The loss of growth-inhibitory responses to transforming growth factor-β (TGF-β) is a frequent consequence of malignant transformation. Smad2, Smad3, and Smad4 proteins are important mediators of the antiproliferative responses to TGF-β and may become inactivated in some human cancers. Epithelial cells harboring oncogenic Ras mutations often exhibit a loss of TGF-β antiproliferative responses. To further investigate the effect of oncogenic Ras in TGF-β signaling, we used an isopropyl-1-thio-β-D-galactopyranoside-inducible expression system to express Ha-RasVal-12 in intestinal epithelial cells. Induction of Ha-RasVal-12 caused a decrease in the level of Smad4 expression, inhibited TGF-β-induced complex formation between Smad2/Smad3 and Smad4, blocked Smad4 nuclear translocation, inhibited the TGF-β-mediated decrease in [3H]thymidine incorporation, and repressed TGF-β-activated transcriptional responses. The withdrawal of isopropyl-1-thio-β-D-galactopyranoside or the addition of an inhibitor of the ubiquitin-proteasome pathway restored the Smad4 level and TGF-β-induced Smad complex formation. Forced expression of Smad4 resulted in partial recovery of the TGF-β-mediated growth inhibition and transcriptional responses in the presence of oncogenic Ras. Further, PD98059, a specific inhibitor of the MEK/ERK/mitogen-activated protein kinase pathway prevented the Ras-induced decrease in Smad4 expression and complex formation. Our results suggest a novel mechanism by which oncogenic Ras represses TGF-β signaling by mitogen-activated protein kinase-dependent down-regulation of Smad4, thereby subverting the tumor suppressor function of TGF-β.

Transforming growth factor-β (TGF-β), the prototypic member of a superfamily of polypeptides, inhibits the proliferation of normal epithelial, endothelial, and hematopoietic cells and thus is important for homeostasis of these tissues. Smad proteins play a key role in the intracellular signaling of the TGF-β family of extracellular polypeptides that initiate signaling from the cell surface through serine/threonine kinase receptors. Binding of the ligand to TGF-β type II receptor results in the recruitment and phosphorylation of the type I receptor, which then phosphorylates Smad2 and Smad3. The phosphorylated Smads form complexes with Smad4 and translocate to the nucleus, where they activate transcription of target genes through cooperative interactions with DNA, transcription factors, coactivators, and corepressors. Inactivating mutations in the components of the TGF-β signal transduction pathway are found in a number of human cancers including colorectal and pancreatic carcinomas that harbor mutations in TGF-β type II receptor, Smad2, or Smad4.

The molecular events required for transforming a normal colon epithelium to an adenomatous polyp and finally to an invasive colon cancer involve the sequential mutation of specific genes. Inactivating mutations in the adenomatous polyposis coli gene and activating mutations in the Ki-ras gene occur early, whereas Smad4 and p53 inactivation occurs generally later during the progression from adenoma to carcinoma in the colon. Smad4 germ line mutations have been identified in juvenile polyposis, which is associated with the increased risk of colorectal cancer. It has been observed that both Smad4 heterozygous and Smad4/adenomatous polyposis coli double-mutant heterozygous mice develop invasive carcinomas (9, 10). Further, Smad4 mutations are relatively infrequent in colorectal adenomas and nonmetastatic carcinomas (11) but are present in more than 30% of invasive metastatic colorectal cancers (12) suggesting that loss of Smad4 function contributes to tumor progression rather than tumor initiation.

TGF-β can override the proliferative effects of Ras-activating mitogens including epidermal growth factor in normal epithelial cells; however, cells transformed by oncogenic Ras often exhibit loss of antiproliferative responses to TGF-β. Activating mutations of the Ki-ras gene occur with high frequency during the progression from adenoma to carcinoma in the colon and probably contribute to tumor progression (7). Phosphorylation in the linker region of Smad3 by activated Ras inhibits TGF-β/Smad-mediated transcriptional responses, Smad nuclear translocation, and growth inhibition (13). However, another report has shown that hepatocyte growth factor/epidermal growth factor-mediated MEK1 activation induces phosphorylation, nuclear translocation and transcriptional activation by Smad2/4 (14). In contrast, other studies have found that constitutively active MEK, or inhibitors for MEK, had no effect on Smad activity in human keratinocytes (15) or that blockade of Smad4 in transformed keratinocytes containing oncogenic Ras leads to hyperactivation of the Ras-dependent ERK signaling pathway (16).

