The Transforming Activity of Ski and SnoN Is Dependent on Their Ability to Repress the Activity of Smad Proteins*

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The regulation of cell growth and differentiation by transforming growth factor-β (TGF-β) is mediated by the Smad proteins. In the nucleus, the Smad proteins are negatively regulated by two closely related nuclear proto-oncoproteins, Ski and SnoN. When overexpressed, Ski and SnoN induce oncogenic transformation of chicken embryo fibroblasts. However, the mechanism of transformation by Ski and SnoN has not been defined. We have previously reported that Ski and SnoN interact directly with Smad2, Smad3, and Smad4 and repress their ability to activate TGF-β target genes through multiple mechanisms. Because Smad proteins are tumor suppressors, we hypothesized that the ability of Ski and SnoN to inactivate Smad function may be responsible for their transforming activity. Here, we show that the receptor regulated Smad proteins (Smad2 and Smad3) and common mediator Smad (Smad4) bind to different regions in Ski and SnoN. Mutation of both regions, but not each region alone, markedly impaired the ability of Ski and SnoN to repress TGF-β-induced transcriptional activation and cell cycle arrest. Moreover, when expressed in chicken embryo fibroblasts, mutant Ski or SnoN defective in binding to the Smad proteins failed to induce oncogenic transformation. These results suggest that the ability of Ski and SnoN to repress the growth inhibitory function of the Smad proteins is required for their transforming activity. This may account for the resistance to TGF-β-induced growth arrest in some human cancer cell lines that express high levels of Ski or SnoN.

ski and sno are members of the ski family of nuclear proto-oncogenes (1–3). Ski was originally identified as the transforming protein of the avian Sloan-Kettering retrovirus (v-Ski) (4, 5). The human cellular homolog c-ski and the closely related snoN were later cloned based on sequence homology to v-ski (1). The amino-terminal region of Ski is highly conserved among Ski family members, including v-Ski (>95% sequence identity) and SnoN (70% identity), whereas the carboxyl-terminal half of the molecule shows little homology among the family members. The N-terminal homology region is necessary and sufficient for the known biological activities of Ski and SnoN (6, 7). Compared with c-Ski, v-Ski is truncated at the carboxyl terminus (5, 8). However, this truncation does not appear to be responsible for the activation of ski as an oncogene because overexpression of wild-type c-Ski results in oncogenic transformation of chicken and quail embryo fibroblasts (9). Thus, the transforming activity of Ski is likely due to overexpression, not truncation, of the c-Ski protein. Consistent with this notion, an elevated level of c-Ski or c-SnoN has been detected in many human tumor cell lines derived from neuroblastoma, melanoma, breast cancer, and carcinomas of the stomach, chorion, thyroid, and epidermoid (1, 10, 11). In addition to up-regulation of ski expression, mislocalization of Ski may also contribute to malignant progression. Ski was found to be present in the nucleus in cells derived from normal skin or early-stage tumors, but localized in the cytoplasm in highly malignant melanoma cells (11). However, the mechanism of transformation by Ski and SnoN has not been defined.

Ski and SnoN are incorporated into the histone deacetylase-1 complex through binding to the nuclear hormone receptor corepressor N-CoR and mSin3A and mediate transcriptional repression of the thyroid hormone receptor, Mad, and pRB (12, 13). Ski has also been shown to interact with the retinoic acid receptor in a ligand-independent manner, as well as with pRb and another transcription factor, Skip (14, 15). However, the role of these interactions in transformation by Ski or SnoN remains to be determined.

