The Oncoprotein Ski Acts as an Antagonist of Transforming Growth Factor-β Signaling by Suppressing Smad2 Phosphorylation*

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The phosphorylation of Smad2 and Smad3 by the transforming growth factor (TGF-β)-activated receptor kinases and their subsequent heterodimerization with Smad4 and translocation to the nucleus form the basis for a model how Smad proteins work to transmit TGF-β signals. The transcriptional activity of Smad2-Smad4 or Smad3-Smad4 complexes can be limited by the corepressor Ski, which is believed to interact with Smad complexes on TGF-β-responsive promoters and represses their ability to activate TGF-β target genes by assembling on DNA a repressor complex containing histone deacetylase. Here we show that Ski can block TGF-β signaling by interfering with the phosphorylation of Smad2 and Smad3 by the activated TGF-β type I receptor. Furthermore, we demonstrate that overexpression of Ski induces the assembly of Smad2-Smad4 and Smad3-Smad4 complexes independent of TGF-β signaling. The ability of Ski to engage Smad proteins in non-productive complexes provides new insights into the molecular mechanism used by Ski for disabling TGF-β signaling.

Members of the transforming growth factor-β (TGF-β)1 superfamily play a critical role in regulating many diverse biological processes, including cell growth regulation, specification of development fate, differentiation, and apoptosis (1–3). TGF-β signaling is initiated when the ligand induces formation of a heteromeric complex, composed of type I (TGF-β receptor-activated) and type II Ser/Thr kinase receptors (4–6). Activation of the receptor complex occurs when the type II receptor transphosphorylates the type I receptor in the GS domain, thus activating the type I kinase that targets downstream substrates, such as the receptor-activated Smads (R-Smads), Smad2 and Smad3 (4–6). Smad2 and Smad3 are specifically recruited to the receptor complex by an adapter molecule called SARA (Smad anchor for receptor activation) (7). Receptor-mediated phosphorylation of Smad2 and Smad3, which occurs within a conserved carboxy-terminal SS/M/V/S motif, induces their association with the common mediator Smad4, and these complexes enter the nucleus, where they activate transcription of specific genes (5).

The ability of Smads to modulate transcription in response to ligand results from a functional cooperativity with the transcriptional coactivators CBP and p300, which are thought to mediate activation of some TGF-β target genes by bringing Smad2 or Smad3 within the proximity of the general transcription machinery and by modifying the chromatin structure through histone acetylation (5, 8). In addition to interacting with coactivators, Smads can bind with nuclear transcriptional repressors as well, including the oncoprotein Evi-1, TGIF, and SNIP1 (9–11). Evi-1 has been shown to interact with Smad3 but not Smad2 and is able to inhibit the activation of TGF-β-responsive promoters by disrupting the binding of the Smad3-Smad4 complex to DNA (9). TGIF is a DNA-binding homeodomain protein that can interact with TGF-β-activated Smads and repress expression of TGF-β target genes (10). Transcriptional repression by TGIF is dependent on its ability to recruit other transcriptional corepressors, including histone deacetylases (HDACs), carboxyl terminus-binding protein, and mSin3A (10, 12, 13). The interaction of TGIF with TGF-β-activated Smads also displaces the coactivators CBP/p300, thus reducing the ability of Smads to activate transcription (10). A similar mechanism has been proposed to explain the action of the corepressors SNIP1, which interacts with Smads on TGF-β-responsive promoters and represses their ability to activate TGF-β target genes by competing with CBP/p300 for Smad interaction (11).

Additional inhibitors of the transcriptional functions of Smad proteins include c-Ski, the cellular counterpart of the v-Ski oncoprotein, and the related protein SnoN (Ski-related novel gene) (14–19). The oncogenic v-Ski was originally identified in avian Sloan-Kettering viruses and found to transform chicken embryo fibroblasts (20). The v-Ski oncoprotein is truncated at the carboxyl terminus by 312 amino acids relative to the c-Ski protein, but this truncation does not play a role in the activation of ski as an oncogene (21). Overexpression of either c-Ski or v-Ski induces morphological transformation and anchorage-independent growth in chicken and quail embryo fibroblasts, indicating that the transforming activity is attributable to overexpression, not truncation, of the c-Ski protein (22, 23). Recombinant c-Ski protein purified from bacteria cannot directly bind to DNA, but c-Ski in nuclear extracts from mammalian cell cultures binds to DNA, suggesting that c-Ski lacks a DNA ability on its own and instead regulates transcription via its ability to associate with other proteins (24, 25). Recently, diverse types of studies converged on the conclusions that Ski binding to DNA is mediated in part through its association with the R-Smad-Smad4 heteromeric complexes (14, 15, 17). Because Ski was found to be a component of the HDAC complex.