To better understand the mechanism by which oncogenic Ras modulates TGF-β/Smad signaling, we have investigated the
effect of activated Ha-Ras on TGF-β signaling by using an inducible system. Upon induction of onecogenic Ras, TGF-β-mediated Smad4 complex formation and transcriptional responses were markedly inhibited. These responses correlated with decreased steady-state levels of Smad4 due to degradation of endogenous Smad4 protein. Reversal of Ras activation or the addition of ubiquitin-proteasome inhibitors prevented the degradation of Smad4 and restored the Smad4 complex formation with Smad2 and Smad3. Furthermore, overexpression of Smad4 largely restored TGF-β-mediated transcriptional responses and TGF-β-mediated growth inhibition in the presence of oncogenic Ras. Our studies demonstrate a mechanism of repression of TGF-β signaling by oncogenic Ras that may contribute to cellular transformation.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Lines—**The RIE:iRas cells with an inducible activated Ha-Rasα12V1-12 cDNA as described previously (17) were treated with IPTG (Life Technologies, Inc.) at 5 mM to induce the expression of Ha-Rasα12V1-12. For the IPTG withdrawal study, cells were treated with IPTG for 72 h, subcultured, and grown in the regular DMEM containing 10% serum (−IPTG).

**Immunoprecipitation and Western Blot Analysis—**Cells were lysed at intervals using Nonidet P-40 lysis buffer containing 50 mM Tris-Cl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 10 mg/ml phenylmethylsulfonyl fluoride, 20 μg/ml FOY-305 (protease inhibitor, Ono Pharmaceutical Co., Osaka, Japan), 1 mM sodium orthovanadate, and 5 mM sodium fluoride. Smad2 and -3 polyclonal antibodies (Zymed Laboratories Inc.) were added to 500 μg of lysates and incubated at 4 °C for 2 h. Protein G plus agarose beads (Santa Cruz Biotechnology) were added to the antigen-antibody mixture and further incubated overnight. Beads were washed thoroughly (4 times) with phosphate-buffered saline containing 0.1% Nonidet P-40 and 0.1% SDS and subjected to Western blot analysis using anti-human Smad4 monoclonal antibody (SC 7966, Santa Cruz Biotechnology), Pan-Ras monoclonal antibodies (Calbiochem), β-actin monoclonal antibodies (Sigma), and phospho-Smad2 antibodies from Upstate Biotechnology (Lake Placid, NY) were used for immunoblotting. In some experiments a ubiquitin-proteasome inhibitor, ALLN (Sigma), and lactacycin (Calbiochem) at 5 μM were added in the presence or absence of IPTG, and selective kinase inhibitors, such as PD98059 (MEK1 inhibitor, 10 μM, Calbiochem) and SB203580 (p38 MAPK inhibitor, 5 μM, Calbiochem), were also used in this study along with IPTG.

**RNA Extraction and Northern Blot Analysis—**The extraction of total cellular RNA at different time points (+/−IPTG) was performed using Tri-Reagent (Molecular Research Center). RNA samples (20 μg) were separated on a formaldehyde-agarose gel and blotted onto a nitrocellulose membrane. The full-length Smad4 cDNA labeled with [α-32P]dCTP by using Rediprime labeling kit (Amersham Pharmacia Biotech). Cyclophilin A message (1B15) was used for equality of loading.

**Metabolic Labeling—**RIE:iRas cells were treated with (+/−)IPTG for 60 h and were labeled with 150 μCi/ml [35S]methionine (Tran35S-label; ICN) for 5 h, and then washed three times with DMEM. Fresh DMEM containing 2 μM unlabeled methionine was added to the culture, and cell lysates were collected at the indicated time points. An equal amount of protein (600 μg) from each sample was immunoprecipitated with anti-Smad4 monoclonal antibody-protein G agarose. The immunoprecipitates were then resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The intensity of the bands was quantitated by densitometry (alpha-Imager).

**Transcriptional Response Assays—**RIE:iRas cells were transiently transfected (+/−)IPTG for 48 h with HA-Smad4 (0.5 and 1 μg), p3TP-Lux (0.7 μg), and pcMV β-gal (0.3 μg) using Superfect (Qiagen). An equal amount of total DNA was used in transfection for each experiment. Twenty-four hours after transfection, cells were treated with or without TGF-β1 (200 pM) for a further 24 h in the medium containing 0.2% fetal bovine serum (+/-IPTG). Cells were then harvested and β-galactosidase activity was measured in an Analytical Luminescence Lab Monolight 2010 luminometer. Luciferase activity was normalized to β-galactosidase activity for differences in the transfection efficiency.

