AIP4 Restricts Transforming Growth Factor-β Signaling through a Ubiquitination-independent Mechanism*

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Smad7 functions as an intracellular antagonist in transforming growth factor-β (TGF-β) signaling. In addition to interacting stably with the activated TGF-β type I receptor (TβRI) to prevent phosphorylation of the receptor-regulated Smads (Smad2 and Smad3), Smad7 also induces degradation of the activated TβRI through association with different E3 ubiquitin ligases. Using the two-hybrid screen, we identified atrophin 1-interacting protein 4 (AIP4) as an E3 ubiquitin ligase that specifically targets Smad7 for ubiquitin-dependent degradation without affecting the turnover of the activated TβRI. Surprisingly, we found that despite the ability to degrade Smad7, AIP4 can inhibit TGF-β signaling, presumably by enhancing the association of Smad7 with the activated TβRI. Consistent with this notion, expression of a catalytic mutant of AIP4, which is unable to induce ubiquitination and degradation of Smad7, also stabilizes the TβRI-Smad7 complex, resulting in inhibition of TGF-β signaling. The ability of AIP4 to enhance the inhibitory function of Smad7 independent of its ubiquitin ligase activity reveals a new mechanism by which E3 ubiquitin ligases may function to turn off TGF-β signaling.

Transforming growth factor-β (TGF-β) is a member of a large family of cytokines that control a broad spectrum of biological processes, including proliferation, differentiation, apoptosis, and extracellular matrix production (1–3). TGF-β initiates signaling from the cell surface by contacting two distantly related transmembrane serine/threonine kinases called TGF-β type I receptor (TβRI) and TGF-β type II receptor. Upon ligand binding, the constitutively active TGF-β type II receptor phosphorylates and activates TβRI, leading to phosphorylation of Smad2 or Smad3 on two serines at the carboxyl terminus, within a highly conserved SSXS motif. Following phosphorylation, Smad2 and Smad3 associate with the shared partner Smad4 and translocate to the nucleus, where Smad complexes, in cooperation with coactivators, participate in transcriptional activation of target genes (1, 4, 5).

Because of its critical role in cell fate determination, TGF-β signaling is subject to many levels of positive and negative regulation, targeting both the receptors and intracellular mediators. For example, TGF-β signaling can be attenuated by the antagonistic Smad, Smad7, which acts by interfering with the activation of Smad2 and Smad3 (2, 6). The mechanism of Smad7-mediated repression of TGF-β signaling has been primarily attributed to the ability of Smad7 to form a stable association with the activated type I receptor, thereby preventing Smad2 and Smad3 from binding to and being phosphorylated by this receptor (7, 8). Recently, several studies have suggested that Smad7 might associate with the E3 ubiquitin ligases Smurf1, Smurf2, and Tiu1 to target the TGF-β receptors for ubiquitin-dependent degradation (9–11).

The ubiquitin conjugation system plays an important role in several biological processes, such as transcriptional regulation, signal transduction, and cell stress responses, and represents one of the most important degradation systems in the cell (12). Alterations in the activity of the ubiquitin system have been implicated in several human disorders, including malignant transformation and genetic diseases (13, 14). Ubiquitination of a protein substrate usually requires three processes involving the ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (12, 13, 15). A target protein must be tagged with a multiqubuitination chain composed of at least four ubiquitins before it can be recognized and degraded by the proteasome system. The specificity of protein ubiquitination is determined by E3 components, which function as adaptors that selectively bind substrates. There are two major classes of E3 ubiquitin ligases. One class carries a RING finger domain, and the other carries a homologous to E6-AP carboxyl terminus (HECT) domain. RING finger E3s function as adaptor proteins, bringing the substrate to the ubiquitin-charged E2, whereas E3s of the HECT domain family participate directly in catalysis by forming a thiolester with ubiquitin during the ubiquitination reaction (12, 13, 15).