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‡ The abbreviations used are: TGF-β, transforming growth factor β; HDAC, histone deacetylases; TβR1, TGF-β type I receptor; R-Smad, receptor-activated Smad; CBP, cAMP-responsive element-binding protein-binding protein; HA, hemagglutinin; NLS, nuclear localization signal; NES, nuclear export signal; HA-TβR act, constitutively active TGF-β type I receptor.
through binding to the nuclear hormone receptor corepressors nuclear hormone receptor corepressor and mSin3A (26, 27), it has been postulated that one of the mechanisms used by Ski to repress Smad signaling involves the formation of a transcriptional repressor complex (14, 15, 17). However, alternative mechanisms of repression of Smad signaling may also exist because Ski has been shown to prevent the formation of a functional R-Smad-Smad4 heteromeric complex, thereby inactivating its ability to activate transcription (28). In this study, we report an additional and heretofore unexpected role of Ski in the negative regulation of Smad activity by showing that Ski can function to prevent the phosphorylation of Smad2 and Smad3 by the activated type I receptor. Our results suggest a new mechanism for the silencing of Smad signaling by the oncprotein Ski.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Culture—The following plasmids have previously been described: EF-FLAG-Smad4, EF-FLAG-Smad4.NES and EF-FLAG-Smad4.NLS (29), pcDNA3–6×Myc-Ski and pcDNA3–6×Myc-Ski.delA (14), pCMV5-FLAG-Ski (15), pCMV5-HA-TβRI, pCMV5-HA-TβRI act, pCMV5–FLAG-Smad2, pCMV5–FLAG-Smad2.SSA, pCMV5–6×Myc-Smad2, and pCMV5-HA-Smad4 (30), and pSC2–6×Myc-Fast1 (31). COS-7, Mv1Lu, and Mv1Lu-Ski13 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 5 mM glutamine, and antibiotics.

Immunoprecipitation and Immunoblotting—COS7 cells transfected with LipofectAMINE Plus (Invitrogen) were lysed at 4 °C in TMNG buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM MgCl2, 10% glycerol, 0.5% Nonidet P-40, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin) and subjected to immunoprecipitation with the appropriate antibody for 2 h, followed by adsorption to Sepharose-coupled protein G for 1 h. The precipitates were washed five times in TMNG, and the immunoprecipitates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the indicated primary antibody. The bands were visualized by an enhanced chemiluminescence detection system according to the manufacturer’s instructions (ECL, Amersham Biosciences). For determination of total protein levels, aliquots of cell lysates were subjected to direct immunoblotting. The following antibodies were used for immunoprecipitation and Western blot analysis: anti-FLAG mouse monoclonal antibody (Clone M2; Sigma), anti-Myc mouse monoclonal antibody (Clone 9E10; Santa Cruz), anti-HA mouse monoclonal antibody (Clone 12C5A; Roche Applied Science), anti-phospho-Smad2 rabbit polyclonal antibody (Zymed Laboratories Inc.), anti-Smad2 rabbit polyclonal antibody (Zymed Laboratories Inc.), and anti-phospho-Smad3 rabbit polyclonal antibody (generous gift from Dr. Ten Dijke).

Immunofluorescence—COS7 cells were plated to semiconfluency and 24 h later transfected with the indicated expression vectors by the LipofectAMINE method. 48 h after transfection, the slides were washed twice in phosphate-buffered saline, fixed in 4% paraformaldehyde for 30 min at room temperature, and permeabilized in 0.1% Triton X-100. The cells were incubated overnight at 4 °C with a mixture of polyclonal anti-FLAG (Sigma) and monoclonal anti-Myc 9E10 antibodies. The cells were washed with phosphate-buffered saline, incubated with a mixture of Texas Red-conjugated goat anti-rabbit and fluorescein isothiocyanate-conjugated goat anti-mouse antibodies, and examined on a Leica fluorescent microscope.

