Smurf1 Interacts with Transforming Growth Factor-β Type I Receptor through Smad7 and Induces Receptor Degradation*

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Smad7 is an inhibitory Smad that acts as a negative regulator of signaling by the transforming growth factor-β (TGF-β) superfamily proteins. Smad7 is induced by TGF-β, stably interacts with activated TGF-β type I receptor (TβR-I), and interferes with the phosphorylation of receptor-regulated Smads. Here we show that Smurf1, an E3 ubiquitin ligase for bone morphogenetic protein-specific Smads, also interacts with Smad7 and induces Smad7 ubiquitination and translocation into the cytoplasm. In addition, Smurf1 associates with TβR-I via Smad7, with subsequent enhancement of turnover of TβR-I and Smad7. These results thus reveal a novel function of Smad7, i.e. induction of degradation of TβR-I through recruitment of an E3 ligase to the receptor.

Members of the transforming growth factor-β (TGF-β) superfamily initiate cellular responses (1) by binding to two different types of serine/threonine kinase receptors, termed type I and type II. Type I receptor is activated by type II receptor upon ligand binding and mediates specific intracellular signals (2). Members of the TGF-β superfamily transduce intracellular signals by Smad proteins. Eight different Smad proteins have been identified in mammals and are classified into three subgroups, i.e. receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads (I-Smads) (3–5).

R-Smads and Co-Smads positively regulate signaling by the TGF-β superfamily (3–5). R-Smads directly interact with type I receptors and become activated through phosphorylation of the C-terminal SSXS motif. R-Smads then form heteromeric complexes with Co-Smads (Smad4) and translocate into the nucleus. Nuclear Smad complexes bind to transcriptional coactivators or corepressors and regulate transcription of target genes. Smad2 and Smad3 act in the TGF-β/activin pathway, whereas Smad1, Smad5, and Smad8 are thought to act as bone morphogenetic protein (BMP)-specific Smads.

The third class of Smads are I-Smads, which include Smad6 and Smad7 in mammals (6–8). I-Smads associate with activated TGF-β superfamily type I receptors, thereby preventing phosphorylation of R-Smads. In addition, Smad6 has been demonstrated to interact with phosphorylated Smad1 to prevent complex formation between Smad1 and Smad4 (9). Smad6 was also reported to interact with Hoxc-8 and function as a transcriptional corepressor for inhibition of BMP signaling (10). Because expression of Smad6 and Smad7 is induced by TGF-β and BMPs, I-Smads inhibit TGF-β superfamily signaling by a negative feedback system (11).

Ubiquitin-dependent protein degradation plays a key role in various biological processes, including signal transduction, cell cycle progression, and transcriptional regulation (12). In the TGF-β signaling pathways, R-Smads, e.g. Smad2 and Smad1/5, have recently been shown to be degraded by the ubiquitin-proteasome pathway. Smad2 activated by TGF-β is degraded by the ubiquitin-proteasome pathway after translocation into the nucleus (13). Smurf1, a member of the HECT family of E3 ubiquitin ligases, ligand-independently induces the ubiquitination and degradation of BMP-specific Smads 1 and 5 through binding to a PY motif in the linker regions (14).

Here we demonstrate a novel function of Smurf1 in receptor degradation in TGF-β superfamily signaling. Inhibitory Smad7 associates with Smurf1 in the nucleus and is exported to the cytoplasm. Smad7 thus recruits Smurf1 to TβR-I, resulting in the degradation and rapid turnover of the TβR-I protein.

**EXPERIMENTAL PROCEDURES**

Transfection, Immunoprecipitation, and Immunoblotting—COS7 cells or 293T cells were transiently transfected using FuGENE6 (Roche Molecular Biochemicals). Immunoprecipitation and immunoblotting were performed as described (15). For inhibition of proteasomal degradation, cells were incubated with 50 μM MG132 (Peptide Institute) or 10 μM lactacystin (Calbiochem) for 4 h. Each experiment has been repeated at least three times with essentially similar results.