Smurf1, Smurf2, and Tiu1 contain the distinctive structural features of the HECT subclass of E3 ubiquitin ligases (16). These features include a phospholipid/calcium-binding C2 domain; WW domains, which mediate protein-protein interactions by binding to PPXY (PY) motifs on partner proteins; and a carboxyl-terminal HECT catalytic domain. By interacting with the PY motif of Smad7, Smurf1, Smurf2, and Tiu1 can be recruited to TGF-β receptors to induce their degradation (9–11). Structure-function analyses have indicated that the ubiquitin ligase activity of Smurf1, Smurf2, and Tiu1 plays an important role in the ubiquitin-dependent degradation of...
TGF-β receptors/Smad7 complexes (9–11). The role of C2 domain in degradation is less clear. Deletion of this domain has no effect on Smad7 ubiquitination but causes a loss of ubiquitin-dependent degradation of the activated type I receptor by Smurf1 (17).

The biochemical and functional characterization of Smurf1, Smurf2, and Tiu1 as partners of Smad7 that target TGF-β receptors for ubiquitin-dependent degradation is yielding a basic view of how E3 ubiquitin ligases may function to turn off TGF-β signaling. Here we identify atrophin 1-interacting pro-tein 4 (AIP4), a member of the HECT subclass of E3 ubiquitin ligases and then immunoblotted with anti-FLAG (α-Flag) or anti-Myc (α-Myc) antibodies. Expression levels of proteins were determined by immunoblotting aliquots of total cell lysates.
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RESULTS

AIP4 Interacts with Smad7—To gain insight into the mechanism of action of Smad7, we performed a yeast two-hybrid screen using the entire mouse Smad7 as bait. By screening \(5 \times 10^6\) clones of a human placental cDNA expression library, we obtained 10 different cDNA species from 106 positive clones. AIP4 was one of the Smad7 interactors isolated, and it exhibited specific and strong binding to Smad7 in the yeast two-hybrid system (data not shown). AIP4 has significant homology to the HECT subclass E3 ubiquitin ligases, which selectively target protein for ubiquitination and degradation by the 26S proteasome (16). Other class E3 ubiquitin ligases include Smurf1, Smurf2, and Tiul1, which associate with Smad7 to target the TGF-β receptor for degradation (9–11, 21, 22). AIP4 shares with Smurf1, Smurf2, and Tiul1 distinctive structural features, including a phospholipid/calcium-binding C2 domain, WW protein-protein interaction domains, and a HECT domain that catalyzes ubiquitination on target proteins (Fig. 1A).

To confirm the interaction of AIP4 and Smad7 in mammalian cells, we expressed a FLAG epitope-tagged version of AIP4 in 293 cells together with a Myc-tagged version of Smad7. Immunoprecipitation of cell lysates with the anti-Myc antibody revealed the presence of FLAG-AIP4, which was absent in a control transfection in which only FLAG-AIP4 was expressed (Fig. 1B). In a reciprocal fashion, the association between AIP4 and Smad7 could also be demonstrated when cell lysates were subjected to immunoprecipitation with the anti-FLAG antibody directed toward AIP4 followed by immunoblotting with anti-Myc antibody for the presence of Smad7 (Fig. 1B). To test the specificity of the interaction of AIP4 with Smad7, 293 cells were transfected with FLAG-AIP4 together with Myc-tagged versions of Smad1, Smad2, Smad3, Smad4, Smad6, and Smad7. In immunoprecipitates of cells expressing either Smad6 or Smad7, efficient coprecipitation of either Smad with the reporter gene assay, cells were seeded into 6-well (35-mm) plates and transfected 24 h later with expression vectors by the Lipofectamine method, serum-starved for 18 h, preincubated for 1 h in Met/Cys-free Dulbecco’s modified Eagle’s medium, and pulsed for 10 min with 100 \(\mu\)g/ml aprotinin, and 2 \(\mu\)g/ml leupeptin) and lysed by sonication. In all cases, lysates were subjected to immunoprecipitation with the appropriate antibody for 2 h, followed by adsorption to Sepharose-coupled protein G for 1 h. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting. For determination of total protein levels, aliquots of cell lysates were subjected to direct immunoblotting. Proteins were electrophoretically transferred to nitrocellulose membranes and probed with the indicated primary antibody. The bands were visualized by an enhanced chemiluminescence detection system according to the manufacturer’s instructions (ECL; American Biosciences).

