Transforming Growth Factor-β (TGF-β1) Activates TAK1 via TAB1-mediated Autophosphorylation, Independent of TGF-β Receptor Kinase Activity in Mesangial Cells

Received for publication, April 11, 2009, and in revised form, June 25, 2009. Published, JBC Papers in Press, June 25, 2009, DOI 10.1074/jbc.M109.007146

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Transforming growth factor-β (TGF-β1) is a multifunctional cytokine that signals through the interaction of type I (TβRI) and type II (TβRII) receptors to activate distinct intracellular pathways. TAK1 is a serine/threonine kinase that is rapidly activated by TGF-β1. However, the molecular mechanism of TAK1 activation is incompletely understood. Here, we propose a mechanism whereby TAK1 is activated by TGF-β1 in primary mouse mesangial cells. Under unstimulated conditions, endogenous TAK1 is stably associated with TβRI. TGF-β1 stimulation causes rapid dissociation from the receptor and induces TAK1 phosphorylation. Deletion mutant analysis indicates that the juxtamembrane region including the GS domain of TβRI is crucial for its interaction with TAK1. Both TβRI-mediated TAK1 phosphorylation and TGF-β1-induced TAK1 phosphorylation do not require kinase activity of TβRI. Moreover, TβRI-mediated TAK1 phosphorylation correlates with the degree of its association with TβRI and requires kinase activity of TAK1. TAB1 does not interact with TGF-β receptors, but TAB1 is indispensable for TGF-β1-induced TAK1 activation. We also show that TRAF6 and TAB2 are required for the interaction of TAK1 with TβRI and TGF-β1-induced TAK1 activation in mouse mesangial cells. Taken together, our data indicate that TGF-β1-induced interaction of TβRI and TβRII triggers dissociation of TAK1 from TβRII, and subsequently TAK1 is phosphorylated through TAB1-mediated autophosphorylation and not by the receptor kinase activity of TβRI.

Members of the transforming growth factor-β (TGF-β) superfamily are key regulators of various biological processes such as cellular differentiation, proliferation, apoptosis, and wound healing (1, 2). TGF-β1, the prototype of TGF-β family, is a potent inducer of extracellular matrix synthesis and is well established as a central mediator in the final common pathway of fibrosis associated with progressive kidney diseases (3, 4). Upon ligand stimulation, TGF-β type 1 (TβRI) and type II (TβRII) receptors form heterotetrameric complexes, by which TβRI is phosphorylated in the GS domain and activated. Smad signaling pathway is well established as a canonical pathway induced by TGF-β1 (5, 6). Receptor-regulated Smads (Smad2 and Smad3) are recruited and activated by the activated TβRI. The phosphorylation in the GS domain (7) and L45 loop (8) of TβRI are crucial for its interaction with receptor-regulated Smads. After phosphorylation, receptor-regulated Smads are rapidly dissociated from TβRI and interact with common Smad (Smad4) followed by nuclear translocation. In addition to the Smad pathway, a recently emerging body of evidence has demonstrated that TGF-β1 also induces various Smad-independent signaling pathways (9–17) by which mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK) (18, 19), p38 MAPK (20–22), and extracellular signal-regulated kinase 1/2 (23, 24) can be activated by TGF-β1.

TAK1, initially identified as a MAPK kinase 7 (MKK7 or MAP3K7) in the TGF-β signaling pathway (11, 12), also can be activated by environmental stress (25), proinflammatory cytokines such as IL-1 and TNF-α (26, 27) and lipopolysaccharide (28). For TAK1 activation, phosphorylation at Thr-187 and Ser-192 in the activation loop of TAK1 is essentially required (29–31). TAK1 can transduce signals to several downstream signaling cascades, including the MAPK kinase (MKK) 4/7-JNK cascade, MKK3/6-p38 MAPK cascade, and nuclear factor κB (NF-κB)-inducing kinase-IκB kinase cascade (26–28). A recent report has shown that TAK1 is also activated by agonists of AMP-activated kinase (AMPK) and ischemia, which in turn activates the LKB1/AMPK pathway, a pivotal energy-sensor pathway (32). TAK1 is also involved in Wnt signaling (33). We and others have previously demonstrated that TAK1 is a major mediator of TGF-β1-induced type I collagen and fibronectin expression through activation of the MKK3-p38 MAPK and MKK4-JNK signaling cascades, respectively.

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3 The abbreviations used are: TGF-β, transforming growth factor-β; TAK1, TGF-β-activated kinase 1; TAB1, TAK1-binding protein; MAPK, mitogen-activated protein kinase; MKK3, MAPK kinase kinase 3; TβRI, type I TGF-β receptor; TβRII, type II TGF-β receptor; TNF-α, tumor necrosis factor α; TRAF, TNF receptor-associated factor; siRNA, small interfering RNA; JNK, c-Jun N-terminal kinase; IL, interleukin; IARAK, IL-1 receptor-associated kinase; XIAP, X-linked inhibitor of apoptosis; HA, hemagglutinin; MMC, mouse mesangial cell; FBS, fetal bovine serum; PBS, phosphate-buffered saline; WT, wild type; IP, immunoprecipitate; IB, immunoblot; BSA, bovine serum albumin; KD, kinase deficient.

4 This work was supported, in whole or in part, by National Institutes of Health Grant R01DK57661 (NIDDK). This work was also supported by an M. James Scherbenske grant from the American Society of Nephrology (to M. E. C.) and Beginning Grant-in-Aid 0665379U from the American Society of Nephrology (to M. E. C.) and Scherbenske grant from the American Society of Nephrology (to M. E. C.).

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.
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(34–37). Furthermore, increased expression and activation of TAK1 enhance p38 phosphorylation and promote interstitial fibrosis in the myocardium from 9-day-old TAK1 transgenic mice (37). These data implicate a crucial role of TAK1 in extracellular matrix production and tissue fibrosis. TAK1 is also implicated in regulation of cell cycle (38), cell apoptosis (39–41), and the Smad signaling pathway (42–44). Thus, TAK1 may function as an important regulator and mediator of TGF-β1-induced Smad-dependent and Smad-independent signaling pathways.

It has been demonstrated that TAK1 can be activated by the interaction with TAK1-binding protein 1 (TAB1) by in vitro binding assays and in overexpression studies (29–31); however, it is not clear whether TAB1 plays a crucial role in ligand-induced TAK1 activation. In embryonic fibroblasts from TAB1 null mice, IL-1 and TNF-α could induce TAK1-mediated NF-κB and JNK activation (45). TAK1 activation induced by TNF-α, IL-1, and T-cell receptor requires TAB2 or its homologous protein TAB3 (46–50). Although many questions still remain, much progress has been made in understanding the activation mechanism of TAK1 by inflammatory cytokines (46, 47, 51–53). Ligand binding of IL-1 receptor (IL-1R) results in recruitment of MyD88, which serves as an adaptor for IL-1 receptor-associated kinase (IRAK) 1 and 4. Subsequently IRAK1 is hyperphosphorylated and induces interaction with TNF-α receptor-associated factor 6 (TRAf6), resulting in TRAF6 oligomerization. After oligomerization of TRAF6, IRAK1-TRAf6 complex is dissociated from the receptor and associated with TAK1, which is mediated by TAB2 (or TAB3). In this process polyubiquitination of TRAF6 by Ubc13/Uev1A is thought to be critical for the association with TAB2 (or TAB3), which links TAK1 activation (46, 54, 55). In the case of TNF-α stimulation, TNF-α receptors form trimers and recruit adaptor proteins, TRAF2/5, and receptor-interacting protein 1 on the membrane. Ubc13/Uev1A- and TRAF2-dependent polyubiquitination of receptor-interacting protein 1 induce association of TAB2 (or TAB3), which then activates TAK1. Thus, TAB2 is required for ubiquitin-dependent activation of TAK1 by TRAFs. On the other hand, it has been demonstrated that hematopoietic progenitor kinase 1 plays a role as an upstream mediator of TGF-β-induced TAK1 activation, which in turn activates the MKK4-JNK signaling cascade in 293T cells (56, 57). Besides hematopoietic progenitor kinase 1, it has been also suggested that X-linked inhibitor of apoptosis (XIAP) might link TAK1 to TGF-β/BMP receptors through the capability of XIAP to interact with TGF-β/BMP receptors and TAB1 (58). Thus, although various molecules participate in the activation of TAK1, the precise mechanism by which TGF-β1 induces TAK1 activation is incompletely understood. Here, we provide evidence that the association of TAB1 with TGF-β receptors is important for TGF-β1-induced activation of TAK1 in mouse mesangial cells. TGF-β1 stimulation induces interaction of TβRI and TβRII, triggering dissociation of TAK1 from TβRI, and subsequently TAK1 is phosphorylated through TAB1-mediated autophosphorylation, independent of receptor kinase activity of TβRI.