We (17, 18, 21) and others (16, 19, 20) have recently shown that Ski and SnoN interact with the Smad proteins to negatively regulate transforming growth factor-β (TGF-β)1 or bone morphogenetic protein signaling; Smad proteins are critical components of the TGF-β signaling pathways (22, 23). In the absence of TGF-β, the two highly homologous R-Smad proteins (Smad2 and Smad3) are distributed mostly in the cytoplasm (24–26). Upon ligand binding, the activated type I TGF-β receptor kinase phosphorylates the R-Smad proteins, allowing them to translocate into the nucleus (25–31) and to form heteromeric complexes with Smad4 (32–35). In the nucleus, the Smad complexes interact with various cellular partners and participate in diverse downstream activities. The Smad proteins can bind to the TGF-β-responsive promoter DNA either directly or in conjunction with other sequence-specific DNA-binding proteins (36). Through the C-terminal Mad homology-2

1 The abbreviations used are: TGF-β, transforming growth factor-β; R-Smad, receptor regulated Smad; Co-Smad, common mediator Smad; MH2, Mad homology-2; CBP, cAMP-responsive element-binding protein-binding protein; CEFs, chicken embryo fibroblasts; HA, hemagglutinin; WT, wild-type; GST, glutathione S-transferase; SBE, Smad-binding DNA element.

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(MH2) domains, the Smad proteins interact with general or promoter-specific transcriptional coactivators and corepressors to regulate the transcription of various TGF-β target genes (36). The Smad proteins play a central role in mediating the growth inhibitory response of TGF-β by activating the expression of cyclin-dependent kinase inhibitors such as p21 and p15INK4A. To study the mechanism of Ski and SnoN-mediated cell cycle arrest, we performed biochemical and structural analyses to map the Smad-binding sites in Ski and SnoN. We recently solved the crystal structure of a Ski fragment bound to the Smad4 MH2 domain (44).

Interaction of Ski or SnoN with the Smad proteins results in disruption of an active heteromeric Smad complex (16, 44), displacement of the transcriptional coactivator p300/CBP from the Smad proteins (44), and recruitment of the nuclear hormone receptor corepressor N-CoR (17, 18). Through these mechanisms, Ski and SnoN repress the activity of the Smad proteins to mediate TGF-β-induced cell cycle arrest. Because the Smad proteins are tumor suppressors, we hypothesized that this ability of Ski and SnoN to inactivate Smad function may be responsible for their transforming activity. To test whether the interaction of Ski/SnoN with the Smad proteins is indeed responsible for the transcriptional repression of the Smad proteins and for the oncogenic activity of Ski and SnoN, we carried out biochemical and structural analyses to map the Smad-binding sites in Ski and SnoN. We recently solved the crystal structure of a Ski fragment bound to the Smad4 MH2 domain (44). Based on this structure and on our mutational analysis, we have identified the amino acid residues in Ski and SnoN that mediate interaction with the Smad proteins. This has allowed us to determine whether Ski/SnoN mutants defective in binding to the Smad proteins can still repress Smad function and induce oncogenic transformation of chicken embryo fibroblasts.

**EXPERIMENTAL PROCEDURES**

**Cells, Antisera, and Constructs—**HeP3B, a human hepatoma cell line (American Type Culture Collection), was maintained in minimal essential medium supplemented with 10% fetal bovine serum. 293T and Phoenix-Eco cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Ba/F3, a pro-B cell line, was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 10% WEHI cell-conditioned medium as a source of interleukin-3 (45). Primary cultures of chicken embryo fibroblasts (CEF) were prepared from 10-day-old embryos and cultured as described (46, 47). CEFs were maintained in Dulbecco’s modified Eagle’s medium/nutrient mixture F-10 supplemented with 2% tryptose phosphate broth, 1% chicken serum, and 1% bovine calf serum.

Antiserum against N-CoR (sc-1609) was purchased from Santa Cruz Biotechnology. Anti-FLAG monoclonal antibody M2 was purchased from Sigma. Alexa Fluor® 488 goat anti-mouse IgG (H + L) was purchased from Molecular Probes, Inc.

Hamaglutinin (HA)-tagged full-length Smad2, Smad3, and Smad4 and FLAG-tagged full-length or truncated Ski and SnoN were cloned into an antibiotic selection plasmid as described previously (17, 18). FLAG-tagged mutants of Ski and SnoN were generated by PCR and subcloned into pCMV5B, pBABE-puro (17, 18), or helper-free RCAS.BP type A avian retroviral vector (48).

