TAK1 MAPK Kinase Kinase Mediates Transforming Growth Factor-β Signaling by Targeting SnoN Oncoprotein for Degradation*

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Transforming growth factor-β (TGF-β) regulates a variety of physiologic processes through essential intracellular mediators Smads. The SnoN oncoprotein is an inhibitor of TGF-β signaling. SnoN recruits transcriptional repressor complex to block Smad-dependent transcriptional activation of TGF-β-responsive genes. Following TGF-β stimulation, SnoN is rapidly degraded, thereby allowing the activation of TGF-β target genes. Here, we report the role of TAK1 as a SnoN protein kinase. TAK1 interacted with and phosphorylated SnoN, and this phosphorylation regulated the stability of SnoN. Inactivation of TAK1 prevented TGF-β-induced SnoN degradation and impaired induction of the TGF-β-responsive genes. These data suggest that TAK1 modulates TGF-β-dependent cellular responses by targeting SnoN for degradation.

Transforming growth factor-β (TGF-β)2 is a multifunctional cytokine involved in the regulation of proliferation, differentiation, migration, and survival of many different cell types (1, 2). TGF-β ligand binds to and activates Ser/Thr kinase receptors (3). This leads to the phosphorylation and activation of the receptor-regulated Smad family (R-Smad). Smad2 and Smad3 (4). Phosphorylated R-Smad forms a functional complex with the co-mediator Smad (Co-Smad), Smad4, and this complex accumulates in the nucleus and modulates expression of the TGF-β-responsive genes such as plasminogen activator inhibitor type-1 (PAI-1) (1, 5, 6). The nuclear Smads complex is maintained in an inactive state via its association with Ski family oncoproteins, Ski and SnoN (7, 8). By binding to Smads, Ski and SnoN recruit transcriptional repressor complexes such as N-CoR/SMRT and mSin3A to TGF-β target promoters and thereby repress transcription of TGF-β-responsive genes (7, 9). Upon TGF-β stimulation, SnoN is immediately down-regulated via the ubiquitin-proteasome pathway induced by anaphase-promoting complex (APC) or Smurf2 E3 ubiquitin-protein isopeptide ligases (10–12). Degradation of SnoN initially allows the Smad heteromeric complex to activate TGF-β target genes (13). However, longer TGF-β treatment leads to higher expression via transcriptional activation of the SnoN gene (14). This functions as a negative feedback circuit to limit the effects of TGF-β. Importantly, overexpression of SnoN results in the loss of TGF-β-induced growth arrest of the cells, suggesting a potential mechanism for SnoN-mediated oncogenesis (8, 14).

TGF-β-activated kinase 1 (TAK1) is a member of mitogen-activated protein kinase kinase kinase (MAPKKK) and functions as a signaling intermediate in several intracellular signaling pathways including the TGF-β and interleukin-1 pathways (15–18). TAK1 is catalytically activated by TGF-β stimulation (15) and plays an essential role in TGF-β-induced p38 activation (17). TAK1 has also been implicated in several TGF-β-induced biological processes including apoptosis (19) and vascular development (20). However little is known about how TAK1 mediates TGF-β signaling. In this study, we found that TAK1 interacts with SnoN and targets it for degradation. The TAK1 regulation of SnoN may participate in TGF-β-induced cellular responses.