Affinity Cross-linking and Immunoprecipitation—Recombinant TGF-β1 (R & D Systems) was iodinated using the chloramine T method. Cross-linking was performed on ice to avoid degradation of the receptors and other proteins. Subsequent immunoprecipitation and analysis by SDS polyacrylamide gel electrophoresis (PAGE) were performed as described (15).

Immunofluorescence Labeling—Immunohistochemical staining of 6My-β-Smad7 in transfected COS7 cells was performed using mouse anti-Myc antibody followed by incubation with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG as described (15). For double staining of Smad7 and Smurf1, immunohistochemical staining of FLAG-Smad7 and 6Myc-Smurfl was performed using mouse anti-
FLAG or rabbit anti-Myc antibody followed by incubation with FITC-labeled goat anti-mouse IgG or rhodamine isothiocyanate (RTIC)-labeled goat anti-rabbit IgG, respectively. Nuclei of the cells were stained by 4,6-diamidino-2-phenylindole. Intracellular localization was determined by confocal laser scanning microscopy.

Pulse-Chase Analysis—Cells were labeled for 10 min at 37 °C with 50 μCi/ml [35S]methionine and cysteine (Amersham Pharmacia Biotech) mined by confocal laser scanning microscopy.

Luciferase Assay—R mutant mink lung epithelial cells were transiently transfected with an appropriate combination of a p3TP-lux promoter-reporter construct, expression plasmids, and pcDNA3. Total amounts of transfected DNAs were the same in each experiment, and values were normalized using Renilla luciferase activity.

RESULTS AND DISCUSSION

**Smurf1 Interacts with Smad6 and Smad7**—Smurf1 has been identified as an E3 ubiquitin ligase for BMP-specific Smads (14). Smurf1 has two WW domains that facilitate protein-protein interactions by binding to the PPXY sequence (PY motif) on partner proteins. Of eight different Smads, not only R-Smads including Smad1 and Smad5 but also I-Smads have a PY motif in their linker regions (Fig. 1A). We therefore examined whether Smurf1 binds to I-Smads. We first analyzed the interaction of Smurf1 with different Smads in transfected COS7 cells. A Smurf1 mutant, Smurf1(C710A), which has a mutation in the HECT domain and fails to recruit ligase activity, was used for this study. Of Smads 1 through 8, Smad6 and Smad7 strongly interacted with Smurf1(C710A) (Fig. 1B). Smurf1(C710A) interacted with Smad1 and Smad5 less efficiently than with Smad6 and Smad7. In contrast, it bound to Smad3 only weakly and failed to bind to Smads 2 and 4. Because Smad8 lacks the PY motif, Smurf1(C710A) did not bind to Smad8 either (Fig. 1B).

The mode of interaction between Smad7 and Smurf1 was further studied. In COS7 cells, weak interaction of wild-type Smad7 (Smad7(WT)) with wild-type Smurf1 (Smurf1(WT)) was detected, and it was slightly facilitated in the presence of the constitutively active TGF-β type I receptor, TβR-I(TD) (Fig. 1C). Moreover, the interaction between Smurf1(WT) and Smad7 was enhanced by the proteasome inhibitor lactacystin. In contrast, a Smad7 deletion mutant that lacks the PY motif (amino acids 207–211) in the linker region (Smad7ΔPY) did not bind to Smurf1.

**Smurf1 Interacts with TβR-I via Smad7**—Smad7 interacts with TβR-I activated by TβR-II, thereby competing with Smad2 and Smad3 for inhibition of TGF-β signaling. We therefore examined in an affinity cross-linking assay whether Smad7 acts as an adapter molecule that links TβR-I to the ubiquitin-proteasome pathway. Although Smurf1 alone did not efficiently bind to TβR-I in transfected COS7 cells, Smad7 dramatically enhanced the interaction between Smurf1 and the TβR-I-TβR-II complex (Fig. 1D, lanes 4 and 5). Moreover, Smurf1 failed to interact with the receptor complex in the presence of Smad7ΔPY (Fig. 1D, lane 6). These results indicate that Smurf1 is recruited to TβR-I through Smad7.

