The Ski protein has been proposed to serve as a corepressor for Smad4 to maintain a transforming growth factor-β (TGF-β)-responsive promoter at a repressed, basal level. However, there have been no reports so far that it indeed acts on a natural promoter. We have previously cloned the human Smad7 promoter and shown that it contains the 8-base pair palindromic Smad-binding element (SBE) necessary for TGF-β induction. In this report, we have characterized the negative regulation of Smad7 promoter basal activity by Ski. We show that Ski inhibits the Smad7 promoter basal activity in a SBE-dependent manner. Mutation of the SBE abrogates the inhibitory effect of Ski on the Smad7 promoter. Moreover, mutation of the SBE increases the Smad7 promoter basal activity. Using the chromatin immunoprecipitation assay, we further show that Ski together with Smad4 binds to the endogenous Smad7 promoter. Finally, we show that RNAi knockdown of Ski increases Smad7 reporter gene activity in transient transfection assays as well as elevating the endogenous level of Smad7 mRNA. Taken together, our results provide the first evidence that Ski is indeed a corepressor for Smad4, which can inhibit a natural TGF-β responsive gene at the basal state.

TGF-β regulates various biological responses through transcriptional regulation of diverse genes (1). Upon TGF-β treatment, Smad2 and Smad3 are phosphorylated by the TGF-β receptor kinase, form complexes with Smad4, and together accumulate in the nucleus to regulate transcription (1). Smad7 antagonizes TGF-β signaling by binding to the receptor and inhibiting it to phosphorylate Smad2 and Smad3 (2–4). Interestingly, Smad7 itself is a direct target gene of Smads (5–10). Its transcription is up-regulated by treatment with TGF-β, thus providing a negative feedback loop control (2–10).

The 8-bp palindromic sequence (GTCTAGAC) was initially identified to be a high affinity binding site for Ski, screened by using nuclear extracts from c-Ski-transformed cells (11). However, Ski cannot bind to this sequence directly. Instead, Ski bound to this site along with certain unidentified cellular proteins (11). Shortly afterward, the same 8-bp palindromic sequence was found to be the consensus binding site for Smad3 and Smad4, identified by a PCR-based selection from random oligonucleotides using recombinant full-length Smad4 or the N-terminal domain of Smad3 (12). This led to the hypothesis that Ski may bind to the GTCTAGAC sequence indirectly through interaction with Smad4 or Smad3. Indeed, several independent studies on Smads subsequently found that Ski and the related SnoN interact with Smads (13–20). Ski and SnoN interaction with Smad4 is constitutive, whereas Ski and SnoN interact with Smad2 and Smad3 in a TGF-β-dependent manner (13–23). Ski and SnoN are corepressors for Smad-mediated transcription (1, 13–23). Ski/SnoN directly binds to the N-CoR and mSin3A, which form a complex with histone deacetylase (24). Ski may also inhibit TGF-β-induced transcriptional responses by competing with Smad2 and Smad3 binding to Smad4 (20, 25). In addition, Ski has also been reported to inhibit TGF-β signaling by suppressing Smad2 phosphorylation by the receptor (26). The transforming activity of Ski and SnoN is dependent on their ability to repress the activity of Smad proteins (27). In addition to Smads, Ski is also required for transcriptional repression by several other proteins, including the Mad repressor, the thyroid hormone receptor-β, the Rb protein, and the Gli3 repressor (19, 28). Thus, Ski appears to be an integral part of the cellular transcriptional repression machinery.

SnoN, and to a lesser extent Ski, is degraded upon TGF-β treatment (16, 17, 19, 20, 29–31). SnoN has been proposed as a nuclear corepressor for Smad4 to maintain TGF-β-responsive genes in a repressed state in the absence of ligand (16, 19). Similarly, Ski may also perform the same function. Additionally, SnoN mRNA is induced by TGF-β treatment (16, 19, 20), which may play a role in turning off the TGF-β signal.

We and others have previously cloned the Smad7 promoter (5–10). Interestingly, the Smad7 promoter represents the first natural promoter in vertebrates that contains the 8-base pair (GTCTAGAC) palindromic Smad-binding element (SBE). We and others have shown that the SBE is necessary for TGF-β induction of the Smad7 promoter (5–10). In this study, we show that Ski, recruited to the SBE by interacting with Smad4, inhibits the basal level of the endogenous Smad7 gene. Our studies provide the first evidence that Ski indeed is a corepressor of Smad4 to repress a natural TGF-β responsive gene at the basal state.