Pulse-chase Analysis—For experiments examining the metabolic stability of Smad7 or TβRI, 293 cells were transfected with expression vectors by the Lipofectamine method, serum-starved for 18 h, preincubated for 1 h in Met/Cys-free Dulbecco’s modified Eagle’s medium, and pulsed for 10 min with 100 \(\mu\)g/ml aprotinin (20 mM Tris (pH 7.5), 10 mM \(\beta\)-glycerophosphate, 2 mM EGTA, 5 mM NaF, 150 mM NaCl, 1 mM Na PP, 1% Nonidet P-40, 10% glycerol, 5 mM dithiothreitol, 1 mM sodium vanadate, 100 ng/ml phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml pepstatin, 2 \(\mu\)g/ml aprotinin, and 2 \(\mu\)g/ml leupeptin) and lysed by sonication. In all cases, lysates were subjected to immunoprecipitation with the specific antibody for 2 h, followed by adsorption to Sepharose-coupled protein G for 1 h. Immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting. For determination of total protein levels, aliquots of cell lysates were subjected to direct immunoblotting. Proteins were electrophoretically transferred to nitrocellulose membranes and probed with the indicated primary antibody. The bands were visualized by an enhanced chemiluminescence detection system according to the manufacturer’s instructions (ECL; American Biosciences).

Gene Expression Analysis—For the reporter gene assays, cells were seeded into 6-well (35-mm) plates and transfected 24 h later with expression vectors by the Lipofectamine method. Cells were subsequently treated with human TGF-β1 (Sigma) at 80 pM for 16 h. Luciferase activity was measured using the luciferase assay system described by the manufacturer (Promega) and normalized for transfection efficiency using a β-galactosidase-expressing vector (pCMV3.LacZ) and the Galacto-Star system (PerkinElmer Life Sciences).

FIG. 2. Deletion mapping of the interaction domains of Smad7 and AIP4. A, schematic diagram of AIP4 constructs used in experiments shown in B. C2, WW, and HECT domains are indicated. B, expression vectors encoding wild-type AIP4 or the indicated deletion mutants fused to the 3xFLAG epitope were transfected into COS-7 cells with HA-Smad7 expression vector. Cell lysates were subjected to immunoprecipitation (IP) with anti-FLAG antibody and then immunoblotted with anti-HA antibody (α-HA). The levels of HA-Smad7 and 3xFLAG-AIP4 constructs were detected by immunoblotting. C, lysates from COS-7 cells transfected with 3xFLAG-AIP4C830A, either alone or together with HA-Smad7, HA-Smad7Y211A, or HA-Smad7ΔPY, were subjected to anti-HA immunoprecipitation (IP) followed by anti-FLAG immunoblotting (α-FLAG). The expression of 3xFLAG-AIP4C830A, HA-Smad7, HA-Smad7Y211A, and HA-Smad7ΔPY was determined by immunoblotting aliquots of total cell lysates.

AIP4 was observed (Fig. 1C). In contrast, none of the other Smads coprecipitated with AIP4, despite efficient expression of these Smad proteins (Fig. 1C). Specific binding of AIP4 to both Smad6 and Smad7 is consistent with the observation that these two proteins possess very closely related MH2 domains and are both inhibitors of TGF-β signaling pathways (2).

