The oncoprotein Ski represses transforming growth factor-β (TGF-β) signaling in an N-CoR-independent manner. However, the molecular mechanisms underlying this event have not been elucidated. Here, we identify an additional domain in Ski that mediates interaction with Smad3 which is important for this repression. This domain is distinct from the previously reported N-terminal Smad binding domain in Ski. Individual alanine substitution of several residues in the domain significantly affected Ski-Smad3 interaction. Furthermore, combined mutations within this domain, together with those in the previously identified Smad3 binding domain, can completely abolish the interaction of Ski with Smad3, while mutation in each domain alone retained partial interaction. By introducing those mutations that abolish direct interaction with Smad3 or Smad4 individually, or in combination, we show that interaction of Ski with either Smad3 or Smad4 is sufficient for Ski-mediated repression of TGF-β signaling. Furthermore our results clearly demonstrate that Ski does not disrupt Smad3-Smad4 heteromer formation, and recruitment of Ski to the Smad3/4 complex through binding to either Smad3 or Smad4 is both necessary and sufficient for repression.

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The oncoprotein Ski can act as a transcriptional repressor primarily due to multiple direct and indirect interactions with histone deacetylase (HDAC) complexes including N-CoR/SMRT/Sin3A corepressors (Ref. 1 and reviewed in Ref. 2). Ski was also shown to act in the nuclear receptor signaling pathways (1, 3, 4) and with tumor suppressors, including Mad, Rb, and PML (1, 5, 6).

Recently, Ski/Sno were demonstrated to mediate negative feedback regulation of TGF-β signaling (7–10) as well as bone morphogenetic protein signaling (11). The mechanism of Ski-mediated repression of TGF-β signaling is primarily attributed to the capacity of Ski to interact with Smad2/3/4 (7–10).

Recently, several studies have proposed structural models of complex formation between Ski and the MH2 domains of Smad3/4 (12, 13). The minimal domain in Ski that defines interaction with Smad3 MH2 domain was mapped to the amino terminus (amino acid residues 17–45 in human c-Ski) and was demonstrated to preferentially bind to Smad3 when it was in the active trimeric complex (12). They also reported that this Ski fragment directly binds to Smad3 allowing Smad3-Smad4 heteromer formation in the complex. In another study the crystal structure of the Smad4 binding domain in Ski in complex with Smad4 MH2 domain was resolved and lead to a model in which Ski competes with Smad3 for binding to Smad4 and thus disrupts the functional integrity of the complex (13). Therefore at the present time studies on the formation of complexes involving Ski and Smads remain controversial, and clearly more work is needed to clarify the interaction of Ski with the Smad3-Smad4 complex.

Here we identify an additional domain in Ski that affects interaction with Smad2/3, which is distinct from the previously identified Smad3 binding domain (12). Our results also provide evidence that binding of Ski to Smad3/4 does not disrupt Smad3-Smad4 heteromer formation, rather the Smad heteromer provides Ski with several discrete sites to mediate interaction with the Smad3/4 complex. Thus interaction of Ski with the Smad3/4 complex through binding to either Smad3 or Smad4 is sufficient for the repression activity of Ski on Smad-dependent transcriptional activation.

EXPERIMENTAL PROCEDURES

Chemicals and Oligonucleotides—All chemicals were purchased from Sigma or Fisher Scientific unless indicated. Oligonucleotides were purchased from Integrated DNA Technologies.

Plasmid Constructs—T7-tagged wt-cSki and mt-cSki (L110P), 6Myc-Smad2, 6Myc-Smad3, 6Myc-Smad4, and constitutively active type I receptor TpR-I(TD) were described previously (4, 8). Site-directed mutagenesis was performed using QuikChange (Stratagene). All mutagenesis and cloning procedures were verified by DNA sequencing.

Cell Culture and Transfection—COS-1 and mink lung epithelial CCL-64 (Mv1Lu) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml). Expression plasmids were introduced into the cells using FuGENE 6 (Roche Applied Science) as described by the manufacturer.

