs in a final concentration of 20 μM using Lipofectamine reagents. At 72 h post-transfection, cells were stimulated.

**Preparation of Cell Extracts**—After transfection or stimulation, whole cell lysates were prepared with lysis buffer (25 mM HEPES, pH 7.7, 0.3 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfon fluoride, 1 mM dithiothreitol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cytoplasmic and nuclear extracts were prepared as described previously with some modifications (18). In brief, cells were suspended in 420 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfon fluoride, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The mixture was kept on ice for 15 min with frequent agitation. Nuclear extracts were prepared by centrifugation at 15,000 rpm for 5 min and stored at −85 °C.

**Immunoprecipitation**—Cell lysates were diluted with an equal volume of dilution buffer (20 mM HEPES, pH 7.7, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfon fluoride, 1 mM dithiothreitol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). After 1 h on ice, whole cell lysates were immunoprecipitated with anti-HA tag antibody (clone 262K or Y-11) or anti-FLAG (M5) antibody on ice for 1.5 h and then rotated with protein G-Sepharose (Amersham Bioscience) at 4 °C for 1.5 h. The Sepharose beads were washed three times with wash buffer (1:1 mixture of whole cell lysate buffer and dilution buffer).

**Immunoblotting**—Cell lysates or immunoprecipitates were resolved by 15% PAGE and transferred to Immobilon-P membrane (Millipore). The membrane was treated with BlockAce (Dainippon Pharmaceutical Co., Ltd, Saita, Japan) and probed with primary antibodies as described above. The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit, anti-mouse, and anti-goat IgG (DAKO), and visualized with the ECL system (Amersham Biosciences). For detection of endogenous phospho-TAK1, anti-phospho-TAK1 (Thr-187) antibody was incubated with the EnVision horseradish peroxidase reagent (DAKO) and anti-normal rabbit serum (DAKO). In competition assays, antibodies were preincubated with 10 μg/ml phosphorylated TAK1 peptides at 4 °C overnight.

**In Vitro Immunocomplex Kinase Assay**—Epitope-tagged TAK1 was immunoprecipitated with anti-FLAG or anti-HA antibody (Y-11, Santa Cruz Biotechnology) and endogenous TAK1 was immunoprecipitated with anti-TAK1 antibody. Immunoprecipitates were analyzed for TAK1 kinase activity using His6-MKK6 as a substrate. Endogenous JNK and IKK were immunoprecipitated with anti-HA tag antibody (clone 262K or Y-11) or anti-FLAG (M5) antibody on ice for 1.5 h and then rotated with protein G-Sepharose (Amersham Bioscience) at 4 °C for 1.5 h. The Sepharose beads were washed three times with wash buffer (1:1 mixture of whole cell lysate buffer and dilution buffer).

**Luciferase Assay**—Cells were cotransfected with expression vectors (as described above) and a luciferase reporter plasmid containing a 4×kB site or AP-1 site (Stratagene). The luciferase activity was measured by using the Dual-Luciferase reporter assay (Promega).

**RESULTS**

**Impaired Activation of NF-κB by TAK1-T187A**—Fig. 1A shows the amino acid sequences between subdomains VII and VIII (the activation loop) of TAK1 in the DNA data base. The protein kinase domains of mammalian TAK1, including human, mouse, and rat, are identical. The activation loop of Xenopus TAK1 (xTAK1) is also completely identical to that of mammalian TAK1. Moreover, Drosophila TAK1 (dTAK1) and MOM-4, a TAK1 homolog in Caenorhabditis elegans, retained the Thr residue corresponding to Thr-187 of mammalian TAK1. Ser-192 of TAK1, another possible phosphorylation site, is also conserved among the species, except for the replacement with Thr in MOM-4.
To characterize the physiological role of Thr-187 phosphorylation, we first tried to investigate the functional importance of Thr-187 in the NF-κB and AP-1 signaling pathways. Overexpression of wild type TAK1 with TAB1 stimulated IKK kinase activity and subsequent degradation of IkBα and nuclear translocation of the NF-κB p65 subunit (Fig. 1B). In contrast, the mutant TAK1-T187A as well as kinase-inactive TAK1-K63W failed to induce these cellular responses. Activation of p38 and JNK is also impaired in cells transfected with TAK1-T187A (Fig. 1C). In addition, the impaired activation of downstream pathways resulted in reduced transcriptional activation of NF-κB and AP-1 by TAK1-T187A (Fig. 1, D and E). These results indicate that Thr-187 is essential for the activation of TAK1 by TAB1.