MATERIALS AND METHODS

Reagents—Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN). Antibodies against HA (Y-11), Hisα, TAK1, TβRI, TβRII, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against MKK3, p-MKK3/6, p-Thr-187-TAK1 (p187-TAK1), p-Thr-184-TAK1, TAK1, and p-Smad3 were obtained from Cell Signaling Technology (Beverly, MA). Anti-p-Smad2 and anti-Smad2/3 antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti-FLAG (M2) and anti-Myc (9E10) antibodies were from Sigma. Cy3-conjugated goat anti-rabbit IgG antibody and fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody and normal goat serum were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-V5 antibodies and pcDNA3.1, pcDNA3.1/ V5-His TOPO TA expression kit, and Lipofectamine Plus™ reagent were purchased from Invitrogen.

Cell Cultures and Transfection—Glomerular mesangial cells from male C57BL/6 mice were isolated and characterized as previously described (22). Primary mouse mesangial cells (MMC) established in culture were maintained in RPMI 1640 medium supplemented with 15% FBS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. Transfection of expression vectors was performed using Lipofectamine Plus™ reagent (Invitrogen) according to the manufacturer’s instructions. In brief, cells grown to ~60% confluence on either 100- or 60-mm dishes were washed with phosphate-buffered saline (PBS) and transfected with 1 µg (for 100-mm dish) or 0.3 µg (for 60-mm dish) of the respective plasmids for 4 h under serum-free conditions. The total amount of DNA was adjusted with empty vector, pcDNA3.1. After transfection, cells were washed with PBS and incubated in medium supplemented with 15% FBS for 16 h before each experiment.

Expression Constructs—Mammalian expression constructs for HA-tagged wild type TAK1 (HA-TAK1) and kinase-deficient mutant TAK1 (HA-TAK1-KD (K63W)), FLAG-TAK1, and Myc-TAB1 were kindly provided by K. Matsumoto (Nagoya University) (60). FLAG-tagged wild type TAK1 (FLAG-TAK1), kinase-deficient mutant TAK1 (FLAG-TAK1-KD (K63W)), and C-terminal-truncated mutant TAK1 (FLAG-TAK1ΔC) were kindly provided by G. Gross (42). To amplify respective cDNAs, various PCR primer sets were synthesized according to corresponding DNA sequences (mouse TAK1, NM_012775; rat TAK1, BC006665; rat TβRI; NM_012775). TAK1 cDNAs for wild type and kinase-deficient mutant were amplified by using PCR with TAK1 primer set (forward primer, 5'-GGATCCGGGATCATGTCGACAGCCCTCCGC-3' ; reverse primer, 5'-CCGCTTACGTAAGTGCCTTGCTTTCG-3') and re-cloned using pcDNA3.1/V5-His TOPO TA expression kit, and V5/His-TAK1 and V5/His-TAK1-KD were produced. The phosphorylation site mutant of TAK1 (V5/His-TAK1-TA (T187A)) and polyubiquitination site mutant of TAK1 (V5/His-TAK1-K34R) were produced by PCR-based mutagenesis with mutagenic primer sets (TAK1-T187A forward primer, 5'-CAAACACACATGGCCAATAAATAAG-3'; TAK1-T187A reverse primer, 5'-CTTATATTGCTATGTTGTTTTG-3'; TAK1-K34R

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forward primer, 5′-CTGGAATTCGAGGAGATCGACT-ACAGGGAGATCGAGGTGGA-A-3′; TAK1-K34R-reverse primer, 5′-CCACCTGATCCCTCGTGGCTGATGCGTCTTC-GAATTCGAGGACC-3′) and the TAK1 primer set. The resulting construct was confirmed by sequencing. HA-tagged wild type TβRI (pCMV5B-HA-TβRI-WT), kinase-deficient mutant TβRI (pCMV5B-HA-TβRI-KD (K232R)), His-tagged TβRI (pCMV5B-His-TβRI-WT), and kinase-deficient TβRI (pCMV5B-His-TβRI-KD (K227R)) were obtained from J. Wrana (62) through Addgene (Cambridge, MA).

V5/His-tagged wild type TβRI (V5/His-TβRI) was re-cloned in pcDNA3.1/V5-His TOPO TA expression vector after PCR using rat TβRI cDNA as a template (62, 63) and RI primer set (RI forward primer, 5′-GCCTCGAGGAGACGGATCC-3′; RI reverse primer, 5′-CCCCAAGCTTTACGATCC-3′). Various point mutations were introduced into V5/His-tagged TβRI by PCR-based mutagenesis with the RI primer set and respective mutagenic primer set. The constitutively active mutant of rat TβRI (V5/His-TβRI-CA (T198D)) was obtained with the mutagenic primer set (RI forward primer, 5′-GATATCCTGCTACAGAAC-3′; RI reverse primer, 5′-GCTTCTCTGGAGCTGATC-3′). The kinase-deficient mutant of rat TβRI (V5/His-His-TβRI (K226R)) was produced with the primer set RI-K226R forward primer (5′-GGAATATTCTCTTCTAGA-3′) and RI-K226R reverse primer (5′-TTCTTCTCAGAAGAGATATTCC-3′). The GS domain mutant of rat TβRI (V5/His-His-TβRI-GS) bearing mutations of five phosphorylation sites in the GS domain (T185V, T186V, S187A, S189A, S191A) was produced with the RI-GS mutagenic primer set (RI forward primer, 5′-GTAGTTGAGGAGGAGGAGAGC-GCGGTATTACCTGTTCA-3′; RI reverse primer, 5′-TTGAACAGAGCTGTTGAAGCCGGCTCTTGAGCC-3′). Each of the PCR products was re-cloned in pcDNA3.1/V5-His TOPO TA expression vector, and correct clones were confirmed by sequencing.

Deletion mutant versions of rat TβRI (V5/His-TβRI-LC and V5/His-TβRI-SC) were generated by PCR with the RI forward primer and respective reverse primers (RI-LC reverse primer, 5′-CCAGCTTTTCTAGGCTAACA-TACAGTCTG-3′ or RI-SC reverse primer, 5′-CCCCAAGCTTACAGGTGTTAAGCCGGCTCTTGAGCC-3′). V5-His-TβRI-SC (amino acids 1–151) contains only nine amino acids with the partial TRAF6 binding motif after the transmembrane domain, whereas V5/His-TβRI-LC (amino acids 1–255) harbors the juxtamembrane region, GS domain, and a portion of the kinase domain excluding the L45 loop. The construction of rat HA-tagged TβRII and C-terminal-truncated mutant TβRII (HA-TβRII and HA-TβRIIΔC) was previously described (63).