**Smad7 Is Translocated to the Cytoplasm by Smurf1**—We next examined the effect of Smurf1 on the subcellular localiza-
tion of Smad7. In the absence of Smurfl, both Smad7(WT) and Smad7ΔPY were predominantly located in the nucleus, although weak staining in the cytoplasm was also detected (Fig. 2A). When transfected alone, Smurfl was detected in the cytoplasm (data not shown). In the presence of Smurfl, Smad7(WT) was mainly observed in the cytoplasm. The cytoplasmic staining of Smad7 was further enhanced by the presence of proteasomal inhibitor MG132 or lactacystin (Fig. 2B). Smad7ΔPY failed to accumulate in the cytoplasm even in the presence of Smurfl, although there is a little leakage of Smad7ΔPY out of the nucleus (Fig. 2A); these results strongly suggest that interaction of Smurfl with Smad7 is required for the cytoplasmic localization of Smad7. Consistent with this, Smurfl and Smad7 colocalized in the cytoplasm (Fig. 2B). Interestingly, similar findings were obtained using Smurfl(C710A), suggesting that recruitment of ligase activity is not required for cytoplasmic translocation of the Smad7-Smurfl complex (Fig. 2B).

An E3 ubiquitin ligase, MDM2, has been reported to promote ubiquitin-dependent degradation and nuclear export of p53 (16, 17). In this case, a mutation within the MDM2 RING-finger domain that cannot induce p53 ubiquitination also lacks the ability to promote the p53 nuclear export. Thus, both Smurfl and MDM2 promote not only ubiquitin-dependent degradation but also nuclear export of the substrates, although the mechanisms of nuclear export appear to differ between them. Itoh et al. (18) reported that Smad7 is predominantly located in the nucleus and that it is exported to the cytoplasm after ligand stimulation. It is possible that Smurfl functions as a carrier protein for Smad7 for nuclear export, although it is currently not known whether ligand stimulation triggers the nuclear export of Smad7 by Smurfl.

Smurfl Induces Ubiquitination of Smad7 and TβR-I—To determine whether Smurfl acts as an E3 ubiquitin ligase for Smad7, ubiquitination of Smad7 by Smurfl was investigated in vivo. Smad7 was transfected into COS7 cells, together with Smurfl and HA-tagged ubiquitin. Smurfl efficiently induced the ubiquitination of Smad7 (Fig. 3A). Notably, Smad7 ubiquitination occurred more efficiently than that of Smad1 or Smad4. Polyubiquitination of Smad7 was not observed when Smad7ΔPY or Smurfl(C710A) was used (Fig. 3B). We also tested the effect of Smad7 on TβR-I ubiquitination by Smurfl in 293T cells. Although Smurfl alone ubiquitinated TβR-I weakly, Smad7 enhanced the receptor ubiquitination by Smurfl (Fig. 3C).

Smurfl Induces Degradation of Smad7 and TβR-I—To investigate whether Smurfl regulates degradation of Smad7 and TβR-I, we analyzed turnover of these proteins by pulse-chase experiments. Smurfl(WT), but not Smurfl(C710A), enhanced the degradation of Smad7 (Fig. 4, A and B), suggesting that Smurfl-induced Smad7 degradation is dependent on the HECT catalytic activity and through the proteasome. Smurfl(WT), but not Smurfl(C710A), was also rapidly degraded (Fig. 4B). Moreover, Smad7 and Smurfl induced the degradation of TβR-I (Fig. 4C). Our results thus demonstrate that Smurfl accelerates turnover of the TβR-I protein by recruitment of an E3 ubiquitin ligase, Smurfl.