**Fig. 1. Roles of Thr-187 in the NF-κB and MAPK signaling pathway.** A, comparison of amino acid sequences of the kinase domain between subdomains VII and VIII of TAK1 from various species. hTAK1, mTAK1, rTAK1, xTAK1, and dTAK1 show human, mouse, rat, C. elegans, and Drosophila TAK1, respectively. Asterisks show conserved possible phosphorylation sites. B and C, HeLa cells (1 × 10⁶ cells/6-cm dish) were transfected with expression vectors for wild type (WT) or point mutated (T187A and K63W) FLAG-tagged TAK1 (1 μg each). Total DNA amounts were adjusted to 2 μg with an empty vector. 24 h after the transfection, IKKa (B) and JNK (C) activities were evaluated by anti-IκBα, and anti-JNK immunocomplex kinase assay, respectively (top panel). Whole cell lysates were immunoblotted (IB) with anti-IκBα, anti-JNK, anti-phospho-p38, and anti-p38 antibodies (middle panels). Nuclear extracts (nucl. ext.) were immunoblotted with the anti-phospho-p65 (third panel in B), cytoplasmic extracts (cyt. ext.) were immunoblotted with anti-IκBα antibody (bottom panel in B). D and E, HeLa cells (1 × 10⁶ cells/3.5-cm dish) were transfected with wild type or point mutated FLAG-tagged TAK1 (1 μg) and HA-tagged TAB1 (1 μg), together with luciferase reporter plasmids driven by NF-κB (D) or AP-1 (E). 24 h after transfection, luciferase activities were determined by dual luciferase reporter assay. Data are the mean ± S.D. of triplicates from a representative experiment. IP, immunoprecipitated.

**TAB1-induced Thr-187 Phosphorylation of TAK1—** To characterize the physiological roles of TAK1 phosphorylation, we developed a novel phosphospecific antibody recognizing TAK1 phosphorylated at Thr-187 (pTAK1-Ab). It has been shown that overexpression of TAK1 with TAB1 resulted in activation of TAK1 (37, 41). Therefore, we first tried to detect Thr-187 phosphorylation of TAK1 in an overexpression experiment. FLAG-TAK1 and HA-TAB1 were expressed in HeLa cells, and then anti-FLAG immunoprecipitates were immunoblotted with pTAK1-Ab (Fig. 2A). The phosphospecific band and MKK6 phosphorylating activity of TAK1 were clearly detected when coexpressed with TAB1 (Fig. 2A). In contrast, no phosphorylation and kinase activity were detected in TAK1-T187A and TAK1-K63W even on the coexpression with TAB1 (Fig. 2A), indicating that pTAK1-Ab specifically recognizes the active form of TAK1. This correlates with the defective activation of the AP-1 and NF-κB signaling pathways by these TAK1 mutants described in Fig. 1.