**EXPERIMENTAL PROCEDURES**

Plasmids and Protein—The mammalian expression vectors for TAK1, HA-TAK1, HA-TAK1(K63W), TAB1, and HA-ubiquitin have been described previously (15, 18, 21, 22). Full-length SnoN and SnoN(1–366) were subcloned into pCMV in-frame with HA tag or FLAG tag at the N terminus. Small interference RNA (siRNA) was produced using the BS/H1 vector to direct expression of the relevant hairpin double-stranded sequence from the H1 promoter. The siRNA target sequences corresponded to nucleotides 88–106 of the TAK1 coding region. Target oligonucleotides were synthesized (5’-GATCCCCGAGTCGACTACAAGGAGATCAGTCAAGGAGATCAGTCCCTTGTAGTCGATCTCCTTTTGGAAA-3’; and 5’-AGCTTTTCAAAAAAGAGATCGACTACAAGGAGA-3’).
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TCTCTTGAATCTCTTGTAGTCGATCTCGGG-3’), annealed, and cloned into BS/H1 between the BglII and HindIII sites using standard molecular cloning techniques. Generation of various point mutations of full-length SnoN and SnoN-(1–366), including full-length SnoN and SnoN-(1–366) mutated at Ser-115, Ser-117, and Thr-119 to Ala or Ser-108, Ser-112, Ser-115, Ser-117, Thr-119, Ser-126, Ser-140, and Ser-141 to Ala, were done using PCR and QuikChange II XL site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing. Bacterially expressed SnoN-(1–366) fused to glutathione S-transferase (GST) (GST-SnoN-(1–366)) was purified using glutathione-Sepharose 4 Fast Flow (Amersham Biosciences) according to manufacturer’s instructions. The retrovirus vectors for HA-SnoN full-length and HA-SnoN mutant full-length were generated by insertion of HA-tagged SnoN cDNAs into pQCXIP vector (Stratagene).

Antibodies and Reagents—The following antibodies were used: anti-HA monoclonal antibody 16B12 (Covance), anti-FLAG monoclonal antibody M2 (Sigma), anti-Sno polyclonal antibody (Upstate Biotechnology), anti-TAK1 antibody (18), anti-p53 monoclonal antibody DO-1 (Santa Cruz Biotechnology), anti-Smad2/3 polyclonal antibody (Upstate Biotechnology), and anti-IκBα monoclonal antibody (Upstate Biotechnology), anti-TAK1 antibody (18), anti-Sno polyclonal antibody (Upstate Biotechnology), anti-Tak1 polyclonal antibody (Upstate Biotechnology), anti-Sno polyclonal antibody C-21 (Santa Cruz Biotechnology), anti-β-catenin monoclonal antibody 14 (BD Biosciences), anti-Smad2/3 polyclonal antibody (Upstate Biotechnology), anti-p53 monoclonal antibody DO-1 (Santa Cruz Biotechnology), and anti-β-actin monoclonal antibody AC-15 (Sigma). Recombinant human TGF-β1 was purchased from Roche Applied Science. The bait plasmid and the library cDNAs were co-transformed into the yeast strain PJ69-4A using the lithium acetate method. The bait to screen a mouse B cell library (in pGAD) (21). Yeast Two-hybrid Screening—Plasmid pGBD-C-TAK1(K63W) was used as bait to screen a mouse B cell library (in pGAD) (21). The bait plasmid and the library cDNAs were co-transformed into the yeast strain PJ69-4A using the lithium acetate method. Transfections of HaCaT cells and HeLa S3 cells were carried out using the calcium phosphate precipitation method. Stable transfections of HaCaT cells and HeLa S3 cells were carried out using TransFastTM (Promega). The retrovirus for expression of HA-SnoN full-length and HA-SonN mutant full-length were generated and infected into HaCaT cells according to the manufacturer’s instruction. Stable cell line selection was achieved using G418, hygromycin B, and cycloheximide (Calbiochem) were used.

Cell Culture, Transfection, and Virus Infection—293 cells, HaCaT cells, and HeLa S3 cells were cultured in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum or bovine growth serum (HyClone). Transfection of 293 cells was carried out using the calcium phosphate precipitation method. Stable transfections of HaCaT cells and HeLa S3 cells were carried out using TransFastTM (Promega). The retrovirus for expression of HA-SnoN full-length and HA-SonN mutant full-length were generated and infected into HaCaT cells according to the manufacturer’s instruction. Stable cell line selection was achieved using G418, hygromycin B, or puromycin.

Yeast Two-hybrid Screening—Plasmid pGBD-C-TAK1(K63W) was used as bait to screen a mouse B cell library (in pGAD) (21). The bait plasmid and the library cDNAs were co-transformed into the yeast strain PJ69-4A using the lithium acetate method. Yeast cells were plated on selective medium plates and allowed to grow at 30 °C. Positive colonies were then restreaked on selective medium plates. Plasmid DNA was rescued from positive colonies that grew on selective medium plates and subject to further sequence analysis.