Immunoprecipitation Assay—Cells were lysed in Nonidet P-40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 10% glycerol) containing phosphatase and protease inhibitors. Pre cleared extracts were incubated with anti-Myc (9E10, Santa Cruz Biotechnology), anti-T7 (Novagen) mouse monoclonal antibodies, or mouse normal IgG (Santa Cruz Biotechnology). Immunocomplexes were recovered using protein G-Sepharose (Amersham Biosciences), separated by SDS-polyacrylamide gels, and transferred to nitrocellulose membranes (PROMTRAN, Schleicher & Schuell). For Western blotting, the primary antibodies used were anti-T7 or anti-Myc. Proteins of interest were detected using chemiluminescence (ECL, Amersham Biosciences).

Reporter Gene Assay—CCL-64 cells in 24-well plate were transfected with luciferase reporter construct 4×SBE-Luc (50 ng/well), β-galactosidase (TK-βGal, 50 ng/well), plus effector plasmids (100 ng/well). Total amount of transfected DNA was equalized with vector (pEGFP-C1).

Luciferase activities were performed 24 h after transfection and normalized to the β-galactosidase activities according to the manufacturer's instructions (Promega). The results are mean values and S.D. from

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SkiL110P could repress Smad-dependent transcriptional activity (4). This finding led us to analyze further how the binding domain (amino acid residues 17–32490) which potentially could provide an indirect link between Ski and Smad3 Binding—In the previous study, analysis of a Ski single point mutant SkiL110P, which was unable to bind to N-CoR, revealed that the Ski/N-CoR interaction was not required for repression of TGF-β signaling, whereas it was for nuclear hormone signaling (4). This finding led us to analyze further how SkiL110P could repress Smad-dependent transcriptional activation without N-CoR/SMRT. As previously reported (4), SkiL110P can associate with Smad4 and Sin3 to a similar extent to the wild-type Ski. Since Smad2/3 are also important factors for Ski-mediated repression, we investigated the interaction of SkiL110P with Smad2/3. We performed co-immunoprecipitation assay using lysates containing Ski (wt-Ski or mt-Ski), Smad2/3, and a constitutively active type I receptor TβR-I(TD) (Fig. 1A). Surprisingly, the mutation L110P significantly (but importantly not completely) reduced the binding capacity to Smad3 (Fig. 1A, upper panels, lane 6) in comparison with the wild-type (Fig. 1A, upper panels, lane 3). Similar results were obtained by using Smad2 (Fig. 1A, lower panels). These results indicate that the mutation L110P in Ski substantially affects interaction with Smad2/3 in addition to N-CoR/SMRT.

Analysis of the proximal amino acid sequence of the Leu110 site revealed that it resides in a hydrophobic residue-rich region that includes a potential amphipathic α-helix highly conserved in Ski/Sno/Dachshund family proteins (Fig. 1B) (14). To test the notion that these residues may facilitate the interaction with Smad3, we performed individual alanine substitution mutagenesis and examined the capacity of these mutants to associate with Smad3. Co-immunoprecipitation assays using lysates containing each Ski mutant, Smad3, and TβR-I(TD) showed that the mutations P107A, Q108A, I109A, V113A, and L114A significantly reduced Ski’s binding ability to Smad3 (Fig. 1C, lanes 4, 5, 6, 8, and 9), indicating that these residues are required for effective Smad3 interaction. Interestingly, the L110A mutation had little effect and even appeared to slightly increase the interaction with Smad3 (Fig. 1C, lane 7), while L110P significantly decreased the interaction (Fig. 1C, lane 10). We obtained similar results using Smad2 (data not shown), and recently using glutathione S-transferase-pull-down assays, this region was also shown to bind to Smad2. Each mutation tested had no effect on Smad4 binding (data not shown). Our data strongly suggest that this domain from amino acids 107–114, which is distinct from the previously identified Smad3 binding domain (amino acid residues 17–45 in human c-Ski) (12), also mediates interaction of Ski with Smad2/3. Analysis of this region in Ski proteins from other species, and also in more distant family members such as SnoN and Dachshund (Dach2), revealed that this region is very highly conserved. Fig. 1B shows that there is 100% protein sequence identity between human, mouse, and frog c-Ski, and this region is also conserved in human SnoN and human Dach2 (Fig. 1B). Thus, this degree of conservation indicates it is likely that this region plays an important role in Ski-Smad2/3 interactions.

