Ski is a transcriptional co-repressor and is involved in the negative regulation of tumor growth factor-β (TGF-β) signaling. To understand more fully the role of Ski in TGF-β signaling, we searched for novel Ski-interacting proteins. The identified C184M protein consists of 189 amino acids and contains the leucine-rich region. An association between Ski and C184M involving the leucine-rich region of C184M and the C-terminal coiled-coil motif of Ski was confirmed by glutathione S-transferase pull-down and immunoprecipitation assays. The C184M protein is located in the cytosol, and the C184M and Ski signals are co-localized in the cytoplasm when C184M was co-expressed with Ski in CV-1 cells. The cytoplasmic C184M-Ski complex inhibited the nuclear translocation of Smad2. Consistent with this, the activity of promoter containing the Smad-binding sites was repressed by C184M, and the TGF-β-induced growth inhibition of mink lung Mv1Lu cells was attenuated by the ectopic expression of C184M. Thus, C184M inhibits TGF-β signaling in concert with Ski. In hepatocytes, which express significant levels of C184M, the Ski signals were found only in the cytoplasm, supporting the notion that C184M forms a complex with Ski in the cytosol.

Transforming growth factor-β (TGF-β) plays an important role in growth, differentiation, adhesion, and apoptosis (for review, see Ref. 1). TGF-β acts as a potent growth inhibitor for most types of cells. Secreted TGF-β binds to the TGF-β type II receptor, which phosphorylates the type I receptor. The activated type I receptor phosphorylates the receptor-regulated Smads (R-Smads), Smad2 and Smad3. Phospho-Smad2 or phospho-Smad3 then forms a complex with Smad4, known as the co-activator Mediator Smad (co-Smad), and migrates into the nucleus, where it regulates the transcription of target genes. In addition to the Smad-mediated pathway, an alternative pathway involving TGF-β-activated kinase (TAK-1) and TAK1-binding protein 1 (TAB1) also mediates TGF-β signaling (2). The TAB1-TAK1 pathway activates the mitogen-activated protein kinase cascade including p38 and c-Jun N-terminal kinase (4, 5). The mitogen-activated protein kinase cascade signals are transmitted to transcriptional regulators, such as ATF-2 and c-Jun (AP-1) that in concert with Smad3/4 induce transcriptional activation of some TGF-β-responsive genes (4–6).

To restrict and terminate the response to the TGF-β signal, the TGF-β signaling is negatively regulated by multiple mechanisms (for review, see Ref. 1). First, the inhibitory Smads (1-Smads), including Smad6 and Smad7, negatively regulate the TGF-β signaling (7–9). Because 1-Smads do not contain phosphorylation sites for TGF-β receptor I, when recruited to the activated type I receptor they do not dissociate from it, which prevents the association of R-Smads with the type I receptor. The expression of Smad7 is induced by the TGF-β stimulus (7, 8), indicating that Smad7 is involved in negative feedback regulation of TGF-β signaling. Second, Smad7 recruits the Smurf2 protein to the TGF-β receptor and induces the degradation of the receptor (10). Smurf2 is a member of the HECT-type ubiquitin-protein isopeptide ligase family, and induces the ubiquitination of the TGF-β receptor, which leads to the degradation of the receptor via the proteasomal- and lysosome-dependent pathways. The third mechanism is the negative regulation of the Smad-dependent transcriptional activation by co-repressors in the nucleus. The phosphorylated Smad2 and Smad3 in the nucleus bind to transcriptional co-repressors such as TGF (11) and members of the Ski protein family (12–15). The co-repressors compete out p300/CBP, the co-activator of Smad2/4 and Smad3/4, and recruit histone deacetylases to the target genes, leading to the inhibition of the Smad2/4- or Smad3/4-induced transcriptional activation.