Immunoprecipitation and Immunoblotting—Whole cell extracts were prepared in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 2 mM dithiothreitol, 1 mM Na₃VO₄, 1 mM phenylmethyisulfonyl fluoride, 100 units/ml aprotinin, 0.5% Triton X-100). Proteins from cell lysates were immunoprecipitated with 1 µg of various antibodies and 15 µl of protein G-Sepharose (Amersham Biosciences). The immune complexes were washed three times with wash buffer containing 20 mM HEPES (pH 7.4), 500 mM NaCl, and 10 mM MgCl₂ and once with rinse buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, and 10 mM MgCl₂ and suspended in 30 µl of rinse buffer. For immunoblotting, the immunoprecipitates or cell lysates were resolved on SDS-PAGE and transferred to Hybrid-P membranes (Amersham Biosciences). The membranes were immunoblotted with various antibodies, and bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against mouse or rabbit IgG using the ECL Western blotting system (Amersham Biosciences).

Cellular Fractionation—To isolate the nuclear and the cytoplasmic fractions, cells in 10-cm dishes were treated with TGF-β (5 ng/ml) and then lysed with 500 µl of hypotonic buffer A (50 mM HEPES (pH 7.4), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 100 units/ml aprotinin) containing 0.1% Nonidet P-40 and homogenized in a Dounce homogenizer (30 strokes). Lysates were then centrifuged at 4,000 × g for 4 min. The supernatant was centrifuged at 12,500 × g for 4 min to obtain the cytosolic fraction. This pellet was resuspended in hypotonic buffer B (buffer A containing 1.7 M sucrose) and then centrifuged at 15,000 × g for 30 min. The pellet (nuclear fraction) was resuspended in 0.5% Triton X-100 lysis buffer and sonicated. All steps were performed on ice or at 4 °C. Protein concentrations were determined using the micro BCA protein assay kit (Pierce). The purity of the cytosolic and nuclear fractions was assessed by immunoblotting of IκB (a cytosolic marker) and lamin B (a nuclear marker).

Real-time Quantitative Reverse Transcription-PCR—Real-time quantitative PCR was performed using 7300 Real-time PCR system (PE Applied Biosystems) and SYBR Premix Ex Taq (Takara Bio Inc.). The cycling conditions were as follows: 95 °C for 10 s; 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The primers for PAI-1 (forward, 5′-GCT ATG GAA CAA GGA TGA GAT-3′; reverse, 5′-CGC CCT GGA CCA GCT TCA G-3′) and β-actin (forward, 5′-GGC GGG ACC TGA CTG ACT AC; reverse, 5′-TCC TTA ATG TCA CGC ACG ATT TC-3′) were designed using Primer Express, version 2.0.

In Vitro Kinase Assay—Ectopically expressed HA-TAK1 was immunoprecipitated with anti-HA antibody as described above. Immunoprecipitates were incubated with or without 1 µg of bacterially expressed SnoN-(1–366) in 10 µl of kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM dithiothreitol, 5 mM MgCl₂, and 5 µCi of γ-32P]ATP (3000 Ci/mmol) at 30 °C for 15 min. Samples were fractionated by 10% SDS-PAGE and visualized by autoradiography.

RESULTS AND DISCUSSION

To study the role of TAK1 in TGF-β signaling, we screened for TAK1-binding proteins using the yeast two-hybrid system. A kinase-inactive mutant of TAK1, TAK1(K63W), was used as the bait to screen a mouse B cell cDNA library. From a total of 2 × 10⁶ transformants, 28 clones were identified as potential interactors. Sequence analysis revealed that one of the positive clones encoded SnoN2 (Fig. 1A). SnoN undergoes alternative splicing, creating four splicing isoform, SnoN, SnoN2, Sno1, and SnoA (7, 24). The N terminus of SnoN, from 1 to 366 amino
acids, is identical among the four isoforms. The SnoN2 sequence is completely identical with that of SnoN except for the C-terminal 46 amino acids (Fig. 1B). As SnoN2 is the less abundantly expressed isoform in human cells (24), we focused on the interaction between TAK1 and SnoN. Interaction of TAK1 with SnoN was further confirmed in mammalian cell by co-immunoprecipitation assays. HA epitope-tagged TAK1 and FLAG-tagged SnoN were transiently co-expressed in 293 human embryonic kidney cells. The cell extracts were immunoprecipitated with anti-FLAG antibody and followed by immunoblotting analysis (Fig. 1C). When FLAG-SnoN was immunoprecipitated, both HA-TAK1 and HA-TAK1(K63W) were co-immunoprecipitated.