To ascertain the physiological relevance of these interactions, we next investigated whether endogenous complexes between AIP4 and Smad7 proteins might be found in mammalian cells. For this, a polyclonal antibody against AIP4 was raised by injecting rabbits with a polypeptide corresponding to the
region of AIP4 from amino acids 220–230. Western blot analysis of 293 cells transfected with FLAG-Smad7, Smad2, Smad3, Smad4, or Smad7 in the absence or presence of Myc-AIP4 were immunoblotted with anti-FLAG (α-Flag) or anti-Myc (α-Myc) antibodies to detect the expression of Smads and AIP4, respectively. C, lysates from 293 cells transfected with FLAG-Smad7 either alone or together with wild-type 3xFLAG-AIP4 or 3xFLAG-AIP4C830A were immunoblotted with anti-FLAG antibody (α-Flag). D, 293 cells, transfected with FLAG-Smad7 either alone or together with Myc-AIP4, were pulse-labeled with [35S]Met/Cys and then chased for the indicated times. 35S-labeled Smad7 was immunoprecipitated and subjected to electrophoresis, and the autoradiographic signals were quantified by phosphorimaging. The levels of 35S-labeled Smad7 in control cells (open oval) and AIP4-expressing cells (filled oval) are plotted relative to the amount present at time 0. E, 293 cells were transfected with HA-Smad7 either alone or together with His-tagged ubiquitin and 3xFLAG-AIP4 or 3xFLAG-AIP4C830A as indicated. Lysates were subjected to immunoprecipitation (IP) with anti-His antibody followed by anti-HA immunoblotting (α-HA) to detect ubiquitinated Smad7. The relative levels of transfected proteins were determined by direct immunoblotting of total cell lysates. F, 293 cells were transfected with either AIP4 siRNA or a scrambled siRNA, and 24 h after transfection, they were treated with or without TGF-β for 1 h. The expression of endogenous AIP4, Smad7, or TβRI was determined by immunoblotting total cell lysates with anti-AIP4 (α-AIP4), anti-Smad7 (α-Smad7), or anti-TβRI (α-TβRI) antibodies. The phosphorylation of Smad2 was assessed by immunoblotting total cell lysates with anti-phospho-Smad2 antibody (α-pSmad2). For comparison, the same membrane was reprobed with an anti-Smad2 antibody (α-Smad2).
with AIP4 binding to Smad7, whereas further deletions that remove the WW1 and WW2 motifs completely abolished the interaction. A similar analysis of carboxyl-terminal truncations revealed that the WW3, WW4, and HECT domains did not associate with AIP4, thereby indicating that the WW1 and WW2 domains in AIP4 are required to mediate binding to Smad7 (Fig. 2B). We also characterized the domains in Smad7 that mediate binding to AIP4 protein. It is noteworthy that Smad7 contains a PPPY sequence, which is a conserved motif (PY motif) that can mediate interaction with WW domains such as those found in AIP4. To determine whether Smad7 associates with AIP4 through its PY motif, we employed two Smad7 PY motif mutants, one (Smad7Y211A) with the conserved Tyr residue in the PY motif mutated to Ala, and another (Smad7ΔPY) with the PPPY sequence deleted. In initial experiments, it was difficult to compare the efficiency of interaction of AIP4 with wild-type Smad7 or Smad7 PY motif mutants because expression of wild-type AIP4 dramatically decreased the steady-state level of wild-type Smad7, presumably by targeting Smad7 for degradation through the proteasome (see below). Consequently, we generated an inactive mutant of AIP4 (AIP4C830A), in which the Cys that is believed to conjugate ubiquitin was replaced by an Ala. In contrast to wild-type AIP4, the Smad7PY motif mutants failed to interact with AIP4C830A (Fig. 2C). Therefore, it is likely that AIP4 associates with Smad7 through interactions between the WW1 and WW2 domains of AIP4 and the PY motif in Smad7.

### AIP4 Specifically Induces the Degradation of Smad7

In the previous experiments, we often observed that the steady-state levels of Smad7 were reduced in cells coexpressing AIP4 (Fig. 1, B and E). This effect of AIP4 depended on the amount of AIP4 protein expressed in the cells (Fig. 3A) and was not observed for Smad2, Smad3, and Smad4 (Fig. 3B), which are defective in their ability to interact with AIP4 (Fig. 1C; see also Fig. 5C). Because expression of the catalytically inactive ubiquitin ligase mutant of AIP4, AIP4C830A, does not alter the steady-state levels of Smad7 (Fig. 3C), we considered the possibility that AIP4 might target Smad7 for ubiquitination, with subsequent degradation through the proteasome. First, we performed an analysis of the half-life of transfected Smad7 by pulse-chase experiments. As shown in Fig. 3D, coexpression of AIP4 dramatically enhanced the turnover of Smad7 protein. We also determined whether AIP4 enhances Smad7 turnover through its ability to induce ubiquitination of Smad7. For this, 293 cells were transfected with a vector coding for His-tagged ubiquitin together with HA-Smad7, in the presence or absence of AIP4. As shown in Fig. 3E, Smad7 ubiquitination occurred exclusively in the presence of His-ubiquitin, and, as expected, AIP4 notably enhanced Smad7 ubiquitination. Under these experimental conditions, cotransfection of similar amounts of the catalytic inactive mutant AIP4C830A did not yield ubiquitinated Smad7 (Fig. 3E), which is consistent with the notion that AIP4 acts as an ubiquitin ligase to induce the degradation of Smad7.