EXPERIMENTAL PROCEDURES

Transfection and Reporter Gene Assay—HaCaT, L17, HepG2, and SW 480.7 cells were transfected using DEAE-dextran (125 μg/ml) for 3–5 h and treated with or without 500 μM TGF-β for 18–24 h. HeLa cells...
were transfected using LipofectAMINE 2000. The Smad7-Luc (−251 to +32) reporter genes containing wild type or mutant Smad7 were used. Very similar results were obtained using the Smad7-Luc (−339 to +641) reporter gene. The results represent at least three independent transfection experiments.

RNAi—The RNAi constructs were built in the pSHAG-1 vector (32). The Ski RNAi #1 construct targets Ski mRNA starting at nucleotide 30. The sequences of the two oligonucleotides are as follows: TCTTCTGCAGGGCTGGGCTGGTGAGAAGCTGGTGCACTGTCGAGAGGAGCCGGGAGCTGAGAG (Smad7 promoter sequence 142 to 165) and AAAGACCAGAGACTCCCCTAAACC (Smad7 5′ UTR 692), served as negative controls. Both pairs amplified the corresponding fragment from the Smad7 plasmid by PCR and yielded essentially the same size PCR product as the primers that span the SBE.

RESULTS AND DISCUSSION

Mutation of the SBE Confers a Higher Basal Activity on the Smad7 Reporter Gene—As we and others have previously reported, the 8-base pair palindromic SBE is essential for TGIF-β induction of the Smad7 promoter (Fig. 1A). Interestingly, mutation of the SBE leads to a significantly higher basal transcriptional activity of the Smad7 reporter gene in HepG2 cells (Fig. 1A). This effect was also observed in a number of other cell types that we examined, such as HaCaT keratinocytes, this effect is modest (Fig. 1B), similar to our previous report (6), which may reflect very low levels of the Ski and SnoN proteins in HaCaT cells (data not shown). In HaCaT keratinocytes, this effect is modest (Fig. 1B), similar to our previous report (6), which may reflect very low levels of the Ski and SnoN proteins in HaCaT cells (data not shown). Overall, these observations suggest that certain factors bind to the 8-bp palindromic SBE and repress the basal transcriptional activity of the Smad7 promoter. When the SBE is mutated, the basal activity then increases because of derepression.

Ectopically Expressed Ski and SnoN Repress the Basal Transcriptional Activity of the Smad7 Reporter Gene in a SBE-dependent Manner—To identify the factors that inhibit the Smad7 basal level, we first asked whether overexpression of Ski or SnoN, which can be recruited to the SBE by Smads, can inhibit the Smad7 reporter construct. As shown in Fig. 2A, overexpression of Ski or SnoN inhibited the Smad7 basal level.
in a dose-dependent manner. When the SBE is mutated, the inhibitory effect of Ski or SnoN is abrogated. Both Ski and SnoN also inhibited TGF-β induction of the Smad7 promoter in a dose-dependent manner (data not shown). TG-interacting factor (TGIF), another Smad corepressor (34), can also inhibit the basal level of the Smad7 reporter gene in a dose-dependent manner, but this effect is independent of the SBE (Fig. 2B).

Conversely, as we reported previously (35), overexpression of PIASy, which can inhibit Smad3 transcriptional activity (35, 36), had little effect on the basal or TGF-β induced level of the Smad7 promoter (35). Thus, inhibition of the Smad7 promoter is a specific feature of the Ski and SnoN proteins. Ski as well as SnoN potentially could be involved in maintaining the Smad7 promoter in a repressed state in the absence of TGF-β signaling.

**Endogenous Ski Together with Smad4 Is Associated with the Smad7 Promoter in the Basal State**—To determine whether endogenous Ski binds to the Smad7 promoter in the natural setting, we performed ChIP assays using HepG2 cells treated with or without TGF-β. As shown in Fig. 3A, Ski was found to bind to the endogenous Smad7 promoter in a dose-dependent manner. Interestingly, overexpression of PIASy, which can inhibit Smad3 transcriptional activity, had little effect on the basal or TGF-β induced level of the Smad7 promoter (35). Thus, inhibition of the Smad7 promoter is a specific feature of the Ski and SnoN proteins. Ski as well as SnoN potentially could be involved in maintaining the Smad7 promoter in a repressed state in the absence of TGF-β signaling.