Ser-192 in the activation loop of TAK1 is another proposed phosphorylation site regulating TAK1 activation (45). We confirmed that replacing Ser-192 with Ala inhibited TAK1 kinase activity, in which Thr-187 phosphorylation was also impaired (Fig. 2A). This result was derived from either impaired Thr-187 phosphorylation in the cells or the requirement of Ser-192 for the antibody binding to TAK1 in vitro. To address this issue, the specificity of pTAK1-Ab to phospho-Thr-187 was evaluated by immunoblotting in the presence of several phospho-TAK1 peptides as competitors (Fig. 2B). Phosphospecific TAK1 was not competed by peptides with no phosphorylation (T-S) and single phosphorylation at Ser-192 (T-pS) and Ser-192 (T-S) and single phosphorylation at Ser-192 (T-pS). In contrast, phospho-Thr-187 peptide (pT-S) and peptide with both phospho-Thr-187 and phospho-Ser-192 (pT-pS) completely blocked the binding of pTAK1-Ab to the active form of TAK1. These results demonstrated that pTAK1-Ab specifically recognizes phospho-Thr-187, and the binding is not dependent on the status of Ser-192 phosphorylation. The specific recognition of phospho-Thr-187 revealed that the mutation of Ser-192 resulted in impaired Thr-187 phosphorylation of TAK1 in vivo.
We next tried to determine whether Thr-187 phosphorylation occurred via an intra- or intermolecular reaction. HeLa cells were transfected with HA epitope-tagged full-length TAK1 or a deletion mutant (HA-TAK1kd) that contains the entire kinase domain (amino acids 1–303) to distinguish it from full-length TAK1, together with TAB1. Full-length TAK1 and TAK1kd, when expressed alone or in combination, induced Thr-187 phosphorylation, indicating that TAK1kd retained kinase activity (Fig. 3A). Of note, the kinase-inactive TAK1 (TAK1-K63W) was also phosphorylated at Thr-187 when coexpressed with the active TAK1kd. These results suggest that Thr-187 phosphorylation is mediated at least in part by an intermolecular reaction.

The intermolecular autophosphorylation of kinase-negative TAK1 made it possible to characterize the significance of Thr-187 and Ser-192 in TAK1 activation. FLAG-tagged full-length TAK1-K63W, TAK1-T187A, TAK1-S192A, and TAK1-T187A/S192A mutants were coexpressed with wild type HA-TAK1kd and HA-TAB1 (Fig. 3B). Immunoblotting of anti-FLAG immunoprecipitates showed that pTAK1-Ab recognized TAK1-S192A as well as TAK1-K63W, both of which conserved the Thr-187 residue, indicating that Ser-192 is not necessary for the recognition of phospho-Thr-187. Although TAK1-S192A had no kinase activity when coexpressed with TAB1 (Fig. 2A), the intermolecular autophosphorylation of TAK1-S192A by TAK1kd potentially induced its kinase activity (Fig. 3B). In contrast, the kinase activity of TAK1-kd187A was severely impaired even on the coexpression of TAK1kd and TAB1. These assays, HA-TAK1kd was not coimmunoprecipitated with full-length TAK1-187A. A, HeLa cells were transfected with expression vectors for wild type (WT) or mutated FLAG-TAK1 (T187A, S192A, and K63W) with or without HA-TAB1 (1 μg each). Immunoprecipitates (IP) with anti-FLAG antibody were immunoblotted (IB) with anti-phospho-TAK1, anti-TAK1, and anti-TAB1 antibodies. The TAK1 kinase activity was measured by an in vitro kinase assay (KA) using M KK6 as a substrate. B, HeLa cells were transfected with expression vectors for FLAG-TAK1 with or without HA-TAB1. 24 h after transfection, whole cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-phospho-TAK1 (Thr-187) antibodies in the absence or presence of 10 μg/ml phospho-TAK1 peptides as competitors (Comp peptide). T187-S192 and pT187-pS192 mean nonphosphorylated and phosphorylated peptide at Thr-187/Ser-192, respectively. The bottom two panels show the expression levels of TAK1 and TAB1.