**Transfection and Retroviral Infection—**293T and HeP3B cells were transiently transfected using the LipofectAMINE Plus protocol (Invitrogen). To generate stable Ba/F3 cell lines expressing doubly tagged proteins, 293T cells were transfected with 2 μg of SnoN, FLAG-tagged wild-type (WT) and mutant Ski and SnoN in the pBABE-puro or pMX-ires-GFP retroviral vector transfected into Phoenix-Eco packaging cells to generate retroviruses. 48 h after transfection, 2.8 μl of viral supernatant was collected and added to 5 × 10^6 Ba/F3 cells in the presence of 6 μg/ml Polybrene. Following centrifugation at 1000 rpm for 2 h at 37°C, the cells were resuspended in 2 ml of RPMI 1640 complete medium and co-cultivated with the transfected Phoenix-Eco packaging cells. 24 h later, the Ba/F3 cells were removed from the Phoenix-Eco cells and cultured in fresh RPMI 1640 complete medium for an additional 24 h. The infected cells were selected in RPMI 1640 medium containing 2 μg/ml paromycin (for pBABEpuro) or by fluorescence-activated cell sorting based on expression of the green fluorescence protein (for pMX-ires-GFP) (Sigma).

**Immunoprecipitation and Western Blotting—**FLAG- and HA-tagged proteins were isolated from transfected 293T cell lysates by immunoprecipitation with anti-FLAG antibody-agarose, followed by elution with the FLAG peptide and analysis by Western blotting as described previously (18). Endogenous N-CoR was isolated from 293T cell lysates by immunoprecipitation with anti-N-CoR antibody.

**Glutathione S-Transferase (GST) Pull-down Assay—**Recombinant WT or mutant Ski was expressed in and purified from Escherichia coli as GST fusion proteins. To test whether these GST-Ski proteins bind to Smad3, 1.5 μg of GST-SnoN immobilized on glutathione-Sepharose (Amersham Bioscience) was blocked at 4°C for 30 min with bacterial cell lysates, followed by 0.2% bovine serum albumin in GST binding buffer (20 mM HEPES, 1% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM NaCl, 0.5% Nonidet P-40, and 0.25 mM KCl) (21). The immobilized GST-Ski protein was then incubated with 293T cell lysate expressing FLAG-Smad3 for 1 h in GST binding buffer at 4°C. After washing, GST-Smad3 associated with GST-Ski was eluted with glutathione and detected by immunoblotting with anti-FLAG monoclonal antibody.

**Soft Agar Colony Assay—**10^5 CEFs were incubated with 10 μg of DNA (FLAG-Ski or FLAG-SnoN in RCAS.BP) in the presence of 30 μg/ml Polybrene. 6 h later, cells were shocked with 25% Me2SO for 3.5 min. The transfected CEFs were passaged for 3 weeks, and the percent transfected cells at this time was ~70%. For the soft agar colony assay, 4 ml of normal growth medium containing 0.66% agar was poured into a 50 dish to form the bottom layer (50). 10^5 cells were then suspended in 2 ml of medium containing 0.44% agar and overlaid on the hardened bottom layer. 4 ml of fresh medium containing 0.44% agar was added to the dish every week. After 3 weeks of incubation, colonies were visualized by staining with 0.1 mg/ml [3-(4,5-dimethylthiazol-2-y)]-2,5-diphenyltetrazolium bromide (Sigma) and scanned on a Hewlett-Packard ScanJet J300C to visualize colonies.

**Immunofluorescence—**To monitor the expression and localization of the introduced WT and mutant Ski and SnoN proteins, CEFs growing on glass coverslips were fixed with 4% paraformaldehyde. The FLAG-Ski and FLAG-SnoN proteins were detected by staining with anti-FLAG monoclonal antibody, followed by Alexa Fluor® 488-conjugated goat anti-mouse antibodies. Nuclei were detected by 4,6-diamidino-2-phenylindole staining. Positively stained cells were scored for each transfected construct and used to calculate the efficiency of transfection/infection.