Two Distinct Domains in Ski Mediate Smad3 Interaction—The partial binding ability shown in the Ski mutants described above (Fig. 1) suggested that two distinct domains in Ski may independently mediate interaction with Smad3. In addition they raised the possibility that the remaining Ski-Smad3 binding found in the mutants could be via Ski-Smad4 interaction, which potentially could provide an indirect link between Ski and Smad3 through binding to the Smad3-Smad4 heteromer. To examine these possibilities, we generated a series of Ski mutants having independent mutations for binding to either Smad3, and/or Smad4, or in combination. Mutation of L19A (equivalent mutation L21A in human c-Ski) was shown previously to abolish interaction with Smad3 (12). Mutation W255E (equivalent mutation W274E in human c-Ski) was shown to disrupt Smad4 interaction (13). Therefore, we introduced mutations L19A, L110P, and W255E individually or in combination to generate targeted disruption of Smad interactions (Fig. 2A).

Co-immunoprecipitation assay was performed using lysates containing Ski, Smad3, and TβR-I(TD) to examine the effect of each mutation on Smad3 binding (Fig. 2B). L19A (LA) alone significantly reduced but did not completely abolish Smad3 binding (Fig. 2B, lane 2). This was similar to the result with L110P (LP) (Fig. 2B, lane 3), albeit to a greater extent. W255E (WE) alone had no effect on Smad3 binding (Fig. 2B, lane 4). Interestingly, combination of mutations LA/LP completely abolished Smad3 interaction (Fig. 2B, lane 5). Mutations LA/WE, LP/WE, and LA/LP/WE showed no noteworthy difference from LA, LP, and LA/LP, (Fig. 2B, lanes 6–8), indicating that mutation WE has no effect on Smad3 interaction. This result was consistent with the previous observations that Ski interacts with Smad3 and Smad4 through discrete binding sites (7, 8, 13). To confirm the effect of the mutations on Smad4 binding, similar co-immunoprecipitation assay was performed using Smad4 in-

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2 L. Li and E. Stavnezer, personal communication.
Smad3 interaction independent of Smad4 binding mutation (LA or LP) is not sufficient, combined mutations (LA/LP) disrupt interaction of Ski with Smad3. Interestingly, LA/LP in the presence of Smad4 allowed this mutant to interact with Smad4 (Fig. 4A, lane 5), in clear contrast to its inability of direct interaction with Smad3 (Fig. 2B, lane 4). Similarly, in the presence of Smad3 the WE Ski mutant could now interact with Smad4 (Fig. 4A, lane 4), in contrast to its inability to directly interact with Smad4 (Fig. 2C, lane 4). However, LA/WE and LP/WE significantly reduced interaction with Smad3 and Smad4 (Fig. 4A, lanes 6 and 7), and LA/WE/P/WE completely abolished the interaction (Fig. 4A, lane 8). The partial interaction shown by LA/WE and LP/WE (Fig. 4A, lanes 6 and 7) is consistent with the partial transcriptional repression activity seen in the luciferase assay (Fig. 3, LA/WE and LP/WE). Clearly indicating that these mutants retain partial repression activity. In contrast, LA/WE/P/WE, which completely abolished individual interaction with Smad3 and Smad4 (Fig. 2B and C), nullified the repression (Fig. 3, LA/WE/P/WE). These results indicate that interaction of Ski with either Smad3 or Smad4 is necessary and sufficient for repression of Smad3/4-dependent transcriptional activation.