**FIG. 2.** TAB1-induced phosphorylation of TAK1 at Thr-187. **A**, HeLa cells (1 × 10⁶ cells/6-cm dish) were transfected with expression vectors for wild type (WT) or mutated FLAG-TAK1 (T187A, S192A, and K63W) with or without HA-TAB1 (1 μg each). Immunoprecipitates (IP) with anti-FLAG antibody were immunoblotted (IB) with anti-phospho-TAK1, anti-TAK1, and anti-TAB1 antibodies. The TAK1 kinase activity was measured by an in vitro kinase assay (KA) using M KK6 as a substrate. **B**, HeLa cells were transfected with expression vectors for FLAG-TAK1 with or without HA-TAB1. 24 h after transfection, whole cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-phospho-TAK1 (Thr-187) antibodies in the absence or presence of 10 μg/ml phospho-TAK1 peptides as competitors (Comp peptide). T187-S192 and pT187-pS192 mean nonphosphorylated and phosphorylated peptide at Thr-187/Ser-192, respectively. The bottom two panels show the expression levels of TAK1 and TAB1.

**FIG. 3.** Intermolecular interaction of TAK1 phosphorylation by TAB1. **A**, HeLa cells were transfected with expression vectors for wild type (WT) HA-TAK1, HA-TAK1-K63W, and HA-tagged TAK1 kinase domain (HA-TAK1-kd) together with HA-TAB1. Whole cell lysates were immunoprecipitated (IP) with anti-HA antibody, and the immunoprecipitates were immunoblotted (IB) with anti-phospho-TAK1, anti-TAK1, and anti-HA antibodies, respectively. **B** and **C**, HeLa cells were transfected with expression vectors for wild type and mutated (K63W, T187A, S192A, and T187A/S192A) FLAG-TAK1 and HA-TAK1-kd together with HA-TAB1. Whole cell lysates were immunoprecipitated with anti-FLAG antibody or anti-HA antibody, and the immunoprecipitates were immunoblotted with anti-phospho-TAK1, anti-TAK1, and anti-TAB1 antibodies, respectively. The TAK1 kinase activity was measured by an anti-FLAG in vitro kinase assay using M KK6 as a substrate.
Regulation of TAK1 phosphorylation by TAB2—TAB2 associates with the COOH-terminal domain of TAK1 and is essential for TAK1 activation in TNF-α and IL-1 signaling pathways. To examine the ability of TAB2 to induce TAK1 phosphorylation, cells were transfected with expression vector for FLAG-TAK1 wild type (WT) and mutated TAK1 with or without HA-TAB1 and T7-TAB2. 24 h after transfection, whole cell lysates were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted (IB) with anti-phospho-TAK1, anti-TAK1, anti-TAB1, and anti-TAB2 antibodies. Immunocomplex kinase assay for TAK1 was carried out using MKK6 as a substrate.

FLAG-TAK1 (data not shown). These results indicate that Thr-187 plays a significant role in TAK1 activation by intermolecular autophosphorylation. However, the kinase activity of TAK1-S192A was lower than that of wild type TAK1 (Fig. 3C), suggesting that Ser-192 plays a role in full activation of TAK1.

**Regulation of TAK1 Phosphorylation by TAB2**—TAB2 associates with the COOH-terminal domain of TAK1 and is essential for TAK1 activation in TNF-α and IL-1 signaling pathways. To examine the ability of TAB2 to induce TAK1 phosphorylation, cells were transfected with expression vector for TAK1 and TAB2. The phosphorylation of wild type TAK1, but not TAK1 mutants, could be detected when coexpressed with TAB2 (Fig. 4A). However, TAB2-induced TAK1 phosphorylation was at a low level compared with TAB1-induced phosphorylation, and no TAK1 kinase activity was detected in the absence of TAB1 (Fig. 4B), supporting that TAB1 is essential for TAK1 kinase activity.