Small Interfering RNAs (siRNAs)—siGENOME SMARTpool targeted against mouse TAB1 (L-042328-00) and siCONTROL Non-Targeting Pool (D-001206-13) were purchased from Dharmacon Inc. Transfection of siRNA using DharmaFECT™4 reagent was carried out according to the manufacturer’s instructions. Briefly, cells were grown to 50% confluence on 60-mm dishes in 3 ml of media supplemented with 15% FBS. Twenty microliters of respective siRNA (total 400 μmol) were added to 380 μl of serum-free media and mixed with 400 μl of serum-free media containing 8 μl of DharmaFECT™4. After 20 min, 3.2 ml of media containing 15% serum were added to prepare siRNA transfection media. For transfection, culture media were replaced with the siRNA transfection media. The final concentration of respective siRNA was 100 nM for each transfection. After transfection, cells were incubated in medium containing 15% FBS for 48 h, then rendered quiescent in medium supplemented with 0.5% FBS for 16 h before each experiment.

Immunoprecipitation and Western Blot Analysis—Cells were washed once with ice-cold PBS and lysed in buffer containing 1% Nonidet P-40, 20 mM Tris (pH 8.0), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl2, 2 mM EGTA, 1 mM NaF, 2 mM diethiothreitol, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 20 μM aprotinin. Cells were disrupted by using sonication and then centrifuged for 15 min at 14,000 × g at 4 °C to remove cellular debris. The protein concentration of cell lysates was determined by BCA protein assay reagent kit (Pierce). For Western blotting, protein samples (50–100 μg) were subjected to 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with either 5% nonfat milk or 5% bovine serum albumin (BSA) for 1 h and then incubated with primary antibodies overnight on a rocker at 4 °C. The membranes were washed 3 times (15 min each) with TBS buffer (10 mM Tris (pH 7.5), 50 mM NaCl, and 0.05% Tween 20) and then incubated with horse-radish peroxidase-conjugated secondary antibodies for 30 min at room temperature. The target proteins were detected with LumiGLO (Cell Signaling Technologies). In the case of immunoprecipitation experiments, 200–500 μg of cell lysates were pre-cleaned with either normal mouse or rabbit IgG with protein A/G-agarose for 2 h at 4 °C. Cell lysates were transferred to fresh tube and reacted with 2 μg of primary antibodies for 30 min at 4 °C followed by precipitation with 20 μl of protein A/G-agarose for 2 h for proteins overexpressed or overnight for endogenous proteins at 4 °C. The immunoprecipitates were then washed three times with the lysis buffer and subjected to Western blotting.

Immunofluorescence Staining—MMC grown to 70% confluency were rendered quiescent in medium supplemented with 0.5% FBS for 16 h, then stimulated with TGF-β1 (2 ng/ml) for the indicated times. Cells were washed 3 times with PBS and then treated with freshly prepared solution containing 2% paraformaldehyde, 0.1% Triton X-100 in PBS for 15 min to fix and permeabilize cells. Cells were washed 3 times with PBS and once with BSA solution (0.5% BSA and 0.15% glycine in PBS). Cells were incubated with blocking solution (5% BSA and 10% normal goat serum in PBS) for 30 min followed by washing 3 times with BSA solution. Cells were incubated with primary antibodies (1:100) in blocking solution at 4 °C overnight. Anti-TAK1 (C-9) mouse monoclonal IgG and anti-TβRI (V-22) rabbit polyclonal IgG antibodies (Santa Cruz Biotechnology) were used as primary antibodies for double staining. Cells were washed 5 times with BSA solution and then treated for 30 min with secondary antibodies conjugated to a fluorochrome (1:400 for Cy3-conjugated goat anti-rabbit IgG antibody and 1:200 for fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody) in BSA solution. Cells were washed three times with
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**RESULTS**

**TAK1 Interacts with TGF-β Receptors**—We have previously demonstrated that TGF-β1 stimulation rapidly activates TAK1 in primary MMC and that PP2A negatively regulates TGF-β1-induced TAK1 activation (64). To understand the mechanism of TAK1 activation by TGF-β1 in MMC, we investigated whether TAK1 interacts with TGF-β receptors. We first determined whether endogenous TAK1 and TGF-β receptors interact. As shown in Fig. 1A, endogenous TAK1 was coimmunoprecipitated with both TβRI and TβRII under unstimulated conditions. To further examine the interaction of TAK1 with the TGF-β receptors, FLAG-TAK1 was coexpressed with V5/His-TβRI or HA-TβRII or both, and immunoprecipitation was carried out with either anti-TβRI or anti-TβRII antibodies followed by immunoblotting with anti-FLAG, anti-V5, and anti-HA antibodies (Fig. 1B, upper group of three panels). The expression of each of the transfected genes was confirmed in whole cell lysates not subjected to immunoprecipitation by Western blotting with corresponding antibodies (Fig. 1B, lower group of three panels). In cells transiently transfected with FLAG-TAK1 and V5/His-TβRI, we observed that FLAG-TAK1 binds to V5/His-TβRI, and similarly, in transiently transfected cells with FLAG-TAK1 and HA-TβRII, FLAG-TAK1 binds to HA-TβRII, although to a lesser degree. However, coexpression of both receptors V5/His-TβRI and HA-TβRII formed TβRI-TβRII receptor complexes and significantly reduced their interaction with FLAG-TAK1. To clarify the effect of the coexpression of TβRII on the interaction of TAK1 with TβRI, reciprocal immunoprecipitation was carried out with anti-TAK1 antibody followed by immunoblotting with anti-V5, anti-HA, and anti-FLAG antibodies, and similar patterns of TAK1 interaction with the receptors were observed (Fig. 1C, upper group of three panels). The expression of each of the transfected genes was confirmed in whole cell lysates not subjected to immunoprecipitation by Western blotting with corresponding antibodies (Fig. 1C, lower group of three panels). Thus, these data indicate that the interaction of TAK1 with TβRII does not require high level coexpression of TβRII, but rather, the coexpression of TβRII interferes with the association of TAK1 with TβRI.

It has been shown previously that the coexpression of TβRI and TβRII promotes formation of TβRI-TβRII complexes and activates TβRI resulting in constitutive signaling (65). Therefore, we next examined whether TGF-β1 stimulation, which induces the TβRI-TβRII heterotetrameric complex formation, also results in reduced endogenous interaction of TAK1 with TβRI. Cell lysates from MMC stimulated with TGF-β1 for varying time periods were subjected to immunoprecipitation with anti-TβRI antibody followed by immunoblotting with anti-TAK1 antibody. To compare the levels of phosphorylation of TAK1 induced by TGF-β1 with the association of TAK1 with TβRI, the same cell lysates were subjected to Western blotting with anti-phospho-TAK1 antibody. Relative equivalence of protein loading was confirmed by immunoblotting the same membrane with anti-TAK1 antibody. As shown in Fig. 1D, treatment with TGF-β1 rapidly and transiently decreased the association of TAK1 with TβRI within 2 and 5 min (top panel), whereas TAK1 phosphorylation was increased at the same time periods (middle panel). Given that total TAK1 levels remained equivalent (lowermost panel), a reduction of TβRI-bound TAK1 indicates that more TAK1 has become unbound and is dissociated from TβRI. In addition, TAK1 and TβRI were colocalized in MMC under unstimulated condition (yellow/orange dots), and TGF-β1 stimulation resulted in a reduction in colocalized TAK1 and TβRI at 5 min and returned to near previous levels prior to stimulation by 15 min (supplemental Fig. S1). Thus, our findings suggest that TGF-β1 stimulation triggers the dissociation of TAK1 from TβRI and induces phosphorylation of TAK1.