The ski gene family was originally identified as oncogenes carried by the Sloan-Kettering virus (16). v-ski and c-ski induce transformation of chicken fibroblasts and muscle differentiation of quail embryo fibroblasts (17, 18). The ski gene family consists of two members, ski and sno (ski-related novel gene) (19). We have shown that the ski and sno gene products (Ski and Sno) act as co-repressors and bind with other co-repressors, N-CoR/SMRT and mSin3A (20). Ski and Sno recruit the histone deacetylase complex to the target promoters via multiple repressors, including Mad, retinoblastoma, thyroid hormone receptor-β, and MeCP2 (20–22). Although Ski and Sno directly bind to Smad2/3/4 and negatively regulate Smad-dependent transcriptional activation (12–15), Sno is rapidly degraded upon TGF-β stimulation (23, 24). Smad2 and Smad3 bind and recruit, respectively, two types of ubiquitin-protein isopeptide ligases, SmurF2 and anaphase-promoting complex/cyclosome, to the Sno protein, which leads to the ubiquitination...
Role of C184M in TGF-β Signaling

**Fig. 1.** The LR of C184M is required for binding to Ski. A, amino acid sequence of the C184M protein. Asterisks indicate the leucine residues that can form the leucine zipper motif. The dotted line above the sequence shows the hydrophobic (leucine-rich) region. The amino acids deleted in the splicing variant form of C184M (a putative MMTV receptor) are underlined. B, schematic representation of the C184M deletion proteins used. The results of the binding assays shown in D are indicated on the right. WT, wild type. C, analysis of the GST-Ski fusion protein containing full-length Ski. The GST-Ski fusion proteins bound to glutathione-Sepharose resin were analyzed by 10% SDS-PAGE followed by Coomassie Brilliant Blue staining. The captured proteins were analyzed by SDS-PAGE, followed by autoradiography. The relative binding activities of various forms of C184M are designated + and −, which indicate the relative binding efficiency to GST-Ski and GST is more than 20-fold and less than 5-fold, respectively. The amount of C184M protein in the input lane was 2.5% that used for the binding assay.

and proteasome-dependent degradation of Sno (25–27). The levels of Sno expression increase markedly 2 h after stimulation with TGF-β (23), indicating that Sno plays a role in the negative feedback regulation of the TGF-β signaling. In contrast, it is not clear whether the Ski protein is degraded upon TGF-β stimulation (24). At least Ski protein degradation does not play any role in the negative feedback regulation of the TGF-β signaling.

In the present study, we have identified a Ski-interacting protein, C184M. C184M induces the cytoplasmic accumulation of Ski, and the cytosolic C184M/Ski complex negatively regulates TGF-β signaling by inhibiting the nuclear translocation of Smad2.

**MATERIALS AND METHODS**

**Yeast Two-hybrid Screening and GST Pull-down Assay—** Yeast two-hybrid screening using a mouse embryonic cDNA library and GST pull-down assays were performed essentially as described previously (29, 22). K buffer (20 mM HEPES (pH 7.9), 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 0.02% skim milk, 50 μM dithiothreitol, 50 μM ZnCl₂, and 0.01% Nonidet P-40) was used for binding between GST-C184M and in vitro translated Ski. For the binding between GST-Ski and in vitro translated C184M, SK-B buffer (20 mM Tris-HCl (pH 8.5), 2.25 mM MgCl₂, 0.02% skim milk/PBS, cells were immunostained with anti-Ski (Santa Cruz Biotechnology) or normal mouse IgG (Santa Cruz Biotechnology) followed by serum. In some experiments, we used an anti-FLAG M2 antibody (Sigma) or normal mouse IgG (Santa Cruz Biotechnology) followed by Western blotting was carried out with an anti-Ski or anti-Sno monoclonal antibody. For the experiments described in Fig. 5, 293T cells were transfected with mixture of plasmids to express N-FLAG-pact-Smad4 (0.65 μg), Smad2 (0.65 μg), Smad5 (0.65 μg), Smad7 (0.65 μg), or SnoN (pact-SnoN) (2.75 μg) and the internal control plasmid β-galactosidase activity, immunoprecipitation was performed with anti-C184M antisera or control serum. In some experiments, we used an anti-FLAG M2 antibody (Sigma) or normal mouse IgG (Santa Cruz Biotechnology) followed by the addition of protein G-Sepharose (Amersham Biosciences). After washing the beads with Harlow-150 buffer (50 mM HEPES (pH 7.5), 0.2 mM EDTA, 10 μM NaCl, 0.5% Nonidet P-40, and 150 mM NaCl), the precipitated proteins were eluted with 2× SDS sample buffer, and Western blotting was carried out with an anti-Ski or anti-Sno monoclonal antibody. For the experiments described in Fig. 5, 293T cells were transfected with mixture of plasmids to express FLAG-C184M (N-FLAG-pact-C184M) (2.75 μg), Smad2 (0.65 μg), Smad4 (N-FLAG-pact-Smad4) (0.65 μg), and the internal control plasmid β-galactosidase (0.3 μg).