**Nuclear Localization of Smad4—**RIE:iRas cells were treated with (+/−)IPTG for 72 h and 30 min with TGF-β1 and fixed in 4% paraformaldehyde for 10 min. Nuclear translocation of Smad4 was visualized by immunofluorescence using Smad4 monoclonal antibodies (SC-7966) and CY-3 conjugated goat antinouse IgG (Sigma).

**TGF-β-mediated [3H]Thymidine Incorporation and Oncogenic Ras Induction—**[3H]Thymidine assays were performed in triplicate. Cells were seeded in 24-well plates and incubated with (+/−)IPTG for 24–72 h. In some experiments adenoviral constructs of either FLAG-Smad4 or Smad2 and Smad3 were added at the multiplicity of infection of 20 with (+/−)IPTG. TGF-β was added for the final 22 h followed by a 2-h incubation with 1 μCi/ml [3H]thymidine (ICN). The radioactivity incorporated into the trichloroacetic acid material was counted by a liquid scintillation counter.

**RESULTS**

Smad4 plays a central role in TGF-β signaling, and its functional inactivation may result in resistance to TGF-β antiproliferative responses. To investigate whether Smad4 is a target of oncogenic Ras in intestinal epithelial cells, an IPTG-inducible vector was used to express Ha-Rasα12V1-12 in RIE-1 cells (RIE:iRas) (17). We observed the increasing expression of oncogenic Ras after treating the cells with IPTG at different time points (Fig. 1A). To determine whether Ras expression alters Smad4 levels, Western blot analysis was performed on cell lysates after treatment with IPTG as shown in Fig. 1B. Endogenous Smad4 was readily detectable, and the level remained unchanged over the period of 72 h of culture in the absence of Ras induction. However, a decrease in the Smad4 level was observed after 24 h with the maximum decrement by 72 h after IPTG treatment. This decrease in Smad4 level was associated with the concomitant increase in the level of a smaller polypeptide, designated Smad4*, which was recognized by the anti-Smad4 monoclonal antibody. No change was observed in the level of Smad4 when RIE-1 parental cells were treated with IPTG (data not shown). Both Smad4* and the full-length Smad4, recognized by anti-Smad4 monoclonal antibody, were also observed in RIE-1 cells stably transfected with Ha-Rasα12V1-12 (RIE-Ha-Ras). In contrast Smad4* was not detected in nontransformed mink lung epithelial or parental RIE-1 cells, but the full-length endogenous Smad4 was abundant (Fig. 1C). No Smad4 expression was detected in SW480.7 colon cancer cells as reported previously (18). With longer exposure additional degradation products smaller than Smad4* were also observed in the IPTG-treated RIE:iRas cells (Fig. 1D). These data suggest that oncogenic Ras causes down-regulation of Smad4 and that the smaller protein, Smad4*, is likely to be a degradation fragment of Smad4 that is recognized by the anti-Smad4 monoclonal antibodies.

The complex formation between Smad2/3 and Smad4 in response to TGF-β is critical for TGF-β-Smad signaling. To investigate whether oncogenic Ras-mediated down-regulation of Smad4 has an effect on the complex formation between Smad2/3 and Smad4, the RIE:iRas cells were treated with or without IPTG either in the absence or presence of TGF-β. Cell lysates were subjected to immunoprecipitation with anti-Smad4 antibody, and each immunoprecipitate was probed with antibodies to Smad4 (Fig. 1E, top panel). At each time point TGF-β induced the association between Smad2/3 with Smad4 both in the presence and absence of IPTG, as expected. However, the amount of coprecipitated Smad4 was reduced significantly with the time of IPTG treatment, and interestingly the Smad4* fragment was also co-precipitated with Smad2/3 during the induction of oncogenic Ras. No change in the level of Smad4 co-precipitated with Smad2/3 was detected in cells that were not treated with IPTG. Ras activation did not inhibit TGF-β-induced accumulation of carboxyl-terminal phosphorylated Smad2 nor did it alter the steady-state levels of Smad2 or Smad3 (Fig. 1E). To determine whether oncogenic Ras can regulate Smad4 mRNA levels, Northern blot analysis was performed after treating RIE-iRas cells with or without IPTG. The Smad4 mRNA level was not altered by Ras activa-
Analyzed. 

Expression in mink, RIE-1, RIE-Ha-Ras, RIE:iRas, and SW 480.7 cells was monitored using monoclonal antibodies and provided as loading control.