**Juxtamembrane Region Including the GS Domain of TβRI Is Crucial for Its Interaction with TAK1**—To determine what region in TβRI is important for its interaction with TAK1, the effect of various TβRI deletion and point mutations (illustrated schematically in Fig. 2A) on this interaction was examined. As shown in Fig. 2B (top panel), coimmunoprecipitation assays show that C-terminal deletion of the kinase domain of TβRI (V5/His-TβRI-LC) had little effect on the interaction of TβRI with TAK1 compared with wild type TβRI (V5/His-TβRI-WT)
when coexpressed with HA-TAK1 in MMC, whereas the deletion of cytoplasmic region including the GS and kinase domains (V5/His-TßRI-SC) dramatically reduced this interaction. On the other hand, mutating the phosphorylation sites in the GS domain (V5/His-TßRI-GS) had little effect on the interaction with TAK1 (Fig. 2B, third lane). The expression of each of the transfected genes was confirmed in whole cell lysates not subjected to immunoprecipitation by Western blotting with the corresponding antibodies (Fig. 2B, lower two panels). These data indicate that the juxtamembrane region including the GS domain, and not the kinase domain, plays a crucial role in the interaction of TßRI with TAK1, but this interaction is not dependent on phosphorylation in the GS domain.

TßRI Kinase Activity Is Not Required for Its Interaction with TAK1—The GS domain phosphorylation has been shown previously to be involved in activation of the TßRI kinase. To further investigate the role of TßRI activity on its interaction with TAK1, we examined the effect of constitutively active mutant of TßRI (V5/His-TßRI-CA) coexpressed with HA-TAK1 in MMC. As shown in Fig. 2C (top panel), the interaction of V5/His-TßRI-CA with TAK1 was significantly reduced compared with wild type TßRI (V5/His-TßRI-WT) or GS domain phosphorylation site mutant TßRI (V5/His-TßRI-GS). The V5/His-TßRI-GS was used as a non-activating mutant of TßRI, as it does not transmit TGF-ß signaling activity in the 3TP-Luc reporter assay (data not shown) as seen with the same TßRI-SC and constitutively active mutant V5/His-TßRI-CA, which we have previously demonstrated to exhibit reduced interaction with TAK1 compared with wild type TßRI and the other mutants, V5/His-TßRI-GS and V5/His-TßRI-LC. Relative equivalence of protein loading and the expression of each of the transfected genes were confirmed by Western blotting with corresponding antibodies (Fig. 2C, lower two panels). These data suggest that the level of TßRI-mediated TAK1 activation may be affected proportionally to the level of TAK1 interaction with TßRI and that it was not dependent on the receptor kinase activity of TßRI. To determine whether TßRI kinase activity is required for TGF-ß1-induced TAK1 phosphorylation, we used SB431542, a chemical inhibitor of TßRI kinase. Treatment of MMC with TGF-ß1 for varying time periods, as shown in Fig. 3B, in the presence of SB431542 did not prevent TGF-ß1-induced TAK1 phosphorylation (top two panels) but completely abrogated Smad2/3 phosphorylation (lower two panels). Thus, our data indicate that the kinase activity of TßRI is not required for activation of TAK1 in response to TGF-ß1 stimulation.

TßRII Interferes with the Interaction of TAK1 with TßRI and Reduces TAK1-mediated TAK1 Phosphorylation—We also examined the role of the kinase activity and the cytoplasmic region of TßRII in its interaction with TAK1. As shown in Fig. 4A (top panel), kinase-deficient K227R mutant of TßRII (KD, third lane) had little effect on its interaction with TAK1 compared with wild type TßRII (WT, second lane). On the other
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**A)**

| HA-TAK1 | V5/His-TβRI | p\(^{187}\)-TAK1 |
|---------|--------------|-----------------|
|         |              |                 |

**B)**

| TGF-β1  | SB431542  |
|---------|-----------|
| 0       | 0         |
| 2       | +         |
| 5       | +         |
| 15 (min)| -         |

FIGURE 3. TAK1 activation is not dependent on receptor kinase activity of TβRI. A, coexpression of TβRI with TAK1 induces TAK1 phosphorylation at Thr-187 independently of the receptor kinase activity of TβRI. HA-TAK1 was coexpressed in MMC with WT or respective mutant versions of V5/His-TβRI (CA constitutively active mutant), GS (phosphorylation site mutant), SC (deletion of the kinase domain), and LC (deletion of the kinase domain and SC (deletion of the GS and kinase domain)). Cell lysates were subjected to IB with anti-p\(^{187}\)-TAK1, anti-HA, and anti-V5 antibodies. B, TGF-β1-induced TAK1 phosphorylation does not require receptor kinase activity of TβRI. MMC grown to subconfluence were rendered quiescent in medium supplemented with 0.5% FBS for 16 h and pretreated with 10 ng/ml TGF-β1 for 1 h before stimulation with TGF-β1 (2 ng/ml) for the indicated times. Cell lysates were subjected to IB with anti-p\(^{187}\)-TAK1 and anti-TAK1 antibodies, respectively. To verify phosphorylation of Smad2 and Smad3, cell lysates were subjected to IB with both anti-p-Smad2 and anti-p-Smad3 antibodies simultaneously. Total Smad2/3 expression was confirmed by IB with anti-Smad2/3 antibody.

hand, deletion of the cytoplasmic region of TβRII (ΔC, fourth lane) eliminated its ability to interact with TAK1. Transiently expressed His-TβRII-WT, His-TβRII-KD, and His-TβRII-ΔC in the precipitates were confirmed by immunoblotting with anti-His antibody (Fig. 4A, middle panel), and the expression of HA-TAK1 was confirmed in whole cell lysates not subjected to immunoprecipitation by Western blotting with anti-HA antibody (Fig. 4A, bottom panel). These findings demonstrate that the cytoplasmic region of TβRII is important for its interaction with TAK1, but this interaction is not dependent on TβRII kinase activity.

We next sought to examine the effect of TβRII on TAK1-TβRI interaction and TAK1 activation. Coimmunoprecipitation assays shown in Fig. 4B (upper group of three panels) demonstrated that coexpression of wild type TβRII with TβRII formed receptor complexes, consistent with our previous data (Fig. 1B), and that TAK1 binding to wild type TβRII was significantly reduced upon coexpression of TβRII (third lane) compared with that observed in the absence of TβRII coexpression (second lane). Interestingly, coexpression of constitutively active mutant of TβRII (fourth and fifth lanes) or GS domain phosphorylation site mutant (sixth and seventh lanes) with TβRII also formed receptor complexes and reduced binding to TAK1. We also observed that the decrease in TAK1 binding upon coexpression of TβRII was associated with decreased levels of phosphorylation of TAK1 at Thr-187 (lower group of four panels). Taken together, our data indicate that coexpression of TβRII with TβRII induces TβRII-TβRII complex formation and reduced TAK1 interaction with TβRI and TAK1 phosphorylation, regardless of TβRII activity.