**Subcellular Localization of C184M, Ski, and Smads—** For the experiments described in Fig. 4, N-FLAG-pact-C184M (1.5 μg) and pact-Ski (1.5 μg) were transfected into CV-1 cells by the CaP₀₆ method. Forty eight hours after transfection, cells were fixed on cover glasses with 2% paraformaldehyde/PBS for 45 min at room temperature and permeabilized by 0.1% Triton X-100/PBS for 12 min. After blocking with 3% skim milk/PBS, cells were immunostained with anti-Ski (Santa Cruz Biotechnology, N-20 or H-329), anti-Sno (Santa Cruz Biotechnology, P-10), or anti-Gal (Santa Cruz Biotechnology, P-14).
H-317), and anti-FLAG M2 antibodies followed by appropriate secondary antibodies conjugated to Alexa488 or TRITC, and analyzed with laser confocal microscopy (Zeiss LSM510). For the experiments described in Fig. 5, the monoclonal anti-Ski, anti-C184M, and anti-Smad2 (Santa Cruz Biotechnology S-20) antibodies, and appropriate secondary antibodies conjugated to Alexa488, TRITC, or Cy5 were used. Cells were counterstained with Hoechst 33258. Preparations were observed with a BX50 microscope (Olympus), and captured images were deblurred by two-dimensional blind deconvolution with AutoDeblur software (Auto-Quant Imaging Inc.).

Growth Properties of Mv1Lu Cells Stably Expressing C184M—The C184M cDNA was inserted into the retrovirus vector pMSCV-IREs-neo (28). Retroviruses were produced in the FLYA13 packaging cell line as described previously (28). Mv1Lu cells were infected with this virus and maintained in 10% fetal calf serum/Dulbecco’s modified Eagle’s medium containing 600 μg/ml G418. G418-resistant cells were expanded in 96-well plates. To measure the growth rate of each cell line, cells (3 × 10^5/well in a 96-well plate) were incubated with various concentrations of TGF-β1 (Sigma) for 24 h, and BrdUrd incorporation was measured using the Cell Proliferation enzyme-linked immunosorbent assay system (Amersham Biosciences) in accordance with the manufacturer’s instructions.

Luciferase Reporter Assay—For the experiments described in Fig. 6C, a mixture of 3TP-Lux (50 ng) and the internal control phRL-TK(int) (Promega; 10 ng) was transfected into the stable Mv1Lu cell lines, 1-2 a mixture of 3TP-Lux (50 ng) and the internal control phRL-TK(int) of TGF-

luciferase assay system (Promega). With 24 h. Firefly luciferase activity was measured together with Renilla luciferase activity (for an internal control) using a dual luciferase assay system (Promega).

Western Blotting—CV-1 cells (5 × 10^5 cells/10-cm dish) were transfected with a mixture of N-FLAG-pact-Ski or SnoN (2 μg), N-FLAG-pact-C184M (8.55 μg), and pact-β-galactosidase (0.75 μg) using LipofectAMINE and incubated for 40 h. Cytoplasmic and nuclear extract were prepared as described previously (30). Nuclear contamination of the cytoplasmic extracts was not significant since we could not detect the nuclear protein, a typical nuclear protein, in Western blotting using the cytoplasmic extract (data not shown).

Histological Analysis—Deparaffinized sections of C57BL/6 mouse liver were stained with an anti-C184M polyclonal antibody (1:50) or a mixture of the anti-Ski monoclonal antibodies 1-1, 9-1, 11-1, and 16-1 (1:50) as described previously (31).