**Electrophoresis Mobility Shift Assay—**Smad2, Smad3, Smad4, or mutant Ski and SnoN were incubated as indicated with the 32P-labeled Smad-binding DNA element (SBE) probe (2 μCpm) at room temperature for 20 min in binding buffer (25 mM Tris-Cl (pH 7.5), 80 mM NaCl, 35 mM KCl, 5 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 15 μg/ml poly(dI-dC), 300 μg/ml bovine serum albumin, and 2% Nonidet P-40) (51). For antibody supershift assays, 2 μg of specific antibodies were preincubated with the protein complex at 4°C for 30 min. The protein-DNA complexes were resolved on a 4% non-denaturing polyacrylamide gel.

**Pulse-Chase Assay—**293T cells growing in p50 dishes were washed twice with Eagle’s medium and incubated in fresh Eagle’s medium/lactate/pyruvate medium and pulsed with 0.2 mM 35S-express for 30 min. The cells were then chased in Dulbecco’s modified Eagle’s complete medium for various periods of time.

**RESULTS**

**Mapping of Smad2-, Smad3-, and Smad4-binding Sites in Ski and SnoN—**A series of deletion mutants of Ski were constructed to identify the Smad3-binding sites in Ski (Fig. 1A, left panel). GST fusions of WT and mutant forms of Ski were immobilized on glutathione-Sepharose and used to precipitate
FLAG-tagged Smad3 from transfected cell lysates. Ski fragments containing either residues 1–241 (Fig. 1A, right panels, lane 5) or 241–441 (lane 9) interacted with Smad3, suggesting that, at least in vitro, there are two Smad3-binding sites in Ski. Because Ski-(1–241) associated with Smad3 with a much higher affinity than Ski-(241–441), the major binding site is located between residues 1 and 241. Further deletion analysis indicated that residues 16–23 are required for binding to Smad3 since fragment 16–728 bound to Smad3 to a similar extent as WT Ski, but fragment 24–728 interacted with Smad3 with a much lower affinity (lanes 1–3 and 8). Furthermore, mutations that changed residues 16–23 to alanine either abolished (16–19A) or greatly impaired (20–23A) this interaction (lanes 6 and 7).

The minor Smad3-binding site has been previously mapped to the region between residues 241 and 323 (17). Further analysis using deletion and point mutants of Ski showed that residues 316–319 are required for this weak interaction (data not shown). Mutant Ski-(241–441) with these residues changed to alanine no longer bound to Smad3 in vitro (Fig. 1A, right panels, lane 10). This minor binding site does not appear to be sufficient to mediate the Ski-Smad interaction or to repress TGF-β signaling in vivo. Mutant Ski or SnoN lacking the major Smad3-binding site but still retaining this minor binding site did not associate with Smad2 or Smad3 in vivo (Fig. 1B and C, right panels), nor was this minor binding site alone able to mediate repression of TGF-β-induced transcriptional activation (data not shown). Therefore, we focused on the major Smad3-binding site in all subsequent binding and functional assays.

To examine whether residues 16–19 in Ski are also required for binding to the Smad proteins in vivo, we mutated these residues to alanine (mS2/3) (Fig. 1B, left panel). Epitope-tagged WT or mS2/3 mutant Ski was cotransfected with HA-tagged Smad proteins, and their interactions were analyzed by co-immunoprecipitation. As shown in Fig. 1B (right panels), mutation of residues 16–19 disrupted the interaction of Ski with both Smad2 and Smad3 (but not Smad4) in vivo, indicating
that Smad2 and Smad3 bind to the same residues in Ski. This is not surprising since Smad2 and Smad3 are 91% identical in amino acid sequence. Furthermore, these residues are well conserved in SnoN because mutation of the corresponding residues in SnoN (residues 85–88, mS293) also disrupted binding of SnoN to Smad2 or Smad3 in vivo (Fig. 1C, right panels).