We also examined the role of TβRII kinase activity in TAK1 receptor interaction and TAK1 phosphorylation. As shown in Fig. 4C, kinase-deficient TβRII also bound deletion mutant V5/His-TβRII-LC and GS domain phosphorylation site mutant V5/His-TβRII-GS (third and fifth lanes in the middle panels) and reduced their interaction with TAK1 (third and fifth lanes in the top two panels), indicating that the TβRI-TβRII interaction does not require TβRI kinase domains or kinase activity of TβRI or TβRII. On the other hand, deletion of the GS and kinase domains of TβRI dramatically reduced interaction with TAK1, and coexpression of kinase-deficient TβRII had little effect on the markedly reduced binding of TAK1 with TβRII mutant V5/His-TβRII-SC (Fig. 4C, sixth and seventh lanes in the top two panels). The expression of each of the transfected genes in the precipitates was confirmed by immunoblotting with corresponding anti-TβRI and anti-TβRII antibodies (Fig. 4C, middle two panels), and the expression of HA-TAK1 was confirmed in whole cell lysates not subjected to immunoprecipitation by Western blotting with anti-HA antibody (Fig. 4C, bottom panel).

Next, we verified the importance of the cytoplasmic region of TβRII in TβRI interaction with TAK1. To this end, V5/His-TβRII-LC and FLAG-TAK1 were expressed in MMC together with wild type TβRII (HA-TβRII-WT) or truncated TβRII lacking the cytoplasmic region (HA-TβRIIΔC). As shown in Fig. 4D, the association of FLAG-TAK1 with V5/His-TβRII-LC was significantly interrupted by wild type TβRII (third lane, top panel) but not by HA-TβRIIΔC (fourth lane, top panel), although HA-TβRIIΔC bound to V5/His-TβRII-LC (fourth lane, third panel from the top). The complex formation of TβRIIΔC with TβRI has been previously shown (67). Taken together, these results suggest that the association of TβRI with TAK1 might be interrupted by interaction of the juxtamembrane region containing the GS domain of TβRI with the cytoplasmic region of TβRII.

TβRII-mediated TAK1 Activation Is Accomplished by Its Autophosphorylation—Given our findings indicating that TAK1 is phosphorylated by coexpression of TβRII regardless of its receptor kinase activity, we sought to determine whether TβRII-mediated TAK1 phosphorylation is accomplished by autophosphorylation. To address this, we first verified autophosphorylation of TAK1 mediated by TAB1. As shown in Fig. 5A, coexpression of wild type TAK1 (V5/His-TAK1-WT) and FLAG-TAB1 strongly induced TAK1 phosphorylation at Thr-184 and Thr-187 in MMC, whereas kinase-deficient mutant (K63W) of TAK1 (V5/His-TAK1-KD) or phosphorylation site mutant (T187A) of TAK1 (V5/His-TAK1-TA) coexpressed with FLAG-TAB1 failed to induce autophosphorylation of TAK1. We then examined the effects of TAK1 kinase activity on TβRII-mediated phosphorylation of TAK1. As shown in Fig. 5B (upper group of panels), the kinase-deficient mutant of TAK1 (FLAG-TAK1-KD) coimmunoprecipitated with V5/His-TβRII (fifth lane), and coexpression of HA-TβRII...
TAB1 Mediates TGF-β1-induced TAK1 Activation—Based on our findings above showing that TβRI-mediated TAK1 phosphorylation is accomplished by its autophosphorylation and TAB1 is a strong inducer of TAK1 autophosphorylation, we next posed the question as to whether TAB1 is involved in TGF-β1-induced TAK1 phosphorylation. We used an approach to knock down endogenous TAB1 in MMC by siRNA specific for mouse TAB1. After transfection of MMC with either control siRNA or TAB1 siRNA, cells were stimulated with TGF-β1 (2 ng/ml) for 5 and 15 min, and knock down of TAB1 was confirmed by Western blot analysis of the cell lysates (Fig. 5C). We also noted that total TAK1 expression was reduced by TAB1 siRNA, but not by control siRNA, perhaps because TAB1 exists in a 1:1 stoichiometric complex with TAK1 (68) and that activation has a correlation with its stability (29). TAB1 siRNA blocked TGF-β1-induced TAK1 phosphorylation at Thr-187 but did not alter TGF-β1-induced Smad2/3 phosphorylation. These data suggest that the activation of TAK1 by TGF-β1 is mediated by TAB1-induced autophosphorylation of TAK1 in MMC.

TAB1 and Phosphorylated TAK1 Do Not Interact with TGF-β Receptors—We next sought to determine the effect of TAB1 on the interaction of TAK1 with TGF-β receptors. Myc-TAB1 was coexpressed with FLAG-TAK1 and either V5/His-TβRI or HA-TβRI in MMC followed by immunoprecipitation with anti-TAK1 antibody and immunoblotting with corresponding antibodies, as indicated in Fig. 6A, upper group of panels. The immunoprecipitation assays demonstrate that coexpression of TAB1 did not alter the association of TAK1 with either TβRI or TβRII. The expression of each of the transfected genes was confirmed in whole cell lysates not subjected to immunoprecipitation by Western blotting with the corresponding antibodies (Fig. 6A, lower group of panels).

To assess whether TAB1 is also associated with TGF-β receptors as a TAK1-TAB1 complex, V5/His-TβRI and HA-TAK1 were coexpressed with or without Myc-TAB1 followed by immunoprecipitation with anti-V5 antibody (Fig. 6B, second to fourth lanes in the upper group of panels), or HA-TβRII and reduced this association (sixth lane), similar to wild type TAK1. However, although we consistently observed TβRI-mediated TAK1 phosphorylation upon coexpression of wild type TAK1 with TβRI and inhibition by coexpression of TβRII, no TβRII-mediated phosphorylation of TAK1 at Thr-187 was observed upon coexpression of the kinase-deficient TAK1 with TβRII either without or with TβRII (Fig. 5B, lower group of panels) even though the kinase-deficient mutant (K63W) of TAK1 (FLAG-TAK1-KD) contains the authentic phosphorylation sites (Thr-184 and Thr-187). Thus, our data indicate that TβRII-mediated TAK1 phosphorylation is accomplished by its own kinase activity.

