TAK1 MAPKKK mediates TGF-β signaling by targeting SnoN oncoprotein for degradation

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Abstract

Transforming growth factor-β (TGF-β) 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 the activation of 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 represses transcription of TGF-β-responsive genes (7,9). Upon TGF-β stimulation, SnoN is immediately downregulated via the ubiquitin-proteasome pathway induced by anaphase-promoting complex (APC) or Smurf2 E3 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 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).

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TGF-β activated kinase (TAK1) is a member of mitogen activated kinase kinase kinase (MAPKKK) and functions as a signaling intermediate in several intracellular signaling pathways including 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′ GATCCCCGAGATCGACTACAAGGAGATCTCCTTGTAGTCGATCTCTTGTAGTCTTT CTTATTGCAAGAGATCGACTACAAGGAGATCTCCTTGTAGTCGATCTCTTGTAG TCTTTTCTACAAAGAGATCGACTACAAGGAGATCTCCTTGTAGTCGATCTCTTGTAGT CGATCTCGGG-3′; and 5′-AGCTTTTCCAAAAAGAGATCGACTACAAGGAGATCTCCTTGTAGTCGATCTCCTTGTAGT CGATCTCGGG-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-112, 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 pQCMXIP 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-IkBa polyclonal antibody C-21 (Santa Cruz), anti-β-catenin monoclonal antibody 14 (BD Science), anti-Smad2/3 polyclonal antibody (Upstate Biotechnology), anti-p53 monoclonal antibody DO-1 (Santa Cruz), anti-β-actin monoclonal antibody AC-15 (Sigma). Recombinant human TGF-β1 was purchased from Roche Applied Science. 5Z-7-oxozeaenol was as described previously (23). G418 (Invitrogen), hygromycin B (A.G. Scientific, Inc.), lambda phosphatase (New England Biolabs), and cycloheximide (Calbiochem) were used.

Cell Culture, Transfection and virus infection

293 cells, HaCaT cells, and HeLa S3 cells were cultured in DMEM plus 10% fetal calf serum (FCS) or bovine growth serum (BGS, 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 TransFast™ (Promega). The retrovirus for expression of HA-SnoN full length and HA-SnoN mutant full length were generated and infected into HaCaT.
cells according to the manufacture’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 DTT, 1 mM Na₃VO₄, 1 mM PMSF, 100 U/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 suspend in 30 μl of rinse buffer. For immunoblotting, the immunoprecipitates or cell lysates were resolved on SDS-PAGE and transferred to Hybond-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 cytoplasm fractions, cells in 10 cm dishes were treated with TGF-β (5ng/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 DTT, 0.2 mM PMSF and 100 U/ml Aprotinin) containing 0.1 % nonidet P-40 and homogenized in a Dounce homogenizer (30 strokes). Lysates were then centrifuged at 4,000 xg for 4 min. The supernatant was centrifuged at 12,500 xg 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 xg 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 cytosolic and nuclear fractions was assessed by immunoblotting of IkB (a cytosolic marker) and lamin B (a nuclear marker).

**Real time qRT-PCR**

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

**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 DTT, 5 mM
MgCl₂, and 5 μCi of [γ-³²P] ATP (3,000 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 x 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 isoforms, SnoN, SnoN2, SnoI 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 keratinocytes 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 but was degraded upon TGF-β stimulation (Fig. 2, left panel). Endogenous TAK1 was localized primarily in the cytoplasm, but also detected in the nuclear fractions. Upon TGF-β stimulation, the amount of nuclear TAK1 was increased. The fractions were reasonably pure, as determined by the presence of IkB only in the cytosolic fraction and not in the nuclear fraction. Conversely, a nuclear protein Lamin B was detected only in the nuclear fraction but not the cytosolic fraction (supplemental Fig. S1). These data raised the possibility that TAK1 is co-localized with SnoN 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 independent 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 amount of endogenous TAK1 but not affect 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 the 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 plasminogen activator inhibitor-1 PAI-1. PAI-1 participates in wound healing processes. The mRNA levels of PAI-1 were increased around 30-60 min, which occur 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) (14,26). 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 have previously shown 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 the 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 slowly migrating band with co-expression of TAK1 and TAB1 (Supplemental Fig. S3B). It has been reported that a member of MAPKKKs phosphorylate substrates within a conserved Ser/Thr-X-X-X-Ser/Thr motif (28). We also generated a 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 or/and Thr-119 are major sites of TAK1-dependent phosphorylation. However, we could still detect a slightly slowly migrating band of SnoN(1-366 AAA) when coexpressed with an active TAK1. It is likely that other sites among the 8 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 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 sties, 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 the Ser-115, Ser-117, and/or Thr-119 (SnoN AAA). Although the basal level of ubiquitination was unchanged in the mutant SnoN (SnoN AAA), TAK1+TAB1-induced increase of ubiquitination was abrogated in the SnoN AAA. These results suggest that phosphorylation of SnoN at Ser-115, 117 and Thr-119 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 (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 not significantly degraded until 2 hr 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-life compare to the wild type SnoN(1-366) in the presence of active TAK1 (TAK1+TAB1). These results suggest that phosphorylation at Ser-115, 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 the SnoN (1-366 AAA) levels decreased slowly compared to 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. The SnoN AAA was decreased slowly compared to 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 demonstrated that mutation of SnoN and SnoN(1-366) at Ser-115, Ser-117 and Thr-119 reduced the TGF-β-induced degradation; however, those were still degraded at 30 min after TGF-β stimulation. This may be consistent with the fact that SnoN(1-366 AAA) is 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 to SnoN(1-366 AAA). Thus, although Ser-115, 117 and Thr-119 may be major sites of TAK1-dependent phosphorylation, phosphorylation at other sites is likely to participate in 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 transcriptional reporter, 3TP-lux, which contains TGF-β-responsive elements of the PAI-1 promoter region (29). At 48h after transfection, cell lysates were prepared, and luciferase activities were measured (supplemental Fig. S2). Constitutive active form of TGF-β receptor ALK5(TD) was
sufficient to induce the expression of 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 compare to 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 determine 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. 6). 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 (APC), which induces the ubiquitination of SnoN on Lys-440, Lys-446, and Lys-449, and its consequent degradation in 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 keratinocytes 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 is inactivated by a small molecule TAK1 inhibitor in keratinocytes HaCaT cells or by siRNA-mediated knockdown in epithelial-like HeLa S3 cells, degradation of endogenous SnoN is impaired. Collectively, these results suggest that TAK1 phosphorylation of SnoN is required for its ubiquitination and the 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 rapidly. We have found that TAK1 phosphorylates SnoN(1-366) at several threonine and serine residues, and SnoN mutant, which lack its phosphorylation sites, is not induced the ubiquitination and 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 as well as 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