To establish the connection between TAK1 and SnoN, we next determined the subcellular localizations of TAK1 and SnoN. We performed biochemical fractionation with human keratinocyte HaCaT cells. TGF-β-stimulated and unstimulated HaCaT cells were fractionated into the nuclear and the cytosolic extracts. Endogenous SnoN was localized only in the nucleus. To examine the interaction between TAK1 and SnoN in the nucleus, endogenous TAK1 was immunoprecipitated following fractionation (Fig. 2, right panel). SnoN was found to be associated with TAK1 in the nuclear fraction independently of TGF-β stimulation, but no association could be detected in the cytosolic fraction. Thus, TGF-β stimulation induces the TAK1 accumulation in the nucleus, and this nuclear TAK1 interacts with SnoN.

The observed association between TAK1 and SnoN suggested that TAK1 is involved in the TGF-β-dependent degradation of SnoN. To test the possibility, we employed siRNA to reduce the levels of endogenous TAK1 (25). We generated two independent HeLa S3 cell lines stably expressing TAK1 siRNA. Expression of TAK1 in each clone was determined by immunoblotting. Expression of TAK1 siRNA greatly reduced the level of endogenous TAK1 but not β-catenin (Fig. 3A). Both TAK1 knockdown cells and parent cells were then treated with TGF-β and subjected to biochemical fractionation. The nuclear fraction was further subjected to immunoblotting with anti-SnoN, anti-TAK1, and anti-Smad2/3. As shown in Fig. 3B, TGF-β-induced degradation of SnoN in HeLa S3 cells, but degradation was impaired in TAK1 knockdown cells. In contrast, TGF-β-induced nuclear accumulation of Smad2/3 was observed to be normal in cells expressing TAK1 siRNA.

To assess the influence of TAK1 knockdown on the TGF-β-dependent biological events, we examined expression of a TGF-β-responsive gene, PAI-1. PAI-1 participates in wound healing processes. The mRNA levels of PAI-1 were increased at around 30–60 min, which occurred subsequent to SnoN degradation and Smad accumulation. The accumulation of PAI-1 mRNA in response to TGF-β was impaired in the TAK1 siRNA-expressing cells (Fig. 3C). These results suggest that TAK1 is involved in TGF-β-dependent biological processes.

We found that the N-terminal region of SnoN-(1–366) was also associated with TAK1 in 293 cells by co-immunoprecipitation assays (Fig. 1C). This region is identical in four isoforms of SnoN and sufficient for transcriptional repression (supplemental Fig. S2 and Refs. 14 and 26). To determine whether the N terminus of SnoN is sufficient for TGF-β-dependent degradation, we generated HaCaT cells stably expressing HA-tagged SnoN-(1–366). We found that TGF-β treatment induced degradation of SnoN-(1–366) (Fig. 4A). We had shown previously that (5Z)-7-oxozeaenol selectively blocks the activity of endogenous TAK1 (23). To determine whether the decrease of the SnoN-(1–366) depends on TAK1 activity, we treated the cells...
with the TAK1 inhibitor. The SnoN degradation was blocked by TAK1 inhibition. Thus, SnoN-(1–366) is likely to be degraded through TAK1-dependent phosphorylation. To further dissect the role of TAK1 on SnoN degradation, we used SnoN-(1–366).