FIGURE 4. TβRII interferes with the interaction of TβRI with TAK1. A, cytoplasmic region of TβRII is responsible for its interaction with TAK1. HA-TAK1 was coexpressed in MMC with respective His-tagged WT, kinase-deficient mutant (KD), or cytoplasmic region truncation mutant (ΔC) of TβRII. Cell lysates were subjected to IP with anti-His antibody followed by IB with anti-HA and anti-His antibodies. The expression of HA-TAK1 in whole cell lysates (CL) not subjected to IP was confirmed by IB with anti-HA antibody. B, coexpression of TβRI and TβRII reduces the interaction of TβRI with TAK1 and TβRII-mediated TAK1 phosphorylation independent of receptor kinase activity of TβRI. FLAG-TAK1 was coexpressed in MMC with respective WT, constitutively active mutant (CA), or phosphorylation site mutant (GS) of TβRII with or without coexpression of HA-TβRII. Cell lysates were subjected to IP with anti-V5 antibody followed by IB with anti-FLAG, anti-His, and anti-HA antibodies. TAK1 phosphorylation and the expression of each exogenous gene in whole cell lysates (CL) not subjected to IP were evaluated by IB with anti-p187-TAK1, anti-FLAG, anti-His, and anti-HA antibodies. C, kinase activity of TβRII is not required for the reduction of the interaction of TβRI with TAK1. HA-TAK1 was coexpressed in MMC with V5/His-tagged WT or mutants of TβRII with or without coexpression of kinase-deficient mutant (KD) of TβRII. Cell lysates were subjected to IP with anti-V5 antibody followed by IB with anti-HA, anti-TRβI, and anti-TAK1 antibodies. The expression level of exogenous TAK1 in whole cell lysates (CL) was confirmed by IB with anti-TAK1 antibody. D, cytoplasmic region of TβRII is required for the reduction of the interaction of TβRI with TAK1. FLAG-TAK1, V5/His-TβRII-LC, and either HA-tagged WT or cytoplasmic region truncation mutant (ΔC) of HA-TβRII were coexpressed in MMC, as indicated. Cell lysates were subjected to IP with anti-V5 antibody followed by IB with anti-FLAG, anti-His, and anti-HA antibodies. The expression of transfected genes in whole cell lysates (CL) not subjected to IP was confirmed by IB with anti-FLAG, anti-His, and anti-HA antibodies. LC, deletion of the kinase domain.
FIGURE 5. TβRI-mediated and TGF-β1-induced TAK1 phosphorylation requires its own kinase activity and TAB1. A, TAB1 induces autophosphorylation of TAK1. FLAG-TAB1 was coexpressed in MMC with WT, kinase-deficient mutant (KD; K63W), or phosphorylation site mutant (TA; T187A) of V5/His-TAK1. Cell lysates were subjected to IB with anti-p187-TAK1, anti-p-p187-TAK1, or anti-V5 antibody to evaluate the expression and the phosphorylation at Thr-187 and Thr-184 of TAK1. B, TβRI-mediated TAK1 phosphorylation is achieved by kinase activity of TAK1. Either WT or kinase-deficient mutant of TAK1 (KD) was coexpressed in MMC with V5/His-TβRI and HA-TβRII, as indicated. Cell lysates were subjected to IP with anti-V5 antibody followed by IB with anti-FLAG, anti-His, and anti-HA antibodies. As a control for IP with anti-V5 antibody, cells coexpressing V5/His-TAK1 and Myc-TAB1 were used (first lane, indicated by dashed box). Normal mouse IgG was used as a negative control. To evaluate the expression level of respective exogenous gene and TAK1 phosphorylation, whole cell lysates (CL) were subjected to IB with anti-V5, anti-HA, anti-Myc, and anti-HA antibodies. C, TAB1 and p187-TAK1 do not associate with TβRI. V5/His-TβRI was coexpressed in MMC with HA-TAK1 and Myc-TAB1 as indicated. Cell lysates were subjected to IP with anti-V5 antibody followed by IB with anti-p187-TAK1, anti-HA, anti-Myc, and anti-HA antibodies. As a control for IP with anti-V5 antibody, cells coexpressing V5/His-TAK1 and Myc-TAB1 were used (first lane, indicated by dashed box). Normal rabbit IgG was used as a negative control. To evaluate the expression level of respective exogenous gene and TAK1 phosphorylation, whole cell lysates (CL) were subjected to IB with anti-p187-TAK1, anti-V5, anti-Myc, and anti-HA antibodies.

V5/His-TAK1 was coexpressed with or without Myc-TAB1 followed by immunoprecipitation with anti-HA antibody (Fig. 6C, second to fourth lanes in the upper group of panels). As a control for the immunoprecipitation assay with anti-V5 anti-body, cells coexpressing V5/His-TAK1 and Myc-TAB1 were used. Conversely, as a control for immunoprecipitation assay with anti-HA antibody, cells coexpressing HA-TAK1 and Myc-TAB1 were used (Fig. 6, B and C, the first lane, indicated by dashed box). We applied normal mouse IgG (Fig. 6B, fifth lane) and rabbit IgG (Fig. 6C, fifth lane) as a negative control for the respective immunoprecipitation assay. Myc-TAB1 was successfully coimmunoprecipitated with V5/His-TAK1 (Fig. 6B, first lane) and with HA-TAK1 (Fig. 6C, first lane) by the corresponding anti-V5 or anti-HA antibodies. We also confirmed that the coexpression of TAK1 and TAB1 induces TAK1 phosphorylation at Thr-187 by Western blot analysis of whole cell lysates (Fig. 6, B and C, lower group of panels), consistent with
These data indicate that TAB1 does not coexist in TAK1-TβRI with either receptor (Fig. 6), though non-phosphorylated TAK1 was coimmunoprecipitated by V5 antibody. Phosphorylation of TAK1 and MKK3 was evaluated by IB with anti-p187-TAK1, anti-TAK1, and anti-p-MKK3 antibodies. Knockdown of TRAF6 was verified by IB with anti-TRAF6 antibody. Phosphorylation of TAK1 and MKK3 was examined by IB of whole cell lysates (Fig. 7C). These data suggest that ubiquitination of TAK1 with TβRI under unstimulated conditions (Fig. 7A) but also the phosphorylation of TAK1 and MKK3, a downstream effector of TAK1 induced by TGF-β1 stimulation in MMC (Fig. 7B). These data indicate that TRAF6 mediates the interaction of TAK1 with TβRI as well as TGF-β1-induced TAK1 activation.

We next confirmed whether ubiquitination of TAK1 by TRAF6 is essential for TβRI-mediated TAK1 phosphorylation. To this end, we generated a single-point mutant of TAK1 in which Lys-34, the polyubiquitination site by TRAF6 (72), was mutated to Arg (V5/His-TAK1-K34R). As shown in Fig. 7C, the polyubiquitination site mutant (V5/His-TAK1-K34R) coimmunoprecipitated with HA-TβRI, similar to wild type TAK-1 (V5/His-TAK1-WT), but failed to be phosphorylated upon coexpression of HA-TβRI. As expected, phosphorylation of V5/His-TAK1-WT resulted in slower migration compared with the V5/His-TAK1-K34R (Fig. 7C, middle panel of the lower group). These data suggest that ubiquitination of TAK1 at Lys-34 by TRAF6 might be crucial for TβRI-mediated TAK1 activation as well as for TGF-β1-induced TAK1 activation. On the other hand, we found that our previous data shown in Fig. 5A, and the phosphorylated TAK1 and TAB1 were readily detectable in the immunoprecipitates pulled down with TAK1 using anti-V5 or anti-HA antibodies (Fig. 6, B and C, first lane in the upper group of panels). However, in immunoprecipitates pulled down with either TβRI or TβRII, neither TAB1 nor phosphorylated TAK1 were coimmunoprecipitated (Fig. 6, B and C, fourth lane) even though non-phosphorylated TAK1 was coimmunoprecipitated with either receptor (Fig. 6, B and C, third and fourth lanes). These data indicate that TAB1 does not coexist in TAK1-TβRI or TAK1-TβRII complexes. Moreover, phosphorylated TAK1 does not associate with TβRI or TβRII. Thus, our findings suggest that TGF-β1-induced TAK1 phosphorylation does not occur on the receptors.

Traf6 Mediates the Interaction of TAK1 with TβRI—Recent evidence has demonstrated that TRAF6 plays a crucial role in TGF-β-induced TAK1 activation (61, 72). However, it is not clear whether TRAF6 mediates the interaction of TAK1 with TβRI. Therefore, we examined whether TRAF6 is implicated in the interaction of TAK1 with TβRI by using transient transfection of TRAF6 siRNA. Knockdown of TRAF6 abrogated not only the interaction of TAK1 with TβRI under unstimulated conditions (Fig. 7A) but also the phosphorylation of TAK1 and MKK3, a downstream effector of TAK1 induced by TGF-β1 stimulation in MMC (Fig. 7B). These data indicate that TRAF6 mediates the interaction of TAK1 with TβRI as well as TGF-β1-induced TAK1 activation.