Smurfl Enhances the Inhibitory Activity of Smad7—To examine the effect of Smurfl on the inhibitory activity of Smad7, we first compared the effect of Smad7ΔPY with that of Smad7(WT) using a TGF-β-responsive promoter-reporter construct, p3TP-lux (Fig. 4D). Smad7ΔPY suppressed activation of the reporter gene in a dose-dependent manner, but its inhibitory effect was less potent than that of Smad7(WT), suggesting that the interaction of Smad7 with Smurfl-like molecules is important for efficient inhibition of TGF-β signaling by Smad7. Next, we tested the effect of Smurfl on the inhibitory activity of Smad7 using p3TP-lux (Fig. 4E). Smurfl(WT), but not Smurfl(C710A), enhanced the inhibitory activity of Smad7. These data indicate that E3 ligase activity of Smurfl is crucial for its effect on the inhibitory activity of Smad7.

Smurfl-like Molecules Target TGF-β Receptors for Degradation via I-Smads—I-Smads have been shown to regulate TGF-β superfamily signaling through multiple mechanisms, e.g., competition with R-Smads for type I receptor interaction, inhibition of complex formation between R-Smads and Co-Smads, and transcriptional repression by interaction with transcription factors, such as Hoxc-8 (6–10). Our present findings revealed a novel mechanism for the inhibitory activity of Smad7.
Smurf1 Induces Degradation of TβR-I by Smad7

Although degradation of receptor complexes by Smurf1 may not be absolutely required for the action of I-Smads, it may play an important role in the negative regulation of TGF-β superfamily signaling by I-Smads. The present findings also suggest that E3 ligases of the Smurf family regulate TGF-β superfamily signaling through dual mechanisms. (i) By interaction with and degradation of R-Smads, Smurf1 negatively regulates BMP signaling. (ii) Smurf1 also interacts with Smad7 and inhibits TGF-β signaling by receptor degradation. Recently, another Smurf, Smurf2, has been suggested to exhibit similar dual specificities. Lin et al. (19) reported that Smurf2 interacts with Smad2, as well as other R-Smads, and induces the degradation of Smad2. Moreover, Kavsak et al. (20) reported that Smurf2 binds to TGF-β receptor complex via Smad7 and causes degradation of receptors and Smad7. It will be important to determine in the future whether there are some functional differences between Smurf1 and Smurf2 in vivo, especially in the interaction with I-Smads or receptors.

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FIG. 3. Ubiquitination of Smad7 and TβR-I by Smurf1 in vivo. COS7 cells (A and B) and 293T cells (C) were transfected with the indicated plasmids and treated with 50 μM MG132 for 4 h before cell lysis. Lysates from cells were subjected to anti-FLAG immunoprecipitation followed by anti-HA immunoblotting. Polyubiquitination species of Smad7 (A and B) [HA-Ub]-FLAG-Smad7 and those of TβR-I (C) [HA-Ub]-FLAG-TβR-I(TD) are indicated in the top panel.

FIG. 4. Smurf1 induces rapid turnover of Smad7 and TβR-I and inhibits transcriptional activity induced by TβR-I. A and B, degradation of Smad7 is enhanced by Smurf1(WT) (A) but not by Smurf1(C710A) (B). In panel B, turnover of Smurf1(WT) or Smurf1(CA) is also shown. COS7 cells were transfected with FLAG-tagged Smad7 with or without FLAG-tagged Smurf1(WT) or Smurf1(CA). Cell lysates were immunoprecipitated by FLAG antibody and analyzed by SDS-PAGE. C, degradation of TβR-I is enhanced in the presence of both Smad7 and Smurf1. COS7 cells were transfected with HA-tagged TβR-I with or without Smad7 and Smurf1. Cell lysates were immunoprecipitated by HA antibody and analyzed by SDS-PAGE. D, Smad7APY is less potent than Smad7(WT) in inhibiting TGF-β signaling. Effects of Smad7(WT) and Smad7APY on the transcriptional activity of constitutively active TβR-I (TβR-I(TD)) were examined. R mutant Mv1Lu cells that lack functional TβR-I were co-transfected with p3TP-lux, together with various combinations of TβR-I(TD) and Smad7 cDNAs. E, Smurf1 enhances inhibitory activity of Smad7. R mutant Mv1Lu cells were co-transfected with p3TP-lux, together with various combinations of TβR-I(TD), Smad7, Smurf1(WT), and Smurf1(CA) cDNAs.
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