**TNF-α-induced Phosphorylation of Thr-187 of Endogenous TAK1**—To characterize the physiological roles of TAK1 phosphorylation, we tried to detect TNF-α-induced phosphorylation of endogenous TAK1. Immunoblotting with pTAK1-Ab demonstrated that three bands at least could be detected in whole cell lysates from TNF-α-treated HeLa cells (Fig. 5A). The faster migrating phospho-TAK1 bands (mainly two bands) were detected at 2–5 min, and additional slower migrating bands were clearly detected at 5 min. Although all of the phosphorylated bands had almost disappeared, the slower migrating forms were detected mainly at 10 min (Fig. 5A). The endogenous phospho-TAK1, but not total TAK1, was also competed out by phospho-Thr-187-containing peptides (pT-S and pT-pS), indicating that these bands were derived from Thr-187-phosphorylated forms of TAK1 (Fig. 5B). This rapid and transient phosphorylation was closely correlated with the inducible TAK1 kinase activity (Fig. 5A). These results indicate that TAK1 received multiple modifications in response to TNF-α and that the status of Thr-187 phosphorylation is the key to trigger and shut off the kinase activity.

In addition to the modifications of TAK1, two TAK1-binding proteins, TAB1 and TAB2, were also phosphorylated from 5 min after the stimulation (Fig. 5A). However, the phosphorylation is not essential for TAK1 activation because the kinase activity was detected at 2 min without phosphorylation of TAB1 and TAB2. Rather, it was detected during the down-regulation phase of TAK1 kinase activity in parallel with the appearance of hypermodified forms of TAK1 at 5–10 min. In fact, it has recently been shown that phosphorylation of TAB1 and TAB2 is involved in the p38α-mediated negative regulation of TAK1 kinase activity (26, 46) described below (see Figs. 7 and 8). Collectively, multiple phosphorylation of the TAK1-TAB1-TAB2 complex is required to regulate the transient TAK1 kinase activity.

**Effects of TAK1-T187A and TAK1 siRNA on JNK, p38, and IKK Pathways**—To investigate the role of TNF-α-induced TAK1 phosphorylation, we first tried to examine the effect of TAK1-T187A on NF-κB transcriptional activity. TNF-α-induced NF-κB-dependent luciferase expression was inhibited by a dominant negative inhibitor of IKKβ (IKKβ-K44M) (Fig. 5C). This result indicates that phosphorylation of Thr-187 is critically involved in the TNF-α-induced TAK1 activation.

To investigate roles of TAK1 in the downstream signaling pathways, we tried to examine the effects of TAK1 siRNA on TNF-α-induced stress signaling pathways. HeLa cells were transfected with TAK1 siRNA or control luciferase (luc) siRNA and then stimulated with TNF-α or high osmotic NaCl (Fig. 5D). TAK1 siRNA, but not luc siRNA, induced an effective knock-down of TAK1 expression without affecting PCNA expression, which was correlated with the decreased phosphorylation of TAK1 (Fig. 5D). This result confirmed the specificity of the phospho-TAK1 antibody to TAK1. In addition, the expression and phosphorylation of TAB1 and TAB2 were inhibited. Furthermore, TAK1 siRNA blocked p38, JNK, and IKK activation (Fig. 5D), demonstrating significant roles of TAK1 in the TNF-α-induced stress signaling pathways. In contrast, osmotic stress induced just a slight phosphorylation of TAK1, whereas phosphorylation of TAB1 and TAB2 was clearly induced (Figs. 5D, 6, and 7B). Interestingly, osmotic stress-induced JNK/p38 activation was not inhibited potently by TAK1 siRNA (Fig. 5D), suggesting that TAK1 is not the main MAP3K controlling the osmotic stress-induced activation of MAPKs. This supports a recent observation made using the RNAi technique that TAK1 is required for maximal activation of JNK by lipopolysaccharide, but not sorbitol-induced osmotic stress in Drosophila S2 cells (47).
is essential for TAK1 function, but Thr-187 phosphorylation of TAK1 is induced in the absence of TAB1.