To further confirm a role for AIP4 in the degradation of Smad7, we employed siRNA to reduce endogenous levels of AIP4. 293 cells were transfected with either a control siRNA or an AIP4-specific siRNA. The AIP4 siRNA effectively reduced AIP4 expression compared with vehicle- or control siRNA-treated cells (Fig. 3F). Western blotting analysis with anti-Smad7 antibody demonstrated that the reduction of AIP4 is associated with an increase in the expression levels of endogenous Smad7 (Fig. 3F), providing further support for the physiological function of AIP4 as a ubiquitin ligase that targets Smad7 for degradation. In the course of these experiments, we also confirmed that the degradation of Smad7 by AIP4 can occur by a mechanism that is independent of TGF-β signaling. Moreover, we observed that expression of AIP4 siRNA had no effect on the expression levels (Fig. 3F) or ubiquitination of the activated TGF-β type 1 receptor (data not shown).

### AIP4 Inhibits the TGF-β Signal Transduction Pathway

Having shown that AIP4 can interact with Smad7 and mediate ubiquitination and proteasomal degradation of this inhibitory Smad, we set out to investigate the functional importance of AIP4 in TGF-β signaling. To test this, we examined the effects of ectopically expressed AIP4 on the luciferase CAGA reporter plasmid (CAGA)₉-Lux, which contains concatamerized CAGA elements previously shown to bind complexes of Smad3 and Smad4 and to be transactivated by both TGF-β and Smad3/Smad4 expression (24). As shown in Fig. 4A, expression of AIP4 resulted in a marked reduction in TGF-β-stimulated luciferase activity. Moreover, coexpression of AIP4 synergizes with Smad7 to inhibit TGF-β-induced (CAGA)₉-Lux activity. A similar conclusion could be drawn when the heterologous GAL4 reporter (G5E1B-Lux), and GAL4-Smad2 was used in these analyses (Fig. 4B). We also investigated the effects of the AIP4 siRNA and found that the reduction in endogenous AIP4 is associated with an increase in TGF-β-induced gene expression from the
AIP4 inhibits TGF-β signaling without affecting the expression levels of the receptor or the R-Smads. A, lysates from 293 cells transfected with the indicated combinations of HA-TβRI, HA-TβRIact, 6xMyc-Smad7, 3xFLAG-AIP4, 3xFLAG-Smurf1, 3xFLAG-Smurf2, and 3x-FLAG-Tiul1 were subjected to Western blot analysis with anti-HA (α-HA), anti-FLAG (α-Flag), or anti-Myc (α-Myc) antibodies. B, 293 cells were transfected with the indicated combinations of HA-TβRIact, 6xMyc-Smad7, and 3xFLAG-AIP4; pulse-labeled with [35S]Met/Cys; and then chased for the indicated times. [35S]-labeled HA-TβRIact or Myc-Smad7 was immunoprecipitated and subjected to electrophoresis, and the autoradiographic signals were quantified by phosphorimaging. The amounts of labeled TβRIact or Smad7 are plotted relative to the amounts present at time 0. C, 293 cells were transfected with 3xFLAG-AIP4 (left panels) or 3xFLAG-Tiul1 (right panels) and 6xMyc-tagged Smads before being treated with or without TGF-β at 80 pM for 1 h. The association of AIP4 or Tiul1 with Smads was analyzed by blotting the Myc immunoprecipitates (IP) with the anti-FLAG antibody (α-Flag). Expression of transfected proteins was monitored by direct Western blotting. D, 293 cells were
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(CAGA)_9-Lux reporter (Fig. 4C). Thus, AIP4 appears to act as an inhibitor of TGF-β-induced Smad-dependent transcription. Previous studies have shown that the E3 ubiquitin ligases Smurfl, Smurf2, and Tiul1 interact with Smad7 to induce ubiquitin-dependent degradation of the activated type I receptor (9–11). As a first step to elucidate the mechanism by which AIP4 induces suppression of TGF-β signaling, we examined whether AIP4 may function similarly to Smurfl, Smurf2, and Tiul1. As shown in Fig. 5A, coexpression of AIP4 and TGF-β type I receptor, in either the presence or absence of Smad7, did not alter steady-state levels of type I receptor. As a control, we observed that Smurfl, Smurf2, or Tiul1 can act in conjunction with Smad7 to target the activated type I receptor for degradation (Fig. 5A). Pulse-chase analysis also confirmed that the turnover rate of the bulk of type I receptor was unaffected by coexpression of Smad7 (Fig. 5B, top panel). In contrast, under the same conditions, Smad7 steady-state levels and half-life were decreased by AIP4 (Fig. 5B, bottom panel). Furthermore, we did not detect any effect of AIP4 on the ubiquitination of TβRI (data not shown). Taken together, these results suggest that TGF-β type I receptor is not efficiently targeted by AIP4 for ubiquitin-dependent degradation.