The SnoN-binding site in Ski and SnoN was determined by mutagenesis and by structural studies. We have previously shown that His222 and Glu233 in Ski are important for binding to SnoN (21). In the crystal structure of a Ski fragment bound to the SnoN MH2 domain, both residues contribute to the extensive interface between SnoN and Ski, with Glu233 generating an intermolecular hydrogen bond and His222 stabilizing the Ski structure (44). In addition, several amino acid residues, including Trp274, Thr271, and Cys272, also make direct contact with SnoN (44). Mutation of these residues either individually (mS45w) or in combination (mS45wt) abolished binding of Ski to SnoN in vitro (mS45w and mS45wt) (Fig. 1A and C, right panels).

Interaction between Ski/SnoN and the Smad Proteins Is Required for Repression of TGF-β-induced Transcriptional Activation—We (17, 18) and others (16, 19, 20) have shown previously that Ski and SnoN can be recruited to the SBEs through the Smad proteins and repress transcriptional activation of TGF-β target genes through multiple mechanisms. In an electrophoretic mobility shift assay, WT Ski or SnoN immunoprecipitated from cells cotransfected with SnoN (Fig. 2A) or Smad2 (data not shown) formed a complex with the SBE that could be supershifted with antibodies directed against the Smad protein or epitope tags of Ski or SnoN (Fig. 2A, lanes 2–4, and 8–10) (16–20). No such complex was detected with mutant forms of Ski or SnoN defective in binding to the Smad proteins (mS45w) (lanes 5–7 and 11–13), indicating that the interaction of Ski or SnoN with the Smad proteins is necessary for binding to the SBE.

When cotransfected with the TGF-β-responsive reporter constructs, WT Ski or SnoN readily repressed TGF-β-induced transcriptional activation of either the natural PAI-I promoter or p3TP-lux, but the mS45 mutants failed to do so (Fig. 2B–D). Interestingly, mutation of either the R-Smad- or SnoN-binding site alone did not significantly affect the repressive activity of SnoN (Fig. 2C, C–D) and Ski (data not shown), suggesting that they can inactivate the activity of the heteromeric Ski and SnoN complex through binding to either Smad protein. This is consistent with our previous structural analysis showing that binding of Ski to either Smad2 or SnoN results in disruption of the heteromeric Ski-SnoN complex (44). Only after both binding sites are mutated is the repression by Ski or SnoN abolished.

To confirm that the lack of repression by mS45w mutant Ski or SnoN is not due to a destabilization of the structure of Ski or SnoN by the mutation, we measured the half-lives of mutant Ski and SnoN and examined their ability to interact with the corepressor N-CoR. Because misfolded proteins are usually degraded rapidly, we hypothesized that if mutation of SnoN binding sites affects the folding of Ski and SnoN, the mS45 mutants should be less stable than WT Ski and SnoN. Their interaction with N-CoR may also be affected by these mutations. However, in a pulse-chase assay (Fig. 3A), the mS45 mutant Ski and SnoN were as stable as their WT counterparts. In fact, the SnoN mutant was even more stable in the presence of the Smad3-dependent polyubiquitination (38). The proteins used in the electrophoresis mobility shift assay reactions were as follows: purified Smad4, 0.2 μg; and WT Ski- or SnoN-bound Smad4, 0.5 μg. Lane 1, GST-Smad4; lane 2, FLAG-tagged Smad4 complex purified from cotransfected 293T cells; lanes 5–7, FLAG-tagged mS45 mutant Smad4 complex; lanes 8–10, FLAG-tagged Ski-Smad4 complex; lanes 11–13, FLAG-tagged mS45 mutant Ski-Smad4 complex. B–D, repression of transcription by the Ski and SnoN proteins. Hepa1C1 cells were transfected with two different concentrations of ski together with 0.5 μg of p3TP-lux (B) or with 2 μg of WT or mutant snoN and 0.5 μg of pLuc800 (C) or p3TP-lux (D). Luciferase activity was measured 48 h later.
Fig. 3. A, stability of the WT and mutant Ski and SnoN proteins. FLAG-tagged WT or mutant Ski or SnoN was transfected into 293T cells either alone or with Smad3 and isolated by immunoprecipitation with anti-FLAG antibody-agarose. The pulse-chase assay was carried out 2 days after transfection as described under "Experimental Procedures." Left panels, WT proteins; right panels, mutant proteins. B, mutant Ski or SnoN proteins defective in Smad binding can still interact with N-CoR. FLAG-tagged WT or mutant Ski or SnoN was transfected into 293T cells. Endogenous N-CoR was isolated by immunoprecipitation (IP) with anti-N-CoR antibody (middle panel). The Ski or SnoN protein associated with N-CoR was visualized by Western blotting of the immunoprecipitates with anti-FLAG monoclonal antibody (upper panel). The expression level of Ski or SnoN was measured by Western blotting of the cell lysates with anti-FLAG monoclonal antibody (lower panel). C, mutant Ski or SnoN defective in Smad binding cannot recruit N-CoR to the Smad proteins. HA-Smad4 was transfected into 293T cells either alone (lanes 1 and 4) or with WT (lanes 3 and 6) or mutant (lanes 2 and 5) SnoN or Ski and isolated by immunoprecipitation with anti-HA antibody. Endogenous N-CoR associated with HA-Smad4 was detected by Western blotting with anti-N-CoR antibody (upper panels). As controls, the anti-HA immunoprecipitates were blotted with anti-FLAG monoclonal antibody for the associated SnoN or Ski proteins (second panels) or with anti-HA antibody for Smad4 (third panels). Total cell lysates were blotted with anti-N-CoR antibody or with anti-FLAG monoclonal antibody to control for the expression of N-CoR and SnoN or Ski protein (fourth and lower panels).