In addition, the siRNA against TAB2 induced an effective knock-down of TAB2 protein, which resulted in impaired phosphorylation and activation of TAK1 (Fig. 6B). Effects of TAB2 siRNA in the TNF-α- and osmotic stress-induced activation of JNK/p38 and IKK were similar to the results from experiments using TAB1 siRNA (Fig. 6B). These results indicated that TAB2 is essential for triggering the Thr-187 phosphorylation of TAK1 and subsequent activation of TAK1.

Role of Thr-187 Phosphorylation in the Feedback Inhibition of TAK1 by p38α—It has been shown recently that phosho-
orylation of TAB1 and TAB2 is correlated with p38α-mediated feedback inhibition of TAK1 activity (26, 46). Here, we investigated the effects of a p38 inhibitor, SB203580, on cellular stress-induced TAK1 phosphorylation. HeLa cells were pretreated with SB203580 for 15 min followed by stimulation with TNF-α (A) or NaCl (B) for the indicated time periods. Whole cell lysates were immunoblotted (IB) with anti-phospho-TAK1 (Thr-187), anti-TAK1, anti-TAB1, and anti-TAB2 antibodies. The TAK1 kinase activity was determined by anti-TAK1 immunocomplex kinase assay using His6-MKK6 as a substrate. In addition, EGFP-TAB1-C3 as a substrate. The TAK1 kinase activity was determined by anti-TAK1 immunocomplex kinase assay using His6-MKK6 as a substrate. C, HeLa cells were transfected with siRNA against 20 nm p38α. At 72 h post-transfection, cells were stimulated with 20 ng/ml TNF-α for the indicated time periods. Immunoblotting (IB) and in vitro kinase assays (IP) were carried out as described in Fig. 5D. IP; immunoprecipitate.

Finally, we tried to examine the functional significance of the proposed p38α-phosphorylation residues, Ser-423 and Thr-431, in TAB1 on Thr-187 phosphorylation of TAK1. HeLa cells were transfected with wild type or mutant TAB1 replacing the residues with Ala (TAB1-SATA), and then stimulated with TNF-α. TNF-α-induced mobility shift of TAB1-SATA was partially impaired compared with wild type of TAB1, confirming that these residues are phosphorylated in response to cytokine. Overexpression of TAB1-SATA resulted in enhanced Thr-187 phosphorylation and kinase activity of TAK1, suggesting that p38α-mediated feedback inhibition of Thr-187 phosphorylation was dependent on the phosphorylation of TAB1.

DISCUSSION

It has been shown that phosphorylation of the Ser/Thr/Tyr residues in the activation loop of protein kinases is important for their catalytic activities. Several stress-responsive MAP3Ks have been shown to be activated by this mechanism. For example, intramolecular cis-autophosphorylation of two Thr residues of MEKK1 is required for the activation (48, 49). The Ser and Thr residues in the activation loop of MLK3 are the key to its activation via both autophosphorylation and trans-phosphorylation by upstream hematopoietic progenitor kinase 1 (50). It has been suggested that the Thr-845 of ASK1 can also be trans-phosphorylated by an unidentified protein kinase in response to H$_2$O$_2$ treatment, which subsequently induces autophosphorylation (51). However, these observations have mainly been made in overexpression experiments; therefore, little is known about the phosphorylation of MAP3Ks in cells exposed to cellular stress under physiological conditions. In this study, we obtained novel insights into the regulation of cellular stress-induced activation of the endogenous TAK1-TAB1-TAB2 complex by monitoring the phosphorylation of Thr-187 in the activation loop of TAK1.