In the above experiments, we found that, unlike Smad7, the PY-containing R-Smads, Smad2 and Smad3, did not interact with AIP4 in unstimulated cells (Fig. 1C). To assess how AIP4 suppresses TGF-β-induced Smad-dependent transcription, we asked whether activation of Smad2 and Smad3 by the type I receptor might regulate their interactions with AIP4, which in turn promotes the degradation of these R-Smads, leading to inhibition of TGF-β signaling. Similar to our previous analysis, we could clearly detect a strong interaction between AIP4 and Smad7 that was unaffected by activation of TGF-β signaling (Fig. 5C). In contrast to Smad7, we were unable to detect any physical association of Smad2, Smad3, or Smad4 with AIP4 in either the absence or presence of TGF-β (Fig. 5C). In a control experiment, we observed that Tiul1 can associate with Smad2, Smad3, Smad4, and Smad7, and the associations of Tiul1 with Smad2 and Smad3 were increased in response to TGF-β signaling (Fig. 5C), similar to our previous findings (11). Because association of AIP4 and Smad2, Smad3, and Smad4 could be transient or unstable, these negative results do not completely rule out direct interactions of AIP4 and Smads. However, analysis of steady-state concentrations of Smad2, Smad3, and Smad4 revealed no significant TGF-β-dependent alterations in concentrations of these Smads in the presence of AIP4 (Fig. 5D). Under these experimental conditions, we confirmed that AIP4 induces the degradation of Smad7 (Fig. 5D). We also investigated whether AIP4 had altered inhibitory activity in luciferase reporter assays using GAL4-Smad2 (ΔPY) and GAL4-Smad3 (ΔPY) constructs lacking the PY motifs that have the potential to stably assemble with proteins with WW motifs such as AIP4. Consistent with our data from steady-state analysis, coexpression of AIP4 suppressed TGF-β-induced activation of GAL4-Smad2 (ΔPY) and GAL4-Smad3 (ΔPY) with activity similar to that of wild-type GAL4-Smad2 or GAL4-Smad3 (Fig. 5E). Thus, it is likely that AIP4 inhibitory activity is not due to an ability to target Smad2 and Smad3 for ubiquitin-dependent degradation.

AIP4 Stabilizes the Smad7/TβRI Complex—In an attempt to elucidate the mechanism underlying the inhibitory function of AIP4, we investigated the possibility that AIP4 might function to suppress TGF-β signaling by facilitating the formation of TβRI-Smad7 complexes. To test this possibility, we expressed in 293 cells Smad7 and wild-type or activated TβRI in the presence and absence of AIP4. In cells cotransfected with wild-type TβRI, a low level of interaction between the receptor and Smad7 could be detected (Fig. 6A). However, in cells expressing the activated TβRI, we observed a marked increase in the amount of type I receptor that coprecipitated with Smad7, similar to previous observations (7). Interestingly, despite the strong decrease in the steady-state levels of Smad7 in the total cellular pool, Smad7 associated with the activated type I receptor appeared to increase in the presence of AIP4 (Fig. 6A), suggesting that AIP4 might stabilize the Smad7-activated TβRI complexes. The ability of AIP4 to induce the association between Smad7 and the activated type I receptor is specific because we found that the amount of the activated TβRI bound to Smad7 was significantly decreased in the presence of Tiul1, Smurfl, or Smurf2 (Fig. 6A), similar to previous observations (9–11). To provide further evidence that AIP4 stabilizes the Smad7/TβRI complex, we investigated the effects of AIP4 on the phosphorylation of Smad2. We observed that expression of the activated TβRI induced a strong phosphorylation of Smad2, and this effect was blocked by coexpression of AIP4 (Fig. 6B). Consistent with this finding, knockdown of AIP4 by siRNA resulted in an increase in phosphorylation of endogenous Smad2 mediated by the activation of endogenous TGF-β receptors (Fig. 3F). Because the association of Smad7 with the activated type I receptor can prevent the access and phosphorylation of Smad2, these results suggest that AIP4 might function in TGF-β signaling by facilitating the association of Smad7 with the activated type I receptor.