**FIGURE 7.** TRAF6 mediates the interaction of TAK1 with TβRI and TGF-β1-induced TAK1 activation. A, a knockdown of TRAF6 by siRNA inhibits TβRI-TAK1 interaction. After transfection of MMC with control siRNA (−) or siRNA specific for TRAF6 (+), cells were incubated for 48 h in medium supplemented with 15% FBS. Cell lysates were subjected to IP with anti-TβRI antibody followed by IB with anti-TAK1 antibody. IP with normal rabbit IgG was used for the negative control. Knockdown of TRAF6 and equivalent loading of each protein sample were verified by IB for TRAF6 and TAK1, respectively. B, TRAF6 mediates TGF-β1-induced TAK1 activation. After transfection of MMC with control siRNA or siRNA specific for TRAF6, cells were incubated for 48 h in medium supplemented with 15% FBS and then rendered quiescent in medium with 0.5% FBS for 16 h followed by stimulation with TGF-β1 (2 ng/ml) for the indicated times. Cell lysates were subjected to IP with anti-TβRI antibody followed by IB with anti-TAK1 antibody. Knockdown of TRAF6 was verified by IB with anti-TRAF6 antibody. Phosphorylation of TAK1 and MKK3 was evaluated by IB with anti-p187-TAK1, anti-TAK1, anti-p-MKK3, and anti-MKK3 antibodies. Equivalent loading of each protein sample was verified by IB for α-tubulin. C, deletion of polyubiquitination site of TAK1 abrogates TAK1 phosphorylation. HA-TβRI was coexpressed with either wild type TAK1 (V5/His-TAK1 WT) or polyubiquitination site mutant of TAK1 (V5/His-TAK1-K34R), as indicated. Cell lysates were subjected to IP with anti-HA antibody followed by IB with anti-V5 antibody. Phosphorylation of TAK1 and the expression of each exogenous gene were confirmed by IB of whole cell lysates (CL) with anti-p187-TAK1, anti-TAK1, and anti-HA antibodies. D, deletion of polyubiquitination site of TAK1 does not affect TAB1-mediated TAK1 phosphorylation. FLAG-TAB1 was coexpressed with either wild type TAK1 (V5/His-TAK1 WT) or polyubiquitination site mutant of TAK1 (V5/His-TAK1-K34R), as indicated. Phosphorylation of TAK1 and the expression of exogenous TAK1 were confirmed by IB of whole cell lysates (CL) with anti-p187-TAK1 and anti-V5 antibodies.

The C Terminus of TAK1 Is Required for Its Association with TβRI—It has been previously reported that, in addition to TAB1, TAB2 is required for TRAF6-mediated TAK1 activation by inflammatory cytokines such as IL-1 and TNF-α (46–50). Unlike TAB1, TAB2 binds to the C terminus of TAK1 (12) and links TAK1 to TRAF6. Given our findings that TRAF6 mediates the interaction of TAK1 with TβRI and TGF-β1-induced TAK1 activation in MMC, we investigated whether TAB2 is associated with TAK1 and TβRI. To address this, cell lysates were subjected to immunoprecipitation with anti-TAK1 antibody and anti-TβRI antibody, respectively, followed by immunoblotting with anti-TAB2 antibody. Immunoprecipitation with normal rabbit IgG was used as a negative control. As shown in Fig. 8A, endogenous TAB2 is associated with TAK1 as well as TβRI under unstimulated condition in MMC. To determine whether interaction of TAB2 is involved in the interaction of TAK1 with TβRI and TAK1 activation, either full-length wild type TAK1 (FLAG-TAK1 WT) or truncated TAK1 lacking

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**EQUATION OF TAK1 BY TGF-β1**
**Activation of TAK1 by TGF-β1**

**FIGURE 8.** The C terminus of TAK1 is required for the interaction of TAK1 with TβRI. An endogenous TAB2 interacts with TAK1 and TβRI. Whole cell lysates from unstimulated MMC were subjected to IP with anti-TβRI or anti-TAK1 antibody, as indicated. Normal rabbit IgG was used as a negative control. Immunoprecipitates and whole cell lysates (CL) not subjected to immunoprecipitation were analyzed by immunoblotting (IB) with anti-TB2 antibody, anti-p187-TAK1, anti-FLAG, and anti-HA antibodies. Whole cell lysates from unstimulated MMC were subjected to IP with anti-TβRI or anti-FLAG antibody, respectively. As shown in Fig. 8A, Flag-TAK1WT coimmunoprecipitated HA-TβRI. HA-TβRI or Flag-TAK1ΔC, deletion of C terminus of TAK1 abrogates its phosphorylation mediated by TβRI. Whole cell lysates (CL) from βB were subjected to IB with anti-157/TAK1, anti-FLAG, and anti-HA antibodies. E, deletion of C terminus of TAK1 does not affect TAB1-mediated TAK1 phosphorylation. Myc-TAB1 was coexpressed withFlag-tagged wild type TAK1 (WT), kinase-deficient mutant TAK1 (K2D), or C-terminal-truncated mutant TAK1 (ΔC). Phosphorylation of TAK1 and the expression of each exogenous TAK1 were confirmed by IB of whole cell lysates (CL) with anti-p187-TAK1 and anti-FLAG antibodies.

The C-terminal region (FLAG-TAK1ΔC) was coexpressed with HA-TβRI in MMC, and the cell lysates were subjected to immunoprecipitation with anti-FLAG antibody (Fig. 8B) and anti-HA antibody (Fig. 8C) followed by immunoblotting with anti-HA antibody or anti-FLAG antibody, respectively. As shown in Fig. 8B, FLAG-TAK1WT coimmunoprecipitated HA-TβRI but not FLAG-TAK1ΔC. These results were confirmed by reciprocal immunoprecipitation experiments shown in Fig. 8C. The deletion of the C-terminal region also abrogated TβRI-mediated TAK1 phosphorylation (Fig. 8D) but did not affect TAB1-mediated TAK1 phosphorylation (Fig. 8E). Thus, these data suggest that TAB2 may link TAK1 to TβRI-TRAF6 complexes.

**DISCUSSION**

TGF-β1 signals through the interaction with heterotetrameric complexes of TβRI and TβRII to activate distinct intracellular pathways that include Smad and non-Smad pathways. The Smad pathway represents a canonical signaling pathway induced by TGF-β1 stimulation that has been well established. However, the mechanisms of TGF-β1-induced non-Smad signaling pathways are incompletely understood. TAK1 was initially identified as a kinase activated by members of the TGF-β and BMP family. However, much of the research investigating the mechanisms of activation of TAK1 has been mainly elucidated from the standpoint of stimulation by pro-inflammatory cytokines such as IL-1β and TNF-α (69). In the present study we demonstrate that TGF-β1-induced TAK1 activation is accomplished by TAB1-mediated autophosphorylation in a TGF-β receptor kinase-independent manner.

We observed that endogenous TAK1 is stably associated with TβRI and TβRII under unstimulated condition in MMC (Fig. 1A). Studies by Watkins et al. (70) show evidence for a direct interaction of TAK1 with TβRII and for an indirect interaction of TAK1 with TβRI in rat cardiomyocytes. On the other hand, XIAP also possibly links TAK1-TAB1 complexes to BMP receptors because of their capability of binding to both TAB1 and BMP receptors (58). Our present findings demonstrate that the interaction of TAK1 with TβRI does not require high level coexpression of TβRII (Fig. 1, B and C), suggesting that TAK1 may associate with TβRI independent of TβRII in MMC. In addition, neither TβRI nor TβRII was detectable as a complex with TAB1, although it was readily found as a complex with TAK1 (Fig. 6), implying that TAK1-TAB1 complexes do not associate with TGF-β receptors. Indeed, although the V5/His-TβRI-LC mutant has no L45 loop required for the interaction with XIAP (59), it could still interact with TAK1, like the wild type TβRI (Fig. 2B, fourth lane). Furthermore, we could not detect an association of endogenous TAK1 with XIAP in MMC (data not shown). Thus, neither TβRII nor XIAP likely mediates the association of TAK1 with TβRI in MMC.