RESULTS

Ski Interacts with Smad4 in the Cytoplasm—We and others have recently reported that Ski is uniformly distributed throughout cells both in the absence and the presence of TGF-β signaling (32, 33). To investigate the potential of a mechanistic relationship between cytoplasmic localization of Ski and Ski-dependent repression of Smad signaling, we further extended the analysis of the interaction of Ski and Smad proteins. To do so, COS-7 cells were transfected with Ski and Smad2 or Smad4, together with either wild-type or constitutively activated TβRI, which contains a substitution of Thr residue 204 to Asp and signals TGF-β responses in the absence of ligand and the type II receptor (34). As shown in Fig. 1A, a weak interaction of Smad2 with Ski could be detected in unstimulated cells, and this interaction was induced by expression of the activated type I receptor, consistent with published results (14, 15, 17, 19). In contrast, the interaction of Ski and Smad4 occurred in both the absence and the presence of TGF-β signaling (Fig. 1B).

Because the mechanism of repression of Smad signaling appears to involve the formation of a transcriptional repressor complex on DNA (14, 15, 17) and because Smad4 shuttles continuously between the cytoplasm and the nucleus in the absence of TGF-β signaling because of the combined activities of nuclear import and export signals present in Smad4 (29), we wanted to investigate whether Ski can form a complex with a Smad4 that is already nuclear prior to TGF-β signaling. To test this possibility, we employed two Smad4 mutants, one (Smad4.NLS) with five conserved Lys residues in the NLS mutated to Ala and another (Smad4.NES) with two conserved Leu residues in the NES mutated to Ala. The Smad4.NLS mutant was completely excluded from the nucleus, whereas the Smad4.NES mutant was exclusively localized in the nucleus (Fig. 2C and Ref. 29). To detect the interaction, cell lysates from transiently transfected COS-7 cells were subjected to immunoprecipitation with anti-FLAG antibody directed to-
ward tagged Smad4 mutants, followed by immunoblotting with anti-Myc antibody for the presence of Ski. Unexpectedly, we found that mutation of NLS in Smad4 induced a strong increase in the association of Ski and Smad4 (Fig. 2A), suggesting that Ski may associate with Smad4 in the cytoplasm. Consistent with this hypothesis, we observed little or no interaction of Ski with Smad4.NES, despite efficient expression of this Smad4 mutant (Fig. 2A). As a control, we investigated the
ability of these Smad4 mutants to associate with Smad2 in response to TGF-β signaling. Similar to wild-type Smad4, the association of Smad4.NLS or Smad4.NES with Smad2 was strongly increased by activation of TGF-β signaling (Fig. 2B), eliminating the possibility that mutations in NLS and NES motifs may affect the folding of Smad4 protein.

To provide further evidence that the association between Ski and Smad4 may take place in the cytoplasm, we transfected COS-7 cells with Ski and wild-type or Smad4 mutants and visualized the proteins by immunofluorescence. In cells expressing c-Ski alone, the Ski immunoreactivity was diffuse and was found throughout the transfected cells (Fig. 2C) as described previously (32, 33). Analysis of cells transfected with wild-type Smad4 and Ski revealed that Ski colocalized with wild-type Smad4, and the distribution of Ski was indistinguishable from cells transfected with Ski alone (Fig. 2C). The distribution of Ski both in the cytoplasm and the nucleus was also not altered by the expression of Smad4.NES, which was found exclusively in the nucleus (Fig. 2C), consistent with our biochemical analysis showing that Smad4.NES is defective in its ability to interact with Ski (Fig. 2A). In contrast, coexpression of Smad4.NLS caused Ski to accumulate predominantly in the cytoplasm, presumably because of the interaction of Ski and Smad4.NLS (Fig. 2C). Similar results were obtained in cells cotransfected with the constitutively activated type I receptor (data not shown), suggesting that the constitutive interaction of Ski and Smad4 may occur exclusively in the cytoplasm independent of TGF-β signaling.