Discussion

Ski and SnoN are expressed in most adult and embryonic cells, albeit at a low level. Under normal physiological conditions, elevation of Ski or SnoN expression occurs only during certain stages of embryogenesis and is under strict controls. When overexpressed, Ski and SnoN induce oncogenic transformation of CEFs. In addition, transformation by Ski has also
be the repression of Smad function impaired. Consistent with this, although v-Ski lacks the R-Smad-binding site due to a truncation of the first 27 amino acid residues from c-Ski, it still represses Smad function and induces potent transformation to a derepression of TGF-β signaling (16–21). To examine whether this is responsible for the transforming activity of Ski or SnoN, we next determined the amino acid residues in Ski or SnoN responsible for interaction with the Smad proteins. Ski and SnoN were found to bind to the R-Smad proteins (Smad2 and Smad3) and Co-Smad (Smad4) through different regions. The presence of two Smad-binding sites suggests that Ski or SnoN can either interact with one of the Smad proteins individually (35, 54, 55) or bind to a heterodimer of R-Smad and Smad4, depending on the absence or presence of ligand and the status of the signaling pathway. Binding of Ski or SnoN to R-Smad or Smad4 individually may result in recruitment of Smad-associated cellular proteins to Ski or SnoN, allowing cross-talk with other intracellular signaling pathways. The ability of Ski or SnoN to repress the transactivation activity of the Smad proteins individually may result in recruitment of Smad-associated cellular proteins to Ski or SnoN, allowing cross-talk with other intracellular signaling pathways. Smad4 (44). Although the disrupted Smad complexes remain bound to Ski, these complexes are not in an active conformation to interact with transcriptional coactivators such as CBP to activate TGF-β target genes (44). Based on this model, binding of Ski or SnoN to one of the Smad molecules, either R-Smad or Smad4, is sufficient for the disruption of the Smad complex and subsequent repression of TGF-β signaling. Indeed, we found that mutation of one of the Smad-binding sites in Ski or SnoN did not affect the ability of Ski or SnoN to repress the transactivation activity of the Smad proteins significantly. Only when both binding sites were abolished was the repression function of Smad4 impaired. Consistent with this, although v-Ski lacks the R-Smad-binding site due to a truncation of the first 27 amino acid residues from c-Ski, it still represses Smad function and induces potent transformation of CEFs, probably because it can still interact with the Smad complex and inactivate it through binding to Smad4.