Although recent evidence has shown that TRAF6 physically interacts with TGF-β receptors and mediates TGF-β-induced TAK1 activation (61, 72), there are some discrepancies among study results. Yamashita et al. (61) suggested that TRAF6 interacts directly with TβRII, whereas its interaction with TβRI is mediated through TβRI. On the other hand, Sorrentino et al. (72) defined the TRAF6 binding motif at the juxtamembrane region of TβRI by which TRAF6 directly binds to TβRI. Our findings also demonstrate that the interaction of endogenous TAK1 with TβRI is mediated by TRAF6 (Fig. 7A, third lane) and that the partial deletion of TRAF6 binding motif in TβRI (V5/His-TβRI-SC mutant) dramatically reduces its interaction with XIAP (59). Another discrepancy concerns the requirement of TβRI kinase activity for TRAF6-mediated TGF-β-induced TAK1 activation. The studies by Sorrentino et al. (72) show that TGF-β-induced TAK1 activation occurs independently of TβRI kinase activity by using selective inhibitors of TβRI kinase (SB505124 and SB431542), whereas Yamashita et al. (61) show by using mutant constructs of TβRI that TGF-β-induced polyubiquitination of TRAF6 leading to TAK1 activation occurs in a TβRI kinase activity-dependent manner. Our present data are consistent with the findings by Sorrentino et al. (72) in that the kinase activity of TβRI is not required for TGF-β-induced TAK1 activation in MMC.

Coexpression of TβRI with TAK1 demonstrates that TβRI induces TAK1 phosphorylation in MMC in growth medium supplemented with 15% FBS. Unexpectedly, however, this TβRI-mediated TAK1 phosphorylation is also observed when
TAK1 is coexpressed with kinase-inactive mutant of TβRI, such as TβRI-G5 and TβRI-LC (Fig. 3A). In contrast, a constitutively active mutant of TβRI (TβRI-CA) exhibited significantly reduced interaction with TAK1 (Fig. 2C) as well as TAK1 phosphorylation (Fig. 3A). Thus, TβRI-mediated TAK1 phosphorylation is not dependent on the receptor kinase activity of TβRI. This notion is further supported by our studies using SB431542, a specific TβRI kinase inhibitor. Treatment of MCF with TGF-β1 in the presence of SB431542 did not prevent TAK1 phosphorylation (Fig. 3B) but prevented phosphorylation of Smad2/3, as expected, indicating that TGF-β1-induced TAK1 activation is not dependent on the kinase activity of TβRI. Moreover, TβRI-mediated TAK1 phosphorylation is regulated proportionally to the level of TAK1 interaction with TβRI (Fig. 3A), suggesting that the association of TAK1 with TβRI may be crucial for its activation.

Our findings consistently demonstrate that TGF-β1 stimulation transiently reduces the association of TAK1 with TβRI (Fig. 1D and supplemental Fig. S1) and rapidly induces TAK1 phosphorylation (Fig. 1D), suggesting that the ligand-induced formation of TβRI-TβRII complex may trigger the dissociation of TAK1 from TβRI, which correlated with TAK1 activation. In addition, coexpression of TβRI and TβRII results in continuous reduction of TAK1 association with TβRI and TβRII-mediated TAK1 phosphorylation (Fig. 4B), suggesting that retaining the receptor complexes might interfere with the re-association of TAK1 with TβRI, resulting in the reduction of TβRI-mediated TAK1 phosphorylation. Moreover, our data also show that the deletion of cytoplasmic domain of either TβRI or TβRII abrogates TβRII-mediated reduction of TAK1 association with TβRI (Fig. 4, C and D), indicating that the interaction of cytoplasmic domains of the receptors interferes with TAK1 association with TβRI. Therefore, it is plausible that the pre-association of TAK1 with TβRI and the ligand-induced receptor complex-dependent dissociation of TAK1 from TβRI might be crucial events for rapid activation of TAK1 by TGF-β1 stimulation. Likewise, Nohe et al. (71) has reported that the activation of TAK1-p38 signaling axis by BMP2 also requires BMP2-induced recruitment of receptors, whereas pre-assembled type I and type II BMP receptor complexes activate the Smad signaling pathway in response to BMP.

Our studies demonstrate that TAB1 mediates TGF-β1-induced TAK1 phosphorylation in MCF (Fig. 5C). However, TAB1 and phosphorylated TAB1 do not interact with TGF-β receptors (Fig. 6, B and C). We also consistently observe that TGF-β1 stimulation triggers the dissociation of TAK1 from TβRI (Fig. 1D and supplemental Fig. S1). TGF-β1 stimulation induces the formation of TβRI-TβRII-KR receptor complexes, and our data indicate that the interaction of cytoplasmic region of TβRII with the juxtamembrane region including the GS domain of TβRI interferes with the interaction of TAK1 with TβRI (Fig. 4, C and D). Taken together, our findings suggest that TGF-β1-induced TAK1 phosphorylation does not occur within the receptor complex but likely occurs after dissociation from the receptors.

We show that TRAF6 mediates the interaction of TAK1 with TβRI as well as TGF-β1-induced TAK1 activation (Fig. 7, A and B). It has been reported that the interaction of TRAF6 with TβRI results in polyubiquitination of TRAF6 and leads to TAB1 activation through direct polyubiquitination at Lys-34 of TAK1 by E3 ubiquitin ligase activity of TRAF6 (72). Similarly, our data show that the polyubiquitination site mutant of TAK1 (TAK1-K34R) interacts normally with TβRI (Fig. 7D), it failed to be phosphorylated by the coexpression with TβRI (Fig. 7C). On the other hand, the TAK1-K34R mutation does not affect TAB1-mediated TAK1 activation (Fig. 7E). It has been previously reported that TAB2 links TAK1 to TRAF6 (46, 69). The binding site of TAB2 is located near the C-terminal end on TAK1 (12), and deletion of the TAB2 binding site eliminates the interaction of TAK1 with TRAF6 as well as TAB2 (46). Our findings demonstrate that TAB2 is stably associated with TAK1 as well as TβRI (Fig. 8A), and deletion of the C-terminal region (TAK1ΔC) including the TAB2 binding site disrupts its interaction with TβRI (Fig. 8, B and C) and TβRI-mediated phosphorylation (Fig. 8D). However, TAK1ΔC can be autophosphorylated by TAB1 (Fig. 8E). Therefore, these data suggest that TAB2 may link TAK1 to TβRI-TRAF6 complexes, and polyubiquitination of TAK1 at Lys-34 by TRAF6 is implicated in TβRI-mediated TAK1 phosphorylation in MCF.

In summary, our data provide evidence that under unstimulated conditions TAK1 physically interacts with TGF-β type I and type II receptors in MCF. TGF-β1 stimulation induces the formation of TβRI-TβRII complexes, and TAK1 is released from the receptor complex and is activated through TAB1-mediated autophosphorylation of TAK1, independent of TGF-β receptor kinase activity (Fig. 9). We also demonstrate that TRAF6 and TAB2 are required for the interaction of TAK1 with TβRI and TGF-β1-induced TAK1 activation in MCF.

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