C

2

IPTG for 0–72 h and then exposed to TGF- 

b

plex formation in RIE:iRas cells. RIE:iRas cells were treated with 

2

IPTG.

I

anti-human Smad4 monoclonal antibodies.

D

Smad4 expression. The same membrane was probed with anti-

12

Smad4* band was abundant after 72 h of IPTG treatment (Fig. 2

A

), indicating that the decrease in the Smad4 protein level was not the result of a decrease in Smad4 mRNA. Taken together, these results suggest that the inhibition of the complex formation between Smad2/3 and Smad4 in response to TGF- 

b

is due to the down-regulation of Smad4 by oncogenic Ras. Further, these results suggest that the smaller protein (Smad4*) can still associate with Smad2/3 in response to oncogenic Ras.

RIE:iRas cells exhibit a morphological transformation between 24 and 48 h after IPTG treatment, and the transformation of these cells could be completely reversed upon withdrawal of IPTG for 72 h (17). The reversibility of Ras-induced down-regulation of Smad4 was examined after withdrawal of IPTG from transformed RIE:iRas cells. RIE:iRas cells were first treated with IPTG for 72 h, then IPTG was withdrawn, and cell lysates were obtained after 24, 48, 72, and 96 h. The full-length Smad4 protein was barely detectable whereas the Smad4* band was abundant after 72 h of IPTG treatment (Fig. 2B, lane1). By 48 h after withdrawal of IPTG, Smad4 expression was restored coincident with the disappearance of the smaller protein band. The increase in Smad4 inversely correlated with the level of oncogenic Ras. The TGF- 

b

-induced complex formation between Smad2/3 and Smad4 was restored along with restoration of the Smad4 level (Fig. 2C). These results demonstrate that the inhibition of complex formation between Smad2/3 and Smad4 occurs through oncogenic Ras-mediated down-regulation of Smad4 in the RIE:iRas cells.

To analyze the effect of oncogenic Ras on TGF- 

b

antiproliferative responses, a [3H]thymidine incorporation assay was performed after treating the RIE:iRas cells with or without IPTG in the presence of TGF- 

b

(Fig. 3A). TGF- 

b

inhibited [3H]thymidine incorporation by ~50% in the absence of IPTG throughout the entire period of study. Induction of activated Ras by IPTG reduced the degree of TGF- 

b

-mediated inhibition of DNA synthesis in a time-dependent manner. By 72 h after IPTG exposure TGF- 

b

inhibited [3H]thymidine incorporation by only 20% of control. However, upon withdrawal of IPTG, a partial rescue of TGF- 

b

-mediated inhibition in [3H]thymidine incorporation was observed. Significant partial recovery of TGF- 

b

-mediated inhibition in [3H]thymidine incorporation occurred when Smad4 was expressed by adenoviral FLAG.
Smad4 transduction in the presence of IPTG (Fig. 3B). Activation of Ras also resulted in a decreased level of exogenously expressed F-Smad4 as shown in Fig. 3C. The incomplete restoration of the TGF-β response may be due to the degradation of both the exogenous F-Smad4 and the endogenous Smad4 in the presence of activated Ras. This experiment confirms that activation of Ras causes the expected loss of TGF-β-growth-inhibitory responses in these cells. These results are consistent with the previously reported findings in cells that are constitutively transformed by activated Ha-Ras (13, 19–20).

Because steady-state levels of Smad4 were decreased after the activation of oncogenic Ras, but the mRNA levels remained unchanged, we examined the degradation rate of Smad4 in the presence and absence of Ras induction. Cells were treated with (+/-)-IPTG for 60 h and pulsed with [35S]methionine and then chased in the absence of exogenous Ras. The half-life of Smad4 in the absence of IPTG was ~3 h (t1/2 ~3 h), whereas the half-life of Smad4 in the presence of IPTG was ~3 h (t1/2 ~3 h). In addition, the steady state level of Smad4 in the absence of IPTG remained almost constant as compared with the decreased level in the presence of IPTG. These results suggest that Smad4 is less stable and undergoes more rapid degradation in the presence of activated Ras.

The majority of protein turnover outside the lysosomal compartment occurs via the 26 S proteasome by ubiquitin-dependent proteolysis (21). Missense mutation in the MH1 domains of Smad2 and Smad4 undergo rapid degradation through the ubiquitin-proteasome pathway (22). To confirm whether the Ras-mediated decrease in the endogenous Smad4 level is through the ubiquitin-dependent proteolysis, the effect of ALLN, an inhibitor of proteolysis by the 26 S proteasome, on IPTG and/or TGF-β-treated RIE:iRas cells was examined. ALLN had no effect on TGF-β-induced complex formation between Smad2/3 and Smad4 (Fig. 4A, top panel, lanes 5 and 6).