RESULTS

Identification of C184M Protein as a Ski-interacting Protein—To search for novel Ski-binding proteins, we performed a yeast two-hybrid screening as described previously (20) using the full-length Ski as the bait and the mouse embryonic cDNA library. From this screen, we identified clones encoding the C184M protein. The c184m gene was originally identified as a gene whose expression is induced during mouse brain development (32). The C184M protein is a small protein consisting of 189 amino acids and contains a hydrophobic amino acid-rich region with a putative leucine zipper motif (leucine-rich region (LR)) (Fig. 1A). A splicing variant of C184M has also been reported to be a putative receptor for the mouse mammary tumor virus (MMTV) (33); this variant does not contain the LR (Fig. 1A and see “Discussion”). To examine the direct interaction between C184M and Ski, we carried out a GST pull-down assay. The in vitro translated C184M protein bound to a GST-Ski fusion protein (Fig. 1, B–D). To identify which region of C184M interacts with Ski, we used deletion mutants of C184M. A small fragment of C184M containing only the LR interacted with GST-Ski, but a ΔLR mutant that lacks the LR did not. Some mutants in which the leucines in the LR were mutated to prolines or alanines exhibited the impaired binding to GST-Ski (data not shown).

To identify the specific region of the Ski protein that interacts with C184M, we performed similar GST pull-down assays using various forms of in vitro translated Ski and a GST-C184M fusion protein (Fig. 2). The full-length Ski protein binds to GST-C184M, and a C-terminal fragment containing the coiled-coil region (residues 556–728) also bound (Fig. 2C). A mutant lacking the C-terminal coiled-coil region (Δ493–728) did not bind to GST-C184M.

In Vivo Interaction between Ski and C184M—To confirm further the interaction between Ski and C184M, we carried out co-immunoprecipitation assays. The expression vectors for both Ski and FLAG-linked C184M were transfected into 293T cells, and whole-cell lysates were prepared for co-immunoprecipitation. The full-length Ski protein was co-precipitated with wild-type C184M but not with the ΔLR mutant (Fig. 3A). The Δ493–728 mutant, which lacks the coiled-coil region of Ski, was not immunoprecipitated with wild-type C184M protein (Fig. 3A). When we used SnoN instead of Ski, SnoN did not co-immunoprecipitate with C184M. Lysates were prepared from 293T cells that were transfected with the expression vectors shown above. Immunoprecipitation was performed with an anti-FLAG antibody or control IgG followed by Western blotting with an anti-Ski (left panel) or anti-SnoN antibody (right panel).

Fig. 3. In vivo association between C184M and Ski. A, co-immunoprecipitation assays. 293T cells were transfected with the Ski and FLAG-linked C184M expression vectors shown above. Whole-cell lysates were subjected to immunoprecipitation (IP) using anti-C184M antibody or the control IgG. The immunocomplexes were analyzed by Western blotting with the anti-Ski or anti-FLAG antibody. In lanes 1, 4, and 7, 20% of the lysates used for immunoprecipitation was directly used for Western blotting. WT, wild type. B, SnoN does not co-immunoprecipitate with C184M. Lysates were prepared from 293T cells that were transfected with the expression vectors shown above. Immunoprecipitation was performed with an anti-FLAG antibody or control IgG followed by Western blotting with an anti-Ski (left panel) or anti-SnoN antibody (right panel).
The ΔLR mutant of C184M alone localized in both the cytoplasm and nucleus in all cells (Fig. 4E). When wild-type Ski was co-expressed with wild-type C184M, the Ski signals were mainly seen in the cytoplasm (Fig. 4F). The Ski signals partially co-localized with C184M signals (Fig. 4F–3). We observed that the intensity of the Ski signals increased with co-expression of C184M, suggesting that C184M not only blocks the nuclear entry of Ski but also increases the levels of cytoplasmic Ski protein (see below).

To confirm the up-regulation of Ski by C184M, we performed Western blotting analysis. The HA-Ski or HA-SnoN expression vector was transfected into CV-1 cells with the C184M expression vector or the control empty vector, and the levels of Ski or SnoN were examined by Western blotting. When Ski was co-expressed with C184M in CV-1 cells, the amounts of Ski in the whole-cell lysates increased (Fig. 4J). This was specific for Ski, because the Sno protein levels were not affected by C184M when similar experiments were performed using the Sno expression vector. When we analyzed the cytoplasmic and nuclear fractions separately, the levels of cytoplasmic Ski were seen to be greatly enhanced by C184M (Fig. 4K). This result is consistent with that of the immunostaining (Fig. 4F–1). The amount of Ski protein in the nuclear extract did not change noticeably (Fig. 4K). The similar cytoplasmic accumulation of Ski by C184M was observed in 293T and Mv1Lu cells (data not shown).