**Conclusions**

Endogenous Ski together with Smad4 is associated with the Smad7 promoter in the basal state, and this association is dependent on the presence of Smad4. These findings suggest a potential role for Ski and SnoN in the regulation of the Smad7 promoter in the absence of TGF-β signaling.
setting, we performed ChIP assays using HepG2 cells. Since the Ski family members do not possess DNA binding activities on their own but instead are presumably recruited to TGF-β-responsive promoters via interaction with Smad proteins, we also included antibodies against Smad4 or Smad2/3 in our ChIP experiments. As shown in Fig. 3, A and B, Ski and Smad4, but not Smad2/3, are bound to the Smad7 promoter at the basal state in the ChIP assay in a SBE-dependent manner. This is consistent with previous notions that among the Smads, only Ski constitutively interacts with Smad4 in the basal state (13). Upon TGF-β treatment, Smad3, and presumably to a significantly lesser extent, Smad2, also bound to the Smad7 promoter (Fig. 3A). Interestingly, Ski is still associated with the Smad7 promoter in the presence of TGF-β (Fig. 3A). This may reflect Ski association with DNA in a complex with Smad4 alone, in a complex with both Smad3 and Smad4, or in a mixture of both complexes. It is possible that Smad3-Smad4, Smad3-Ski, Smad4-Ski, and Smad3-Smad4-Ski complexes may coexist in TGF-β-treated cells and that the Smad3-Smad4 complex leads to the activation of target genes, including the Smad7 gene. Future studies will be required to resolve these complex issues.

To determine whether Ski is recruited by Smad4 to the Smad7 promoter, we performed the ChIP assay using SW480.7 cells, which do not express Smad4 protein. As shown in Fig. 3C, left, Ski had no detectable binding to the Smad7 promoter in the absence of Smad4. When retroviral Smad4 was introduced into the SW480.7 cells, Ski binding was then detected. Furthermore, this led to reduced mRNA level of Smad7 (Fig. 3C, right). It is noteworthy that when Smad4 is overexpressed at high
levels through transient LipofectAMINE-mediated transfection of large amounts of Smad4 plasmid, Smad4 can lead to the binding of not only Ski but also Sno3 to the Smad7 promoter in the absence of TGF-β signaling; the net effect is an increase of the Smad7 mRNA level (data not shown).

To provide further evidence that Ski is recruited by Smad4 to the Smad7 promoter, we asked whether the inhibitory effect of Ski is dependent on the presence of Smad4. As shown in Fig. 3D, overexpression of Ski does not inhibit the Smad7 reporter gene in SW480.7 cells unless Smad4 is cotransfected. Taken together, these experiments suggest that Ski, recruited to the endogenous Smad7 promoter by interaction with Smad4, represses Smad7 expression in the basal state.

Although overexpression of SnoN also inhibits Smad7 (Fig. 2A), we could not detect SnoN binding to the Smad7 promoter in the ChIP assay by using a few different SnoN antibodies (Fig. 3E and data not shown). It is possible that Ski and SnoN have overlapping as well as distinct promoter specificities in natural settings. It appears that Ski is better suited than SnoN to bind the endogenous Ski protein indeed represses the Smad7 promoter, we analyzed whether the endogenous Ski and/or SnoN repress the Smad7 promoter. What is the physiological significance of Ski repression of the Smad7 gene? Since Smad7 binds and inhibits TGF-β receptor phosphorylation of Smad2 and Smad3, the low level of Smad7 maintained by the repressive action of Ski may greatly facilitate, at least in the initial stage, propagation of the TGF-β signal.

Smad7 expression needs to be tightly regulated. Aberrant expression of Smad7 is involved in the pathology of several diseases. For example, Smad7 is overexpressed in inflammatory bowel disease mucosa and purified mucosal T cells. Both whole tissue and isolated cells exhibited defective TGF-β signaling (37). In addition, overexpression of Smad7 in transgenic mice results in severe pathological alterations in multiple epithelial tissues (38). Conversely, deficient Smad7 expression is causally linked to scleroderma (39). Decreased Smad7 expression also contributes to cardiac fibrosis in the infarcted rat heart (40). Thus, the appropriate Smad7 level is critical for balanced TGF-β activity, and deregulated Smad7 activity can lead to the development of disease.

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