To confirm whether TAK1 phosphorylates SnoN-(1–366), we prepared bacterially expressed recombinant GST-SnoN-(1–366) and performed in vitro kinase assay using immunoprecipitated HA-TAK1 from 293 cells co-transfected with HA-TAK1 and TAB1. TAK1 is activated by co-expression of TAB1 (21, 27). We found that TAK1, but not the kinase-inactive TAK1(K63W), could phosphorylate SnoN-(1–366) in vitro (Fig. 4B). To further confirm the phosphorylation of SnoN-(1–366) by TAK1 in vivo, we performed immunoblotting analysis and looked for a mobility shift in SDS-PAGE as an indicator of phosphorylation. In 293 cells, co-expression of TAK1 and TAB1 led to the appearance of a slower migrating form of SnoN-(1–366) (Fig. 4C). This shift in migration was reversed by treatment with phosphatase. These results suggest that TAK1 phosphorylates the N terminus of SnoN in vivo. To define the approximate region of SnoN phosphorylated by TAK1, we generated several truncated versions of SnoN-(1–366). Among the short regions of SnoN, we found that SnoN-(101–214) showed a slower migrating band with co-expression of TAK1 and TAB1 (supplemental Fig. S3A). We then generated a series of SnoN-(101–214) mutants that contain several amino acid substitutions from Ser or Thr to Ala. The analysis suggests that Ser-108, Ser-112, Ser-115, Ser-117, Thr-119, Ser-126, Ser-140, and/or Ser-141 are potential phosphorylation sites. We generated a mutant SnoN-(1–366) containing the Ser/Thr to Ala substitutions at Ser-108, Ser-112, Ser-115, Ser-117, Thr-119, Ser-126, Ser-140, and Ser-141 (SnoN-(1–366 8A)), which did not show slow migration band with co-expression of TAK1 and TAB1 (supplemental Fig. S3B). It has been reported that a member of the MAPKKK family phosphorylates the nuclear factor in vivo (28). We also generated SnoN(1–366 AAA), containing mutations of Ser-115, Ser-117, and Thr-119 to Ala and examined TAK1-dependent phosphorylation. SnoN(1–366 AAA) exhibited decreased phosphorylation by TAK1 (Fig. 4C and supplemental Fig. S3B). These results suggest that Ser-115, Ser-117, and Thr-119 are major sites of TAK1-dependent phosphorylation. However, we could still detect a slightly slower migrating band of SnoN-(1–366 AAA) when coexpressed with an active TAK1. It is likely that other sites among the eight amino acid residues may also be phosphorylated by TAK1.

SnoN is ubiquitinated and degraded upon TGF-β stimulation (11, 13). Our finding raised the possibility that TAK1-dependent phosphorylation of SnoN may induce SnoN ubiquitination and degradation. To investigate the relationship between SnoN phosphorylation and ubiquitination, we asked.
whether TAK1 induces SnoN ubiquitination. 293 cells were transfected with HA-tagged SnoN or SnoN-(1–366), TAK1, TAB1, and FLAG-ubiquitin. We immunoprecipitated SnoN followed by immunoblotting for ubiquitin (anti-FLAG) (Fig. 5A). The full-length SnoN was ubiquitinated to some extent in the absence of TAK1, and the level of ubiquitination was increased by co-expression of TAK1 + TAB1. However, the kinase-inactive TAK1(K63W) could not increase the ubiquitination. This suggests that TAK1-dependent phosphorylation of SnoN can trigger its ubiquitination. To verify the role of TAK1-dependent phosphorylation, we used the SnoN mutant lacking the phosphorylation sites SnoN(1–366 AAA). 293 cells were transfected with FLAG-tagged SnoN-(1–366), TAK1, TAB1, and HA-tagged ubiquitin. SnoN-(1–366) was immunoprecipitated with anti-FLAG antibody, and ubiquitinated SnoN-(1–366) was detected by immunoblotting with anti-HA. 

**FIGURE 5.** TAK1 mediates ubiquitination of SnoN. A, TAK1-dependent ubiquitination of SnoN. 293 cells were transfected with TAK1 wild type (WT) or K63W, TAB1, HA-SnoN, HA-SnoN-(1–366), and FLAG-ubiquitin (Ub). Cell lysates were immunoprecipitated (IP) with anti-HA and immunoblotted (IB) with anti-FLAG (upper panel). Whole cell extracts (WCE) were immunoblotted with anti-HA (lower panel). The asterisk indicates the position of HA-SnoN that is non-specifically detected by anti-FLAG. B, phosphorylation-dependent ubiquitination of SnoN-(1–366). 293 cells were transfected with TAK1, TAB1, FLAG-SnoN-(1–366) wild type or AAA mutant, and HA-ubiquitin. Cell lysates were immunoprecipitated with anti-FLAG and immunoblotted. C, phosphorylation-dependent ubiquitination of SnoN. 293 cells were transfected with TAK1, TAB1, FLAG-SnoN wild type or AAA mutant, and HA-ubiquitin. Cell lysates were immunoprecipitated with anti-FLAG and immunoblotted. The asterisk indicates the position of FLAG-SnoN that is below the smallest major bands detected by anti-HA.