As described above, down-regulation of Smad4 expression and Smad4 complex formation by oncogenic Ras was accompanied
by an increase in the level of Smad4* (lane 7). However, Smad4 degradation and the decrease in Smad2/3 and Smad4 complex formation were prevented by treatment of the cells with ALLN in presence of IPTG (lane 8). The TGF-β-induced carboxyl-terminal phosphorylation of Smad2 and the relative expression levels of Smad2 or Smad3 remained unchanged under identical experimental conditions (Fig. 5A). In a similar experiment, lactacystin, a highly specific inhibitor of the 26S proteasome, was used (25). Addition of lactacystin restored the level of Smad4* (lane 8) for the final 20 h along with the IPTG in 0.2% serum-containing medium. Cell lysates were analyzed for luciferase activity and were normalized to the β-galactosidase activity. Lower panel, RIE:iRas cells were transiently transfected with HA-Smad4 in the presence or absence of IPTG. After 48 h, cells were lysed and immunoprecipitated with anti-HA antibody, and precipitates were analyzed with anti-Smad4 monoclonal antibodies.

Oncogenic Ras propagates a signal through the activation of protein kinases including the ERK-MAP kinases. Treatment of the cells with PD98059 (an inhibitor of the MEK/ERK pathway) completely prevented Ras-induced degradation of Smad4 and restored TGF-β-mediated complex formation between Smad4 and Smad2/3 (Fig. 4C, lanes 3, 4, 7, and 8). In contrast, SB203580 (an inhibitor of p38 MAPK activity) failed to inhibit Smad4 degradation and subsequent restoration of its complex formation (lanes 11 and 12). Another MEK inhibitor, U0126, showed similar results as PD98059 (data not shown). These results indicate that the MEK/ERK pathway is involved in Smad4 degradation after Ras activation.

The ability of Smads to regulate specific transcriptional programs in response to TGF-β requires nuclear translocation of Smad4 to functionally cooperate with other transcription factors (24). To test whether oncogenic Ras-mediated degradation of endogenous Smad4 can interfere with its nuclear accumulation, immunofluorescence microscopy was performed on RIE:iRas cells treated with or without IPTG and/or TGF-β. As expected, nuclear localization of Smad4 in response to TGF-β was observed in the absence of IPTG (Fig. 6A). However, nuclear accumulation of Smad4 in response to TGF-β was dramatically reduced from 89 to 29% after induction of oncogenic Ras by IPTG. This translocation of Smad4 to the nucleus was restored 72 h after withdrawal of IPTG, correlating with the restoration of complex formation between Smad4 and Smad2/3. These observations indicate that Ras activation causes degra-
dation of Smad4, resulting in the inhibition of Smad4 complex formation and decreased Smad4 nuclear translocation.

To investigate whether activated Ras-mediated inhibition in the nuclear accumulation of Smad4 has any functional consequences on transcriptional activation, RIE:iRas cells were cotransfected with p3TP-Lux reporter (25), which is highly responsive to TGF-β. Treatment of the cells with TGF-β enhanced the promoter activity in the RIE:iRas cells in the absence of IPTG (Fig. 6B, top panel). IPTG treatment of the cells significantly reduced the TGF-β-induced reporter activity. Cotransfection with Smad4 partially restored TGF-β-mediated induction in 3TP-Lux promoter activity in the presence of IPTG in a dose-dependent manner (Fig. 6B). Interestingly, the exogously expressed HA-tagged Smad4 levels were also decreased in the presence of activated Ras (Fig. 6B, bottom panel). Taken together, these results suggest a mechanism by which oncogenic Ras suppresses TGF-β transcriptional signaling and growth inhibition by targeting Smad4 for degradation.