Ski Induces a Ligand-independent Association of Smad2 or Smad3 with Smad4—The observations that Ski constitutively interacts with Smad4 in the cytoplasm suggested the possibility that Ski might act to exert its inhibitory function by interfering with the assembly of R-Smad-Smad4 heteromeric complexes in response to TGF-β signaling. To test this hypothesis, COS-7 cells were transfected with Smad2 and Smad4 in the presence or absence of Ski and wild-type or activated TβRI. As shown in Fig. 3A, association of Smad2 with Smad4 was strongly increased by the activated type I receptor, similar to previous observation (35, 36). Surprisingly, cotransfection of Ski resulted in a ligand-independent increase in the amount of Smad2 bound to Smad4 (Fig. 3A). A similar conclusion could be drawn when Smad3 was used instead of Smad2, suggesting that Ski can support the formation of R-Smad-Smad4 heteromeric complexes in a manner independent of TGF-β signaling.

The phosphorylation of Smad2 and Smad3 at the carboxy-terminal SS(M/V)S motif by the activated TGF-β receptor is required for subsequent interaction with Smad4 and the nuclear translocation of the complexes (5, 8). In principle, the formation of the Smad2-Smad4 complex by coexpression of Ski could be achieved either by the ability of Ski to induce phosphorylation of Smad2 at the carboxy-terminal SSMS motif through a yet to be identified mechanism or, alternatively, to induce the formation of Smad2-Smad4 complexes without affecting the phosphorylation of Smad2. To distinguish between these two possibilities, we determined the effect of Ski on the association of Smad4 with either wild-type Smad2 or Smad2(3SA) that contains Ala mutations in the three Ser of the sequence SSMS. As anticipated, coexpression of the activated receptor resulted in a strong increase in the amount of wild-type Smad2 that coprecipitated with Smad4, whereas Smad2(3SA)-Smad4 complexes were not detected (Fig. 3B). In contrast, in cells expressing Ski, Smad2(3SA) strongly associates with Smad4 with an efficiency approaching that elicited by transfection of wild-type Smad2 (Fig. 3B), indicating that the ability of Ski to induce the association of Smad2 and Smad4 is not due to activation of endogenous TGF-β receptors, which in turn phosphorylates Smad2, leading to the formation of Smad2-Smad4 complexes.

Ski Blocks the Phosphorylation of Smad2 and Smad3—The finding that Ski induces the formation of Smad2-Smad4 complexes independent of TGF-β signaling raised questions about the ability of the activated TGF-β receptor to recognize and phosphorylate R-Smads that are already engaged in complexes with Ski and Smad4. To investigate whether Ski could interfere with the ligand-dependent phosphorylation of Smad2, we transfected COS-7 cells with 6×Myc-Smad2 and FLAG-Ski together with either wild-type or constitutively activated TβRI.
Suppression of Smad2 Phosphorylation by Ski

and assessed the phosphorylation of 6×Myc-Smad2 by an anti-phospho-Smad2 antibody that specifically recognizes TGF-β receptor-phosphorylated Smad2 (30). As described previously (30), coexpression of Smad2 with activated TβRII resulted in a strong increase in Smad2 phosphorylation (Fig. 4A). In contrast, overexpression of Ski completely inhibited the phosphorylation of Smad2 in response to TGF-β signaling (Fig. 4A). The inhibitory effect of Ski on TGF-β-mediated phosphorylation of Smad2 depended on the amount of Ski expressed in the cells (Fig. 4B). A similar inhibitory effect of Ski was observed in cells cotransfected with 6×Myc-Smad3, suggesting that Ski may function to inhibit TGF-β signaling by preventing the ligand-dependent phosphorylation of Smad2 and Smad3 (Fig. 4C).

In the course of these experiments, we found that when the phosphorylation of 6×Myc-Smad2 was monitored by immunoprecipitation followed by Western blotting with anti-phospho-Smad2 antibody, a protein that was the size expected for endogenous Smad2 associated with 6×Myc-Smad2 and became phosphorylated by activated TβRII, and this TGF-β-dependent phosphorylation was blocked by overexpression of Ski (Fig. 4A). Because Smad2 protein can form homodimer in response to phosphorylation, this result suggested that Ski might also prevent the phosphorylation of endogenous Smad2 in response to TGF-β signaling. To examine this directly, we analyzed the phosphorylation of endogenous Smad2 in response to activation of endogenous TGF-β receptors using the previously defined cell line Mv1Lu-Ski13, in which the stable expression of Ski suppressed the ability of Smads to mediate TGF-β-induced growth arrest and transcriptional responses (17). Western blotting analysis with anti-phospho-Smad2 antibody from the parental cell line Mv1Lu that was untreated or treated with TGF-β for 15 min revealed a ligand-dependent phosphorylation of endogenous Smad2 (Fig. 4D). In contrast, in the Mv1Lu-Ski13 cell line, we could not observe any effect of TGF-β on Smad2 phosphorylation (Fig. 4D). Thus, in stably transfected cells and in transient transfection assays, the presence of Ski caused a loss of Smad2 phosphorylation in response to TGF-β signaling.