TAK1-dependent phosphorylation, we used the SnoN mutant lacking the phosphorylation sites SnoN(1–366 AAA). 293 cells were transfected with FLAG-tagged SnoN-(1–366), TAK1, TAB1, and HA-tagged ubiquitin. SnoN-(1–366) was immunoprecipitated with anti-FLAG antibody, and ubiquitinated SnoN-(1–366) was detected by immunoblotting with anti-HA antibody (Fig. 5B). Co-expression of TAK1 and TAB1 resulted in a marked increase in the ubiquitination of SnoN-(1–366). In contrast, SnoN-(1–366 AAA) mutant, which lacks major phosphorylation sites, showed almost no ubiquitination under the same conditions. To further investigate the effect of TAK1 phosphorylation on the full-length SnoN, we generated a mutant full-length SnoN carrying the mutation at Ser-115, Ser-117, and/or Thr-119 (SnoN AAA). Although the basal level of ubiquitination was unchanged in the mutant SnoN (SnoN AAA), the TAK1 + TAB1-induced increase of ubiquitination was abrogated in SnoN AAA. These results suggest that phosphorylation of SnoN at Ser-115, Ser-117, and/or Thr-119 by TAK1 is important for SnoN ubiquitination.

We next examined the effect of SnoN phosphorylation on its degradation. 293 cells were transfected with SnoN-(1–366), SnoN-(1–366) mutant SnoN(1–366 AAA), TAK1, and TAB1, and the half-life of SnoN-(1–366) was determined following cycloheximide treatment (Fig. 6A). In the absence of the active TAK1, SnoN-(1–366) was stable and was not significantly degraded until 2 h after the cycloheximide treatment, whereas the half-life was significantly shortened when TAK1 was activated. The SnoN-(1–366 AAA) mutant showed a longer half-
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life compared with the wild type SnoN-(1–366) in the presence of active TAK1 (TAK1 + TAB1). These results suggest that phosphorylation at Ser-115, Ser-117, and Thr-119 is important for degradation of SnoN.

To investigate whether phosphorylation of SnoN was required for TGF-β-induced degradation, we generated HaCaT cells stably expressing SnoN-(1–366), and SnoN-(1–366 AAA) mutant. Whereas SnoN-(1–366) was rapidly decreased in response to TGF-β stimulation, the SnoN-(1–366 AAA) levels decreased slowly compared with the wild type (Fig. 6B). Finally, we tested the TGF-β-induced degradation of the full-length SnoN. We generated HaCaT cells stably expressing SnoN wild type and AAA mutant and examined their levels upon TGF-β treatment (Fig. 6C). We used two independent stable clones that express SnoN or SnoN AAA at different levels. SnoN AAA decreased slowly compared with the wild type SnoN in both clones. These results suggest that TAK1-dependent phosphorylation is important for the TGF-β-induced degradation of SnoN.

Our data have demonstrated that mutation of SnoN and SnoN-(1–366) at Ser-115, Ser-117, and Thr-119 reduced TGF-β-induced degradation; however, SnoN AAA and SnoN-(1–366 AAA) were still degraded at 30 min after TGF-β stimulation. This may be consistent with the fact that SnoN-(1–366 AAA) was still phosphorylated to some extent by TAK1 (Fig. 4C). Other phosphorylation sites may participate in SnoN degradation. To examine this possibility, we determined the half-life of SnoN-(1–366 8A) (supplemental Fig. S3C). SnoN-(1–366 8A) was stable even when TAK1 was activated. We also generated HaCaT cells stably expressing SnoN-(1–366 8A) and examined the effect of TGF-β treatment (supplemental Fig. S3D). SnoN-(1–366 8A) was not degraded by TGF-β and was much more stable compared with SnoN-(1–366 AAA). Thus, although Ser-115, Ser-117, and Thr-119 may be major sites of TAK1-dependent phosphorylation, phosphorylation at other sites is likely to participate in the induction of SnoN degradation.