The Cytoplasmic C184M-Ski Complex Inhibits the Nuclear Translocation of Smad2—Because the Ski protein has been reported to bind to Smad2 and Smad4 (12–15), it is possible that the C184M-Ski complex in the cytoplasm prevents the translocation of Smad2 into the nucleus. To test this possibility, we performed immunostaining experiments and investigated the localization of Smad2 in the presence and absence of C184M and Ski. When Smad2 and Smad4 expression vectors were co-transfected into CV-1 cells together with ALK5α (a constitutively active form of TIRβ), Smad2 was condensed in the nucleus (Fig. 5A–2). When C184M was co-expressed with either C184M or Ski, the localization of Smad2 was not affected, and Smad2 was condensed in the nucleus (Fig. 5B–1–4 and Fig. 5C–1–4). When Smad2/4 was co-expressed with both C184M and Ski, Smad2 accumulated largely in the cytoplasm despite the presence of ALK5α (Fig. 5D1). In this case, both C184M and Ski signals were also present in the cytoplasm.
Smad2 appeared to co-localize with C184M and Ski (Fig. 5D–5). These results suggest that the cytoplasmic C184M-Ski complex inhibits the nuclear translocation of Smad2. The association between Samd2 and C184M appeared to be mediated by the Ski protein, because Smad2 did not bind to GST-C184M in the GST pull-down assay (data not shown).

To confirm further the interaction between Smad2 and C184M via Ski, we carried out co-immunoprecipitation assays. The expression vectors for both FLAG-Smad2/4 and C184M were transfected into 293T cells together with the HA-Ski expression vector or the control empty vector, and whole-cell lysates were prepared for co-immunoprecipitation. When Ski was co-expressed, the anti-C184M antibody efficiently co-precipitated the Smad2/4 proteins both in the presence and absence of ALK5 (Fig. 5E). In contrast, when Ski was not expressed, Smad2/4 was not co-precipitated with C184M at all in the absence of ALK5*, and only a small amount of Smad2/4 was co-precipitated in the presence of ALK5*. Thus, Smad2/4 is co-precipitated with C184M in the presence but not in the absence of Ski.

C184M Negatively Regulates TGF-β Signaling—Ski is known to be a negative regulator of TGF-β signaling that antagonizes TGF-β-mediated growth inhibition (12–15). Therefore, C184M, which binds to Ski, might play some role in the regulation of TGF-β signaling. To explore this possibility, we generated stable mink lung epithelial cell (Mv1Lu) lines that expressed different levels of C184M protein (Fig. 6A), and we measured BrdUrd incorporation to investigate their responsiveness to TGF-β (Fig. 6B). TGF-β1 at various concentrations suppressed the growth of the control cell line harboring the


empty vector (clone 1-2). The TGF-β1-induced growth inhibition was completely or partly impaired in cell lines expressing wild-type C184M (2-8, 2-11, and 2-15 in Fig. 6 B). Interestingly, the degree to which TGF-β1-induced growth inhibition was abrogated correlated with the level of C184M expression. By using the Mv1Lu cell clones, BrdUrd incorporation was measured in the presence of various concentrations of TGF-β1. The average of three independent experiments is shown. C.184M inhibits the TGF-β1-induced transcriptional activation. The Mv1Lu cell clones were transfected with the 3TP-Lux reporter, and luciferase assays were performed. Open, black, and gray bars represent the data with clones 1-2, 2-8, and 3-18, respectively. The average of three experiments is indicated with the standard deviation.

The TGF-β signals are mediated mainly by Smad proteins. Therefore, we carried out luciferase assays to test whether C184M represses the trans-activation of transcription by Smad2 and Smad4. We used the 3TP-Lux reporter, a fusion promoter consisting of the PAI-1 and collagenase promoters that is activated by Smad2/4 or Smad3/4 and ATF-2 (4). The stable Mv1Lu cell lines expressing exogenous C184M were transfected with the 3TP-Lux reporter, and the luciferase activity was measured. In the TGF-β1-treated clone 2-8, which expresses ectopic wild-type 184M protein, the luciferase expression levels from the 3TP-Lux reporter were lower than that of the control clones harboring the empty vector (clone 1-2) or expressing the ΔLR protein (clone 3-18) (Fig. 6 C).