Because Ski appears to prevent the ligand-dependent phosphorylation of Smad2 on the SSMS motif (Fig. 4, A, B, and D), we investigated how the formation of a complex between Ski and Smad2 is increased in response to TGF-β signaling (Fig. 1A). To do this, we analyzed Ski interaction with wild-type Smad2 or the Smad2(3SA) mutant, which is defective in its ability to be phosphorylated by the activated type I receptor. As with wild-type Smad2, we observed a strong increase in the amount of Smad2(3SA) that associates with Ski upon activation of TGF-β signaling (Fig. 4E). These data suggest that TGF-β signaling can induce the association of Ski and Smad2 independently of Smad2 phosphorylation by the activated type I receptor.

The Suppression of Smad2 Phosphorylation by Ski Is Linked to the Association of Smad4 with Ski—Previous studies have shown that Ski contains discrete binding sites for Smad2 and Smad4 (14, 15, 28). The availability of a mutant of Ski (Ski.delA) that interacts with Smad2 but fails to bind to Smad4 (14) allowed us to address the question of whether Ski repressing TGF-β-mediated activation of Smad2 is dependent on the presence of Smad4. We first examined the ability of Ski.delA to modulate the association of Smad2 and Smad4 in response to TGF-β signaling. Consistent with our previous analysis (Fig. 3A), overexpression of wild-type Ski resulted in a strong increase in the association of Smad2 and Smad4 independent of TGF-β signaling (Fig. 5A). In contrast to wild-type Ski, Ski.delA, which was expressed efficiently, had little or no ability to induce the association of Smad2 and Smad4 in the absence of TGF-β signaling (Fig. 5A). More importantly, in the presence of TGF-β signaling, we found an ~3-fold increase in the association of Smad2 and Smad4 in cells transfected with Ski.delA relative to cells transfected with control vector (Fig. 5A), raising the interesting possibility that deletion of the Smad4 binding site might generate a specific dominant inhibitory form of Ski. From these results, it can be suggested that Ski might mediate the ligand-independent formation of Smad2-Smad4 heteromeric complex through its association with Smad4.

We next evaluated the effect of Ski.delA on the ability of the activated type I receptor to induce the phosphorylation of Smad2. As described above (Fig. 4, A and D), coexpression of wild-type Ski with Smad2 resulted in a marked reduction in Smad2 phosphorylation induced by the constitutively activated type I receptor (Fig. 5B). In contrast, expression of Ski.delA along with Smad2 increased the sensitivity of the cells, yielding a ~3–4-fold increase in receptor-induced Smad2 phosphorylation (Fig. 5B). Together, these results suggest that the ability of Ski to prevent TGF-β-dependent phosphorylation of Smad2 is linked to the association of Ski with Smad4 and further suggest that Ski.delA might act as a dominant-negative inhibitor by blocking the function of endogenous Ski protein.