SnoN represses TGF-β signaling by recruiting transcriptional repressors to Smad complex (3, 14). We next examined whether the phosphorylation of SnoN modulates its transcriptional activity. Transient transfection experiments were performed in 293 cells with a transcriptional reporter, 3TP-lux, which contains TGF-β-responsive elements of the PAI-1 promoter region (29). At 48 h after transfection, cell lysates were prepared, and luciferase activities were measured (supplemental Fig. S2). The constitutively active form of TGF-β receptor ALK5(TD) was sufficient to induce the expression of a TGF-β-responsive gene in 293 cells, and overexpressed full-length SnoN as well as SnoN-(1–366) suppressed the TGF-β-dependent transcription as reported previously (3, 14). SnoN-(1–366 AAA and 8A) mutants could also reduce the TGF-β-dependent transcription, suggesting that the mutation does not affect binding to Smads or to transcriptional co-repressors. SnoN-(1–366) mutants may be a more potent inhibitor compared with SnoN-(1–366), because it is stable upon TGF-β stimulation. However, in the transiently transfection experiments, we could not detect the difference between SnoN-(1–366) and SnoN-(1–366 AAA and 8A), which is likely because they were highly expressed and ALK5(TD) could not effectively reduce the amount of SnoN-(1–366). Collectively, our results suggest that SnoN-(1–366 AAA and 8A) can bind to and inhibit Smads but is resistant to TAK1-mediated degradation. Therefore, TAK1 is likely to inhibit SnoN by modulating SnoN stability.

In this report, we have determined the role of TAK1 MAPKKK in TGF-β. Previous works had shown that TAK1 is activated by TGF-β (15) and that SnoN, an inhibitor of Smads, is degraded upon TGF-β stimulation (13). This study links these two observations and suggests that TAK1 contributes to the induction of TGF-β-responsive genes by inducing the degradation of SnoN (Fig. 7). SnoN has been shown to be the important negative regulator of TGF-β signaling via its interaction with Smad proteins (7, 8). Upon TGF-β stimulation, SnoN is rapidly degraded by ubiquitin-dependent proteasome pathway (13, 14). Two ubiquitin ligases are reported as SnoN ubiquitin ligases. One is anaphase-promoting complex, which induces the ubiquitination of SnoN on Lys-440, Lys-446, and Lys-449 and its consequent degradation in a Smad3-dependent manner (11, 12). Another ubiquitin ligase is Smurf2, which is recruited to SnoN by Smad2, resulting in the ubiquitination and degradation of SnoN (10). SnoN-(1–366), which lacks sites ubiquitinated by APC or Smurf2, is neither ubiquitinated nor degraded in Ba/F3 pro-B cells (14). However, we show that TGF-β induces degradation of SnoN-(1–366) in human keratinocyte HaCaT cells in a manner dependent on TAK1-induced phosphorylation and ubiquitination. Mutation of TAK1-dependent phosphorylation sites on SnoN-(1–366) blocked TGF-β-dependent degradation. Moreover, when endogenous TAK1 was inactivated by a small molecule TAK1 inhibitor in keratinocyte HaCaT cells or by siRNA-mediated knockdown in epithelial-like HeLa S3 cells, degradation of endogenous SnoN was impaired. Collectively, these results suggest that TAK1 phosphorylation of SnoN is required for its ubiquitination and degradation in some epithelial cells. These results further suggest that several different pathways induce SnoN degradation, depending on the cell type.

Phosphorylation-induced degradation of proteins is a widely used mechanism by which protein levels can be modulated rap-
idly. We have found that TAK1 phosphorylates SnoN-(1–366) at several threonine and serine residues and that the SnoN mutant, which lacks its phosphorylation sites, did not undergo ubiquitination or degradation. We should note that the mutant SnoN is still capable of inhibiting TGF-β-induced transcription (supplemental Fig. S2). This indicates that the mutations at the phosphorylation sites do not interfere with interaction of SnoN with Smads or with transcriptional co-repressors. TAK1 is likely to inhibit SnoN solely by modulating SnoN stability. TAK1 is the first kinase demonstrated to phosphorylate SnoN and target it for ubiquitin-dependent proteasomal degradation.

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