TAK1, TGF-β activated kinase 1; MAPKKK, mitogen-activated protein kinase kinase kinase; TGF-β, Transforming growth factor-β; APC, anaphase-promoting complex; HA, hemagglutinin; TAB1, TAK1-binding protein 1; siRNA, small interfering RNA; GST, glutathione S-transferase.

REFERENCE

Figure 1. TAK1 interacts with SnoN
(A) Two-hybrid assay for the interaction of TAK1 with SnoN2. TAK1 KW, TAK1(K63W).
(B) Domain organization of SnoN. (C) Interaction of TAK1 with SnoN in 293 cells. 293 cells were transfected with expression vectors for HA-TAK1 and Flag-SnoN. Cell lysates and anti-Flag immunoprecipitates were analyzed by immunoblotting.
Figure 2. Nuclear TAK1 interacts with SnoN
Localization and interaction of endogenous TAK1 and SnoN. HaCaT cells were treated with or without TGF-β. Cell lysates were fractionated and immunoprecipitated with anti-TAK1 (T) or control IgG (C). Immunoprecipitates were analyzed by immunoblotting with indicated antibodies. Cyto, cytosolic fraction. Nuc, nuclear fraction. The two faint bands indicated by asterisk (*) in left upper panel in the cytosolic faction are non-specific bands.
Figure 3. TAK1 is essential for TGF-β-dependent SnoN degradation and PAI-1 expression

(A) HeLa S3 stable transformants expressing TAK1 siRNA. Expression levels of TAK1 and β-catenin were determined by immunoblotting. Equal protein loadings were confirmed by β-catenin levels. (B) TGF-β-dependent SnoN degradation. HeLa S3 parent and two independent TAK1 siRNA cells (#1 and #2) were treated with TGF-β. The nuclear fractions were analyzed by immunoblotting. Anti-p53 was used as a loading control. (C) TGF-β-dependent PAI-1 expression. HeLa S3 parent and TAK1 siRNA cells were treated with TGF-β, and PAI-1 mRNA levels were analyzed by real time qRT-PCR. mRNA levels relative to β-actin mRNA are shown. Data show the means of three independent samples and SD.
Figure 4. Phosphorylation of SnoN
(A) Effect of TAK1 inhibitor. HaCaT cells stably expressing HA-SnoN(1-366) were treated with 5Z-7-oxozeaenol (500 nM) followed by TGF-β stimulation for 30 min. SnoN(1-366) was detected by immunoblotting. (B) In vitro kinase assay. 293 cells were transfected with expression vectors for HA-TAK1, and TAB1. TAK1 was immunoprecipitated with anti-HA. Kinase reactions of immunoprecipitated TAK1 were performed with [γ-32P] ATP and purified GST-SnoN(1-366) as a substrate. (C) Phosphorylation of SnoN(1-366). 293 cells were transfected with expression vector for TAK1, TAB1, Flag-SnoN(1-366) wild type, or AAA mutant. Cell lysates were incubated in either absence or presence of lambda protein phosphatase and analyzed by immunoblotting with anti-Flag.
Figure 5. TAK1 mediates ubiquitination of SnoN

(A) TAK1-dependent ubiquitination of SnoN. 293 cells were transfected with TAK1 wild type or K63W, TAB1, HA-SnoN, HA-SnoN(1-366), and Flag-ubiquitin. Cell lysates were immunoprecipitated with anti-HA and immunoblotted 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.

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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.
Figure 6. TAK1 mediates degradation of SnoN

(A) Stability of SnoN. 293 cells were transfected with TAK1, TAB1, and Flag-SnoN(1-366) wild type, or AAA mutant. Cells were treated with 50 μg/ml cycloheximide. SnoN was detected by immunoblotting. (B) TGF-β-induced degradation of SnoN(1-366). HaCaT cells stably expressing HA-SnoN(1-366) wild type, or AAA mutant (two independent clones, AAA-1 and AAA-2) were treated with TGF-β. Cell lysates were analyzed by immunoblotting with anti-HA. β-actin is shown as a loading control. (C) TGF-β-induced degradation of SnoN. HaCaT cells stably expressing HA-SnoN wild type or HA-SnoN AAA mutant were treated with TGF-β. Two independent clones expressing HA-SnoN (WT-1 and WT-2) and HA-SnoN(AAA) (AAA-1 and AAA-2) at different levels were used. Cell lysates were analyzed by immunoblotting with anti-HA. β-actin is shown as a loading control.
Figure 7. Model for TGF-β signaling