The suppression by AIP4 of TGF-β-induced Smad-dependent transcription may be, at least in part, due to the ability of AIP4 to facilitate the interaction of activated type I receptor with Smad7. We therefore considered whether suppression of TGF-β signaling by AIP4 is independent of its ubiquitin ligase activity. To approach this question, we first examined whether the inactive form AIP4C830A may function to stabilize the Smad7-activated TβRI complex. Similar to wild-type AIP4, expression of AIP4C830A enhanced the interaction of Smad7 with the activated type I receptor (Fig. 6C). The specificity of this effect of AIP4C830A was evident because we were unable to detect an increase in the association of Smad7 and the activated TβRI when Tiul1C890a was coexpressed instead of AIP4C830A (Fig. 6C). Thus, we concluded that AIP4 functions to stabilize the TβRI-Smad7 complex by a mechanism that is independent of its ubiquitin ligase activity.

We next examined the effect of AIP4C830A on TGF-β-mediated expression from the (CAGA)_9-Lux reporter. As shown in Fig. 6D, coexpression of AIP4C830A inhibited TGF-β-dependent transcription with an efficiency approaching that elicited by transfection of wild-type AIP4, supporting the notion that AIP4 inhibits TGF-β signaling by enhancing the association of Smad7 and the activated type I receptor. This mechanism of inhibition is specifically mediated by AIP4 because we were unable to detect a significant inhibition of TGF-β-mediated luciferase activity by expression of the catalytically inactive mutant Tiul1C890A (Fig. 6D), which is consistent with our observation that Tiul1C890A cannot stabilize the Smad7-TβRI complex.

transfected with FLAG-tagged Smads in either the presence or absence of Myc-AIP4 before being treated with or without TGF-β at 80 ps for 1 h. Aliquots of total cell lysates were immunoblotted with anti-FLAG (α-Flag) or anti-Myc (α-Myc) antibodies to detect the expression of Smads and AIP4, respectively. E, 293 cells were transfected with G5E1B-Lux together with wild-type or ΔPY versions of GAL4-Smad2 (left panel) or GAL4-Smad3 (right panel) in either the absence or presence of AIP4. Cells were treated with (●) or without (□) TGF-β for 16 h prior to lysis and then assayed for luciferase activity. Luciferase activity was normalized to β-galactosidase activity and expressed as mean ± S.D. of triplicates from a representative experiment performed at least three times.
complex (Fig. 6C). Taken together, these results strongly suggest that AIP4 functions as an intracellular antagonist of TGF-β signaling by a mechanism that is independent of its ubiquitination ligase activity.

**DISCUSSION**

In this study, we have identified the HECT domain E3 ubiquitin ligase AIP4 as a protein that interacts with Smad7 and inhibits TGF-β signaling. Furthermore, we demonstrate that AIP4 functions by enhancing the association of Smad7 with the activated type I receptor independent of its ubiquitin ligase activity. To the best of our knowledge, this seems to represent a novel mechanism for the negative regulation of TGF-β signaling by the E3 ubiquitin ligases.