Expression of c184m mRNA Is Induced in Response to TGF-β Signal—Because C184M negatively regulates TGF-β signaling, we examined whether the expression of C184M is enhanced by stimulation with TGF-β. We examined the c184m mRNA level by Northern blotting. Mv1Lu cells were treated with 200 pM TGF-β1 for the various times indicated above, and Northern blotting was performed. β-Actin mRNA was examined as the control (lower panel). Asterisk indicates the non-specific band corresponding to 28S ribosomal RNA. B, cytoplasmic localization of Ski and C184M in mouse liver. Deparfaffinized sections of C57BL/6 mouse liver were stained with anti-C184M and anti-Ski antibodies. C184M is expressed in the cytosol and nuclei of hepatic cells, whereas Ski is expressed mainly in the cytosol.

DISCUSSION

In this study we have identified the Ski-interacting protein, C184M, that acts with Ski as a negative regulator of TGF-β signaling.

The c184m gene was originally identified as a gene whose expression was enhanced in developing mouse brain (32), but its function has not been known. The splicing variant of C184M, which lacks the LR (Fig. 1 A), was reported to be a putative cellular receptor for MMTV (33); however, a data base search of mouse ESTs of C184M suggests that this splicing variant is a minor transcript compared with full-length C184M. This is supported by the fact that we could not amplify the cDNA fragment encoding this variant by reverse transcriptase-PCR using mouse brain mRNA (data not shown). The functional
relationships between the MMTV receptor and the Ski-binding C184M protein are unclear. However, we have observed that the ΔLR mutant of C184M enhances the formation of microfilaments (F-actin), whereas wild-type C184M reduces their formation (data not shown). Similar observations were obtained when we examined microfilaments in a stable clone of Mv1Lu cells expressing wild-type or ΔLR C184M (data not shown).

Therefore, one possibility is that overexpression of the splice variant of C184M regulates cytoskeletal organization, which then leads to increased endocytosis of MMTV.

Although the Ski proteins are localized in the nucleus when they are overexpressed alone in cultured cells, we found that Ski is mainly localized in the cytoplasm of hepatocytes that also express significant levels of C184M (Fig. 7B). Smad proteins are already activated to some extent in the quiescent liver, although they are further activated during liver regeneration (35).

Therefore, one possibility is that C184M and Ski play a role in keeping the Smad activity at a low level to maintain hepatocytes in the G0 phase. Recently, Reed et al. (34) reported that Ski localizes mainly to the cytoplasm in human preinvasive melanoma cells and that nuclear translocation of Smad3 is inhibited in these cells. Therefore, it is possible that C184M is involved in the cytoplasmic localization of Ski in melanomas, although it is unknown whether C184M is expressed in these melanoma cells. Recently, Grimm and Gurdon (36) showed that the loss of mesodermal competence during the early development of Xenopus laevis is controlled by the nuclear exclusion of Smad2. Thus, the cytosolic retention of Smad proteins may be a common mechanism for the negative regulation of TGF-β signaling, and C184M might be involved in this regulation.

Ski has been reported to interact with Smad1–4 to repress the signaling by the TGF-β superfamily, including bone morphogenetic protein and activin (12–15, 37). Therefore, the cytoplasmic Ski-C184M complex could interact with Smad1 and Smad3 in addition to Smad2. In fact, we have observed that C184M inhibits the nuclear translocation of Smad3 in CV-1 cells, similar to what we see with Smad2, and also inhibits the Smad3/4-induced trans-activation (data not shown). Thus, the C184M-Ski complex targets Smad3 as well as Smad2 to repress Smad3/4-induced trans-activation (data not shown). Thus, the role of C184M in bone morphogenetic protein signaling remains to be elucidated, it is possible that C184M is involved in this regulation.
The Ski-binding Protein C184M Negatively Regulates Tumor Growth Factor-β Signaling by Sequestering the Smad Proteins in the Cytoplasm
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