**DISCUSSION**

Activating mutations of the Ki-ras gene occur with high frequency during the progression from adenoma to carcinoma in the colon and probably contribute to tumor progression (7). Cells harboring oncogenic Ras mutations often acquire a loss of TGF-β antiproliferative responses. Although previous data have suggested a mechanism for oncogenic Ras-mediated inhibition of TGF-β signaling by down-regulating Smad2/3 nuclear accumulation (13), they do not appear to fully explain the broad spectrum of the effects of activated Ras on TGF-β signaling. Ras and TGF-β interactions may contribute to the oncogenic transformation of epithelial cells. Here we have further investigated the molecular mechanism by which oncogenic Ras inhibits TGF-β signaling. Our results demonstrated that Smad4 was degraded in the presence of activated Ras through the MEK/ERK pathway. This decrease in Smad4 resulted in decreased complex formation between Smad2/3 and Smad4 and a decrease in Smad4 nuclear translocation. Reversal of activated Ras restored Smad4 levels and partially restored TGF-β growth-inhibitory responses. Exogenous expression of Smad4 largely restored the TGF-β-mediated growth inhibition and TGF-β-mediated transcriptional responses.

Previous studies have demonstrated a significant role for proteasome-mediated regulation of Smad proteins. Smurf2, a member of the HECT family of E3 ubiquitin ligase, was shown to target both Smad1 and Smad2 for ubiquitination and proteasome-mediated degradation (26, 27). Previous studies have shown that a missense mutation in the MH1 domains of Smad2 and Smad4 identified in some colorectal and pancreatic cancers results in rapid degradation of these mutant Smads through the ubiquitin-proteasome pathway (22). The half-life of Smad4 containing an arginine mutation in the MH1 domain was reported as 1.5 h as compared with the 6-h half-life of wild-type Smad4 when overexpressed in COS1 cells (22). In the present study we observed that the half-life of wild-type Smad4 was decreased from 8 to 3 h as a result of oncogenic Ras expression. Our results showing that the Ras-mediated decrease in Smad4 levels is abrogated by the treatment with ubiquitin-proteasome inhibitors suggest that the decrease in Smad4 is mediated by proteasomal degradation. Thus targeting of Smads for ubiquitin-mediated degradation may be a common mechanism whereby cancer cells abrogate the Smad tumor suppressor function. The appearance of the smaller molecular weight protein band (Smad4*) that is recognized by the anti-Smad4 monoclonal antibody after induction of Ras is consistent with the process of protein degradation, and this Smad4* also coprecipitates with Smad2/Smad3. Whether this fragment may have any dominant negative effect on TGF-β/Smad signaling is an interesting question that is currently under investigation.

We observed a near-complete degradation of endogenous Smad4 by 72 h after the induction of Ras. This degradation appears to be dependent on ERK/MAP kinase activation as suggested by the abrogation of this effect with two different MEK inhibitors. Smad4 functions as an inhibitor of Ras-dependent ERK signaling activity in Ras-transformed keratinocytes, and loss of Smad4 function in these cells results in hyperactivation of ERK signaling and progression to undifferentiated carcinomas (16). Thus, by causing Smad4 degradation, Ras activation may prevent the inhibitory modulation of the Ras/ERK signaling pathway. This may provide an autostimulatory mechanism that could result in excessive activation of Ras/MEK/MAP kinase and thereby promote a more aggressive malignant phenotype.

Previous reports have described that neoplastic transformation of intestinal, lung, liver, or mammary epithelial cells confers resistance to antiproliferative responses to TGF-β (28). We have shown that degradation of Smad4 by activation of Ras results in a dramatic decrease in the complex formation with Smad2/3, and subsequently translocation of Smad4 to the nucleus is inhibited. Smad signaling is essential for TGF-β-mediated growth inhibition (28). Our studies indicated that over-expression of Smad4 results in a significant recovery of TGF-β-mediated inhibition in [3H]thymidine incorporation and TGF-β-induced transcriptional responses despite the presence of the oncogenic Ras. This incomplete recovery of TGF-β responses may be due to the partial degradation of the exogously expressed Smad4 along with the endogenous Smad4 and/or as a result of the persistent inhibitory phosphorylation of Smad2/3 in the linker region as described previously by Kretzschmar et al. (13). In addition, transformation of the RIE-1 cells with the ras oncogene causes other effects such as an increase in cyclin D1 that may be sufficient to partially overcome TGF-β growth-inhibitory effects (29, 30). We conclude that oncogenic Ras may block Smad-dependent antiproliferative responses to TGF-β in epithelial cells by two different mechanisms: 1) by early termination of Smad signaling through Smad4 degradation (our data) and 2) by negative regulation of Smad2/3 through phosphorylation in the linker region (13).

**Acknowledgments**—We thank Brian Law and Harold L. Moses for adenoviral constructs of F-Smad4 and β-gal and Hongmiao Sheng and Mark De Cuestecker for helpful discussions.

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J. Biol. Chem. 2001, 276:29531-29537. doi: 10.1074/jbc.M100069200 originally published online May 22, 2001

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