AIP4 is the founding member of a group of E3 enzymes that regulate the degradation of several cellular proteins involved in a variety of cellular processes, including signal transduction. For example, AIP4 targets latent membrane protein 2A (LMP2A) of Epstein-Barr virus for degradation through specific interaction between its WW domains and PY motifs in LMP2A (25). In addition, AIP4, through interaction with LMP2A, can be recruited to the tyrosine kinases Lyn and Syk, leading to their degradation (26). Our study demonstrates that AIP4 interacts with Smad7 to trigger its ubiquitination, with subsequent degradation through the proteasome. In marked contrast to Smurf1, Smurf2, and Tiul1, we found here that ectopic expression of AIP4 induces ubiquitin-dependent degradation of Smad7 in both the absence and presence of TGF-β signaling and has no effect on the turnover of activated TjIR. Another remarkable difference between AIP4 and the three ubiquitin ligases Smurf1, Smurf2, and Tiul1 is that AIP4 functions to inhibit TGF-β signaling by stabilizing the association between Smad7 and the activated type I receptor, which is critical for Smad7 to prevent access of Smad2 and Smad3 to the receptor (7). Consistent with this, expression of the catalytically inactive mutant AIP4C830A also inhibits TGF-β-mediated transcriptional responses. This mutant retained its ability to stabilize the Smad7 receptor complexes, indicating that AIP4 synergizes with Smad7 in the inhibition of TGF-β signaling by a mechanism that is independent of its ubiquitin ligase activity. These findings suggest that the functional cooperation between Smad7 and AIP4 could be an important mechanism for controlling the activity of TGF-β. Thus, AIP4 would provide additional mechanisms to allow quantitative inhibition by Smad7 in the presence of high levels of TGF-β signaling.

In addition to targeting Smad7, Smurf1 can interact with the PY motifs of the bone morphogenic protein-regulated Smads, Smad1 and Smad5, and can mediate ubiquitination and proteasomal degradation of these R-Smads (27). Smurf2 can also target Smad1 and Smad5 as well as activated Smad2 for ubiquitin-dependent degradation (21, 22). Furthermore, Tiul1 can degrade not only the activated type I receptor in association with Smad7 but also Smad2 in association with the corepressor TG-interacting factor (TGIF) (11). Our studies indicate that the regulatory role of AIP4 in TGF-β signaling is specific to Smad7. We observed that AIP4 strongly associates with Smad7. In contrast, AIP4 did not show any interaction with Smad1, Smad2, Smad3, or Smad4 even when expressed at high levels in 293 cells. Consistent with the physical interaction, expression of AIP4 in cultured cells resulted in ubiquitination and degradation of Smad7, whereas it had no effect on Smad2, Smad3, or Smad4. Interestingly, Smad6, which also functions as an antagonist of both TGF-β and bone morphogenic protein receptors, is closely related to Smad7 and interacts with AIP4. Although this has not yet been investigated, it is likely that AIP4 may function similarly to target Smad6 for ubiquitin-mediated degradation by the proteasome. Because AIP4 binds
stably with Smad6 and Smad7, but not to R-Smads, which contain PY motifs in their linker region and have the potential to stably assemble with WW motif-containing proteins, these data indicate that the WW motifs of AIP4 may contain residues that confer specificity for the Smad6 and Smad7 PY domains. Alternatively, other PY motif- and WW domain-containing proteins might also bind AIP4 and Smad6 or Smad7, respectively, thus providing additional mechanisms to control the association between these proteins.

The identification of AIP4 as a Smad7-interacting protein provides new insight into the mechanism by which Smad7 mediates inhibition of TGF-β signaling. By facilitating the association of Smad7 with the activated type I receptor, AIP4 functions as an inhibitor at a very early step in the TGF-β signaling pathway. It is striking that AIP4 also targets Smad7 for ubiquitin-dependent degradation. The exact role of ubiquitin-dependent down-regulation of Smad7 remains to be defined. The expression of Smad7 is increased in response to TGF-β in several cell types, which provides a negative feedback loop to control TGF-β activity (2). The expression of Smad7 can also be induced by interferon-γ (28) and tumor necrosis-α (29), raising the interesting possibility that Smad7 may fulfill other cellular functions independent of its inhibitory role. Consistent with this, Smad7 has been shown to cooperate with TGF-β in inhibiting adipocyte differentiation and to promote apoptosis stimulated by TGF-β in epithelial cells (30–33). Moreover, overexpression of Smad7 has been reported to sensitize various cell types to many forms of cell death, including serum withdrawal and anoikis (18, 31).

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