Ski Induces the Formation of Inactive Smad2-Smad4 Complexes—Following TGF-β-mediated phosphorylation and association with Smad4, Smad2 moves to the nucleus and activates expression of specific genes through cooperative interactions with specific DNA-binding proteins. For example, activation of the Mix2 gene by TGF-β or activin-related ligands require the formation of a Smad2-Smad4-Fast1 complex that binds to a specific promoter sequence known as the activin response element (37, 38). To provide further evidence that Ski can block the TGF-β-dependent phosphorylation of Smad2, we tested whether it could interfere with the assembly of Smad2-Fast1 complex in response to TGF-β signaling. For this, COS-7 cells were transfected with Fast1 and Smad2 in the presence or absence of Ski and wild-type or activated TβRII. In the absence of TGF-β signaling, weak interaction between Fast1 and Smad2 could be detected (Fig. 6). However, coexpression with the activated TβRII enhanced the interaction of Fast1 with Smad2 (Fig. 6), supporting the notion that receptor-dependent phosphorylation of Smad2 is required for subsequent association with Fast1 (37, 38). Interestingly, in cells expressing Ski, we observed almost a complete block in the formation of the Smad2-Fast1 complex in response to TGF-β signaling (Fig. 6). To determine whether Ski inhibits the TGF-β-dependent association of Smad2 and Fast1 through its constitutive association with Smad4, we investigated the function of the mutant Ski.delA, which interacts with Smad2 but not with Smad4. In contrast to wild-type Ski, expression of Ski.delA enhanced the ability of the activated type I receptor to induce the association of Smad2 and Fast1 (Fig. 6), consistent with the notion that Ski.delA might act as a dominant-negative inhibitor.

DISCUSSION

TGF-β is a potent natural antiproliferative agent that plays an important role in suppressing tumorigenicity (39). Tumor cells can acquire resistance to the antiproliferative effect of TGF-β by a number of different mechanisms including defects in TGF-β cell surface receptors and mutational inactivation of downstream effector components of the signaling pathways, such as Smad2 and Smad4 (2, 39). Elevated expression of the proto-oncogene c-Ski may also provide an alternative mechanism whereby tumor cells down-regulate TGF-β responsiveness and escape its tumor suppressor function. Consistent with this notion, overexpression of Ski resulted in suppression of TGF-β-induced transcriptional activation and caused cells to
FIG. 4. Ski prevents receptor-dependent phosphorylation of Smad2 and Smad3. A, COS-7 cells were transfected with the indicated combinations of 6×Myc-Smad2, HA-TβRI, or HA-TβRI act either in the absence or the presence of FLAG-Ski. The cell lysates were subjected to anti-Myc immunoprecipitation (IP) and then immunoblotted with anti-phospho-Smad2 antibody (α-p-Smad2). The phosphorylation of Smad2 was also assessed by immunoblotting total cell lysates with anti-phospho-Smad2 antibody (α-p-Smad2). Expression of transfected DNA was monitored
resist the growth inhibition by TGF-β (14, 15, 17, 19). The proposed mechanisms of Ski repression of TGF-β signaling appears to depend on its ability to recruit a nuclear repressor complex consisting of nuclear hormone receptor corepressor, mSin3A, and HDAC1 to TGF-β-responsive promoter elements through its interaction with Smad proteins (15, 17). Interaction of Ski with TGF-β-activated Smads also prevents interaction with the coactivator CBP/p300, thus reducing the ability of Smads to activate transcription (14, 28). In addition to the recruitment of a transcriptional complex and dissociation of CBP/p300, binding of Ski to the Smads converts the Smad heterocomplex into an inactive conformation (28). Recently, we and others have reported that Ski can also reside in the cytoplasm in a TGF-β-dependent manner. Con- sistent with this, mutations in Smad4 NLS motif that completely excluded Smad4 from the nucleus strongly enhance the association of Smad4 and Ski. Further evidence for the association of Ski and Smad4 in the cytoplasm is provided by the inability of Ski to interact with the Smad4.NES mutant, which is exclusively localized in the nucleus.

The present work also demonstrates that Ski can induce the formation of inactive R-Smad-Smad4 heteromeric complexes independent of TGF-β signaling. These studies thus provide a novel mechanism for the suppression of TGF-β signaling by the Ski oncoprotein.

In the current model for TGF-β signaling, Smad4 is assumed to be retained in the cytoplasm in the absence of a signal, but it accumulates in the nucleus following stimulation of cells with TGF-β, presumably because of formation of complexes with the R-Smads Smad2 and Smad3 (5, 6). However, Ski was shown to constitutively associate with Smad4 in both the presence and the absence of TGF-β signaling. Our study indicates that this constitutive interaction of Ski with Smad4 occurs in the cytoplasm in a TGF-β-independent manner. Consistent with this, mutations in Smad4 NLS motif that completely excluded Smad4 from the nucleus strongly enhance the association of Smad4 and Ski. Further evidence for the association of Ski and Smad4 in the cytoplasm is provided by the inability of Ski to interact with the Smad4.NES mutant, which is exclusively localized in the nucleus.