There have been reported two possible activating phosphorylation sites of TAK1 (42, 45). We confirmed previous observations that mutations of Thr-187 or Ser-192 with Ala inhibited TAK1 kinase activity and Thr-187 phosphorylation (Fig. 2A). In addition, we found that autophosphorylation of Thr-187 occurs intermolecularly, and Thr-187 plays a significant role for TAK1 activation (Fig. 3). Although Ser-192 phosphorylation...
The hypothesis is supported by the findings that SB203580 and bly corresponded to the inducible kinase activity of TAK1. This synchronized after the stimulation, and the lower bands possibly corresponded to the inducible kinase activity of TAK1. This hypothesis is supported by the findings that SB203580- and p38α siRNA-mediated enhancement of TAK1 kinase activity correlates with increased formation of the lower bands (Fig. 7). On the other hand, we hypothesized that the upper migrating bands of TAK1 detected at 5 min after the TNF-α stimulation are inactivated forms (Fig. 2A), which is clearly supported by TAB1 RNAi experiment (Fig. 6A). This is also supported by the overexpression experiment showing impaired kinase activity of TAB1-C3-activating TAK1 with reduced mobility, compared with the TAK1/TAB1 complex (Fig. 8B). These results raise the possibility that additional modifications of Thr-187-phosphorylated TAK1 negatively control the TAK1 kinase activity to suppress the TNF-α-induced kinase activity of TAK1 rapidly. If this is correct, phospho-TAK1 antibody can recognize the inactivated forms as well as the active forms. It has been shown recently that replacement of Ser-1034 with Ala in the COOH-terminal domain enhanced ASK1 kinase activity, suggesting a negative control mechanism of ASK1 activity through phosphorylation of Ser-1034 (52). In this study, we demonstrated that Thr-187 phosphorylation in response to IL-1 attenuates IL-1-induced TAK1 kinase activity. PP2Cα, a novel member of the PP2C family, has also been shown to associate with TAK1, and they transiently dissociate during the activation of TAK1 in response to IL-1 (54). These findings raise the possibility that PP2Cs are Thr-187 protein phosphatases to suppress TAK1 kinase activity. Future identification of Thr-187 protein phosphatases will provide the mechanistic insights into the turnover of TAK1 kinase activity.

The phosphorylation of Ser/Thr/Tyr in the activation loop of protein kinases is mediated by different mechanisms. It has been well known that the TXY motif of MAPks is phosphorylated by upstream MAP2ks. As mentioned above, the activating phosphorylation of some MAP3ks such as ASK1 and MEKK1 has been shown to be mediated by autophosphorylation (48–51). In this study, we demonstrated that Thr-187 phosphorylation in vitro took place through an intramolecular mechanism (Fig. 3A). In contrast, Kishimoto et al. (45) reported previously that in vitro phosphorylation of the immunoprecipitated TAK1/TAB1 complex from cell lysates transfected with their expression vectors is mediated through intramolecular autophosphorylation. The immunoprecipitated TAK1 might be already phosphorylated at Thr-187 in the cells; therefore, its in vitro phosphorylation occurred at other unknown residues. These observations strongly suggest that the intermolecular...
trans-autophosphorylation of Thr-187 largely contributes to the expression of active TAK1 in response to cellular stress. However, the first event whereby the TAK1-TAB1-TAB2 complex causes the phosphorylation of Thr-187 is not clear. The dissociation of PP2Cs and TAB2-mediated intracellular redistribution is a possible mechanism. Furthermore, we could not rule out the existence of a Thr-187 kinase.

Komatsu et al. (56) demonstrated that embryonic fibroblasts from TAB1-deficient mice displayed drastically reduced TAK1 kinase activity and decreased sensitivity to TGF-β stimulation. However, there is no evidence directly demonstrating a role for TAB1 in cellular stress-induced TAK1 activation. In the present study, the RNAi experiment clarified a critical role of TAB1 in TNF-α-induced TAK1 activation and subsequent JNK/p38 activation (Fig. 6A). However, TAB1 siRNA did not fully block the TNF-α-induced phosphorylation of Thr-187, with slowly migrating forms mainly detected (Fig. 6A). A possible explanation of this finding is that a weakly expressed TAB1 ignored the phosphorylation of Thr-187 and the small amount of activated TAK1-TAB1 complex participated in the trans-autophosphorylation of orphaned TAK1. Therefore, unphosphorylated TAB1 may play a role in keeping TAK1 active to prevent the inactivating modification. It is interesting that inhibition of p38α-mediated TAB1 phosphorylation resulted in the increased formation of lower migrating forms of phospho-TAK1 (Figs. 7 and 8), raising the possibility that the feedback control by p38α-mediated phosphorylation of TAB1 triggers the negative modification of TAK1 to suppress TAK1 kinase activity. On the other hand, the depletion of TAB2 completely abolished the activation and phosphorylation of TAK1 (Fig. 6B), suggesting that intracellular recruitment to the corresponding TRAF proteins is essential for phosphorylation of Thr-187. Recently, several groups independently identified a TAB2-related protein, TAB3, which has been shown to play a role in TNF-α and IL-1 signaling pathways (27, 46, 57–59). Therefore, characterization of TAB3 in the phosphorylation of Thr-187 as well as TRAF-mediated ubiquitination of TAB2 and TAB3 is the next step to clarify the fine tuning of TAK1 activation by TAB2 and TAB3.