**Ski Allows Direct Smad3-Smad4 Heteromer Formation, Enabling Recruitment of Ski to the Smad3/4 Heteromeric Complex through Either Smad3 or Smad4 Binding—Smad3 and Smad4 form heteromers in response to TGF-β signaling (16). Thus, it is possible that Ski mutants, which lack direct interaction with either Smad3 or Smad4, can be recruited to Smad complex through the Smad3-Smad4 heteromer. To test this notion, we performed co-immunoprecipitation assay (Fig. 2, B and C) in the presence of both Smad3 and Smad4 (Fig. 4A). Interestingly LA/WE in the presence of Smad4 allowed this mutant to interact with Smad3 (Fig. 4A, lane 5), in clear contrast to its inability of direct interaction with Smad3 (Fig. 2B, lane 4). Similarly, in the presence of Smad3 the WE Ski mutant could now interact with Smad4 (Fig. 4A, lane 4), in contrast to its inability to directly interact with Smad4 (Fig. 2C, lane 4). However, LA/WE and LP/WE significantly reduced interaction with Smad3 and Smad4 (Fig. 4A, lanes 6 and 7), and LA/WE/P/WE completely abolished the interaction (Fig. 4A, lane 8). The partial interaction shown by LA/WE and LP/WE (Fig. 4A, lanes 6 and 7) is consistent with the partial transcriptional repression activity seen in the luciferase assay (Fig. 3, LA/WE and LP/WE). It is possible that exogenously expressed mutant Ski can dimerize with endogenous Ski through its C-terminal coiled-coil domain (17); thus, the presence of endogenous wild-type Ski may contribute to recruit Smad3/4 to the complex. However, this possibility is ruled out, as the interactions are not observed in our co-immunoprecipitation assays using the triple mutant form of Ski (LA/WE/P/WE), which retains this coiled-coil domain (lanes 8, Fig. 4A and Fig. 2, B and C). These results provide evidence that binding of Ski still permits
were detected by Western blotting using anti-Myc or anti-T7 antibody. The immuno complexes (IP) (upper panels) and 10% input (lower panels) were detected by Western blotting using anti-Myc or anti-T7 antibody.

B. A proposed model for Ski-mediated repression of Smad3/4-dependent transcriptional activation. Smad3-Smad4 heteromer formation (8) and thus may function by inhibiting the binding of coactivators rather than recruiting corepressors. Hence these lines of repression by Ski cannot be ruled out either. Regardless of the details our data clearly show that binding of Ski to the Smad3/4 complex is central to its ability to repress.

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**Fig. 4.** Ski does not disrupt but allows direct Smad3-Smad4 heteromer formation. A. Ski allows direct Smad3-Smad4 heteromer formation. Myc-Smad3 and Myc-Smad4 were co-immunoprecipitated with T7-tagged Ski mutants as indicated using anti-T7 antibody. The immune complexes (IP) (upper panels) and 10% input (lower panels) were detected by Western blotting using anti-Myc or anti-T7 antibody. B. A proposed model for Ski-mediated repression of Smad3/4-dependent transcriptional activation. Smad3-Smad4 heteromer formation, and interaction with either Smad3 or Smad4 is necessary and sufficient for Ski-mediated transcriptional repression in TGF-β signaling.

Our finding that there are two distinct domains in Ski involved in Smad2/3 interaction allowed us to generate a series of informative Ski mutants completely lacking direct binding to either Smad3 (LA/LP), Smad4 (WE), or both (LA/LP/WE) (Fig. 4B). These Ski mutants have provided strong evidence that either Smad3 or Smad4 interaction with Ski is necessary and sufficient for repression of TGF-β signaling. This is mediated by the Smad3-Smad4 heteromer formation, resulting in full repression activity as WT on Smad3/4-dependent transcriptional activation. The Ski mutant lacking direct binding to both Smad3 and Smad4 (LA/LP/WE) no longer retains repression activity because of the inability to be recruited to the Smad3/4 complex.

Ski allows direct Smad3-Smad4 heteromer formation, and interaction with either Smad3 or Smad4 is necessary and sufficient for Ski-mediated transcriptional repression in TGF-β signaling. As previously shown by several groups using gel shift assays (7–10, 18), Ski can be recruited to the Smad-binding elements (SBE) on DNA through interaction with Smads. However, how Ski can exert its repression activity on Smads remains unknown. We recently reported that this Ski-mediated repression is N-CoR-independent (4). Ski was shown to link Smad3 and Smad4, forming a complex including HDAC1 (8). Thus it is possible that corepressors other than N-CoR play a role in Ski-mediated repression in TGF-β signaling. Furthermore, Ski was suggested to interfere with Smad-CBP/p300 coactivator complex formation (8) and thus may function by inhibiting the binding of coactivators rather than recruiting corepressors. Hence these lines of repression by Ski cannot be ruled out either. Regardless of the details our data clearly show that binding of Ski to the Smad3/4 complex is central to its ability to repress.