The present work also demonstrates that Ski can induce the formation of inactive R-Smad-Smad4 heteromeric complexes independent of TGF-β signaling, thereby interfering with the

by direct immunoblotting. B, COS-7 cells were transfected with 6×Myc-Smad2 and the indicated amounts of FLAG-Ski together with HA-TβR1 or HA-TβR1 act, and total cell lysates were subjected to immunoblotting with anti-phospho-Smad2 antibody (α-p-Smad2). The expression of transfected DNA was determined by immunoblotting total cell lysates using anti-FLAG (α-FLAG), anti-Myc (α-Myc), anti-HA (α-HA) antibodies.
phosphorylation of Smad2 and Smad3 by the activated TGF-β type I receptor. The Smad proteins contain two well conserved domains, the amino-terminal MH1 domain and the carboxy-terminal MH2 domain, which are separated by a proline-rich linker that differs substantially between the different classes of Smad (5). Receptor-mediated phosphorylation appears to relieve these two domains from a mutually inhibitory interactions, and this conformation is required for the formation of R-Smad-MH4 heteromeric complexes (41). One possibility is that conformational changes in unphosphorylated R-Smad, as a consequence of their constitutive interaction with Smad4 caused by overexpression of Ski, may in turn interfere with the access of R-Smad to the activated receptor and lead to the suppression of R-Smad phosphorylation. Another not mutually exclusive possibility is that Ski-mediated association of unphosphorylated R-Smad with Smad4 may alter the affinity of R-Smad to the adaptor molecule SARA, which recruits unphosphorylated R-Smad to the receptors for phosphorylation (7). Indeed, recent evidence has suggested that the binding of Smad4 to R-Smads serves as a dual signal for dissociation of R-Smad from SARA and subsequent accumulation of the Smad heterocomplexes in the nucleus (7).

Because the interaction of Ski with R-Smad was increased in response to TGF-β signaling, it is tempting to speculate that the ligand-dependent phosphorylation of Smad2 at the carboxy-terminal serines that serve as TGF-β receptor phosphorylation sites is required for their association with Ski (14, 15, 17, 19). Paradoxically, we found that expression of Ski completely blocks the TjRI-dependent phosphorylation of Smad2. This discrepancy is resolved by the evidence presented here that activation of TGF-β can also lead to the association of Ski and Smad23/3A), which is defective in its ability to be phosphorylated by the activated type I receptor. These observations not only suggest that Ski could interact with the unphosphorylated form of R-Smad but also raised the interesting possibility that TGF-β signaling might enhance the association of Ski and Smad2 independent of phosphorylation of Smad2 by the activated type I receptor. At present we do not understand the mechanism whereby activation of TGF-β signaling induces the association of Ski with the unphosphorylated form of Smad2.

It was recently proposed that Ski interacts with Smads on TGF-β-responsive promoters and represses their ability to activate TGF-β target genes, based on the reported ability of Ski to associate with nuclear hormone receptor corepressor, mSin3A, and HDAC1 (14, 15, 17, 26, 27). In contrast, our molecular model and the supporting data strongly suggest that Ski suppresses TGF-β-mediated phosphorylation of R-Smad through its ability to interfere with the phosphorylation of R-Smad by the activated type I receptor. Because overexpression of Ski suppressed the majority of TGF-β-mediated phosphorylation of R-Smad, we suggest that perhaps only a small proportion of R-Smad proteins is incorporated into a transcriptional corepressor complex containing Ski and its associated HDAC. Consistent with this interpretation, suppressing the activity of HDAC by the inhibitor trichostatin A did not interfere with the ability of Ski to repress a TGF-β-inducible promoters containing the Smad3/4-binding element SBE (19). Furthermore, overexpression of Ski in melanomas cells has been shown to prevent Smad3 nuclear translocation in response to TGF-β signaling (32), reinforcing the notion that the inhibitory effect of Ski on TGF-β signaling is not attributable to transcriptional repression in the nucleus.

From these molecular studies, we concluded that the inhibitory effects of the oncogenic Ski on TGF-β signaling derive in part through its ability to prevent the ligand-dependent phosphorylation of R-Smads.

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