The residues in Ski that mediate Smad4 binding are well defined. We found that mutation of one of the Smad-binding sites in Ski or SnoN did not affect the ability of Ski or SnoN to repress the transactivation activity of the Smad proteins significantly. Only when both binding sites were abolished was the repression function of Smad4 impaired. Consistent with this, although v-Ski lacks the R-Smad-binding site due to a truncation of the first 27 amino acid residues from c-Ski, it still represses Smad function and induces potent transformation of CEFs, probably because it can still interact with the Smad complex and inactivate it through binding to Smad4.
conserved in all Ski family members, including v-Ski, c-Ski, and c-SnoN and its isoforms (SnoN2, Snof, and SnoA), and the region required for R-Smad binding is present in all but v-Ski. A careful comparison of the amino acid sequences within the two Smad-binding sites did not reveal any extensive homology between the two regions. These Smad-binding sites do not show any obvious similarity to the PPXY-containing motif found to mediate Smad2-binding in FAST1 and in the Milk family of proteins or to the Smad-binding domains of SARA, type I TGF-β receptor, and Smurfr2 (58–62). Consistent with these observations, different domains or residues in Smad2 may mediate binding to these proteins. For example, a basic amino acid residue stretch in the L3 loop of Smad2 interacts with activated type I TGF-β receptor, whereas the PY motif in the linker region of Smad2 recognizes the WW domain in Smurfr2 (54). Thus, Smad2 and Smad3 may recognize multiple sequence motifs through different amino acid residues. Interestingly, the R-Smad-binding site is not 100% conserved between Ski and SnoN. This partial difference may contribute to the different affinity of Ski and SnoN for the bone morphogenic protein Smad proteins (17, 18). A thorough understanding of the Ski-Smad and SnoN-Smad protein interactions will require a detailed analysis of the three-dimensional crystal structures of these complexes.

Because Smad proteins are important tumor suppressors, we hypothesized that the ability of Ski and SnoN to inactivate Smad function may be responsible for their transforming activity. In this study, we employed CEFs as a model system to investigate the transforming activity of Ski and SnoN because Ski was originally identified in CEFs as a viral oncogene by virtue of its ability to transform CEFs. Unlike many mammalian fibroblast cell lines that proliferate in response to TGF-β, CEFs, like many epithelial cells, undergo growth arrest in the presence of TGF-β (data not shown). Thus, overexpression of Ski or SnoN blocks TGF-β-induced growth arrest (data not shown), and this may be responsible for the transformation of CEFs. Indeed, we have shown here that the interaction of Ski and SnoN with Smad proteins is required for the antagonism of TGF-β signaling and, more importantly, for their transforming activity. Mutant Ski and SnoN lacking the Smad-binding sites are defective in repression of TGF-β-induced cell cycle arrest and fail to induce anchorage-independent growth of CEFs. Extrapolating from this, the high level expression of Ski or SnoN in some human cancer cells may be responsible for the resistance of these cancer cells to TGF-β-induced growth arrest, a key step in the malignant progression of mammalian tumor cells.

TGF-β signaling pathways are considered to be both tumor suppressor pathways and promoters of tumor progression and invasion. TGF-β1, TGF-β receptors, and the Smad proteins are expressed in virtually all tissues and cell types. Activation of TGF-β1 and TGF-β signaling in vivo can be regulated at both the levels of extracellular ligand activation and intracellular signal transduction. These highly regulated processes regulate the differentiation or proliferation state of a given cell type or tissue. In normal cells and at early stages of tumorigenesis, activation of TGF-β and Smad proteins inhibits cell growth. Perturbation of this growth inhibitory pathway by activation or overexpression of oncopgenes such as ski and snoN results in a diminished growth inhibitory response, leading to rapid tumor growth and clonal expansion and permitting the accumulation of additional mutations and tumor progression. As tumor cells lose their ability to be inhibited by TGF-β and progress to a more malignant stage, stimulation by TGF-β causes these cells to undergo epithelial-to-mesenchymal transdifferentiation, leading to increased tumor metastasis and invasion. Thus, the activity of Ski and SnoN may function to promote the switch of the tumor cell responses to TGF-β from growth inhibition to accelerated malignant progression.

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