Feedback inhibition of TAK1 by p38α-mediated phosphorylation of TAB1/TAB2 is a recent advance in the regulation of TAK1 activation (26, 46). In this study, we confirmed the enhancing effect of SB203580 and p38α siRNA on TNF-α- and osmotic stress-induced TAK1 kinase activity with reduced phosphorylation of TAB1/TAB2, and newly found down-regulation of Thr-187 phosphorylation in the feedback control (Fig. 7). In addition, we detected enhanced phosphorylation and activation of TAK1 by TAB1-C lacking the p38α binding and PP2C-like domains (Fig. 8B) and TAB1-S/A/T substituting p38α-phosphorylating residues with Ala (Fig. 8C). These results suggest that the down-regulation of Thr-187 phosphorylation is mediated via TAB1 and TAB2. Although PP2CB and PP2Ce are strong candidates for the phosphatase carrying the dephosphorylation of Thr-187, none of the data rules out a role of the PP2C-like domain of TAB1 in p38α-mediated dephosphorylation of Thr-187. On the other hand, it has recently been shown that p38α is activated by MAPKK-independent and TAB1-dependent autophosphorylation of p38α (43, 44). Therefore, it is necessary to characterize the functional relationship between TAB1-TAB1-TAB2 and p38α further.

We and others have shown that TAK1 is a kinase-regulating IKK complex in mammalian cells. Mutations in the Drosophila dTAK1 gene reveal a conserved function in the control of Rel/NFκB-dependent innate immune responses (33). The RNAi approach revealed that knock-down of TAK1 resulted in the impaired phosphorylation of IκBo and p65 in HeLa cells (25, 60). In this study, we confirmed the inhibitory effect of TAK1 siRNA on the TNF-α-induced activation of IKK (Fig. 5D). In contrast, although siRNAs against TAB1 and TAB2 showed inhibitory effects on the cellular stress-induced activation of JNK/p38, they could not block the activation of IKK (Fig. 6). The gene targeting of TAB2 clarified that it is essential for embryonic development through prevention of liver apoptosis but not for the IL-1 receptor-mediated signaling pathway in embryonic fibroblasts (38). One possible explanation of this observation is that TAB3 is redundant in the activation of TAK1. On the other hand, TAB1 siRNA almost completely inhibited TAK1 kinase activity but not Thr-187 phosphorylation, suggesting that the Thr-187 phosphorylation is enough for the activation of IKK. In any case, further investigation is necessary for understanding the precise function of TAK1, TAB1, TAB2, and TAB3 in the TNF-α-induced IKK activation.

In summary, we demonstrated the regulatory mechanisms of the activating phosphorylation of TAK1 in a signaling complex with TAB1 and TAB2. The findings in this study provide new insight into the regulation of the rapid and transient activation of TAK1 and functional importance of the complex in TNF-α-induced stress signaling pathways. However, it is necessary to characterize further that the regulatory mechanisms found in this study are generally applied to other cells in the diverse cellular functions of TAK1.

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