Smurf2 is a Ubiquitin E3 Ligase Mediating Proteasome-dependent Degradation of Smad2 in Transforming Growth Factor-β Signaling*§

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Smads are important intracellular signaling effectors for transforming growth factor-β (TGF-β) and related factors. Proper TGF-β signaling requires precise control of Smad functions. In this study, we have identified a novel HECT class ubiquitin E3 ligase, designated Smurf2, that negatively regulates Smad2 signaling. In both yeast two-hybrid and in vitro binding assays, we found that Smurf2 could interact with receptor-activated Smads (R-Smads), including Smad1, Smad2, and Smad3 but not Smad4. Ectopic expression of Smurf2 was sufficient to reduce the steady-state levels of Smad1 and Smad2 but not Smad3 or Smad4. Significantly, Smurf2 displayed preference to Smad2 as its target for degradation. Furthermore, Smurf2 exhibited higher binding affinity to activated Smad2 upon TGF-β stimulation. The ability of Smurf2 to promote Smad2 destruction required the HECT catalytic activity of Smurf2 and depended on the proteasome-dependent pathway. Consistent with these results, Smurf2 potently reduced the transcriptional activity of Smad2. These data suggest that a ubiquitin/proteasome-dependent mechanism is important for proper regulation of TGF-β signaling.

EXPERIMENTAL PROCEDURES

GenBank™ Search—The amino acid sequence of Smurf1 was used to search GenBank™ for Smurf1 homologues. In deposited EST sequences and human genomic sequences in GenBank™ data bases, we found multiple clones (e.g. GenBank™ accession numbers AI273639, AA253311, AA148064, AA566447, W45583, AA630312, and AC009994) with strong similarity with Smurf1. Through pairwise comparison among one another, we found these sequences were likely derived from a single putative gene. Therefore, we designated this gene smurf2 and assembled an electronic copy of its complementary DNA (cDNA) sequence encoding the coding sequence of Smurf2.

Reverse Transcription-PCR—Total RNA was prepared from exponen-
Smurf2 is a Smad2-Ubiquitin E3 Ligase

Fig. 1. Structural features of human Smurf2 and Smurf2-Smad2 interactions. A, schematic representation of Smurf2 structure. C2, WW, and HECT domains are indicated. B, yeast two-hybrid assay. Three growing colonies from yeast cells transformed with indicated bait plasmid (pEG202-Smad), the prey plasmid pJG4–5/Smurf2, and a β-galactosidase reporter plasmid were first selected and streaked onto agarose plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Blue staining indicates Smurf2-Smad interaction. C, GST in vitro binding assay. GST-Smad1, -2, -3, or -4 was tested for direct interaction with in vitro translated 35S-Met-labeled full-length Smurf2. An equal amount of GST-Smad fusions was used, as shown by Coomassie Blue staining (lower panel). D, mapping of Smad-interacting domains of Smurf2. Yeast two-hybrid assays were used to map the domains of Smurf2 responsible for Smad-Smurfr2 interactions. – marks lack of detectable interaction, whereas +++ marks a very strong interaction. The structural organization of Smurf2 is shown with motifs indicated.

Amplified Smurf2 cDNA was digested with EcoRI and HindIII and subcloned into the EcoRI-HindIII sites of the CMV-driven expression plasmid pXP2F. The Smurf2 sequences from two independent RT-PCRs were analyzed.

Yeast Two-hybrid Assays—LexA-based yeast two-hybrid assays (33) were used to detect interactions between Smads in bait plasmid pEG202 (34) and Smurf2 in prey plasmid pJG4–5. A series of fragments of Smurf2 were obtained by PCR and subcloned in pJG4–5. Plasmids were transformed into yeast EGY48 using alkali cation (BIO 101, Inc.), and protein interactions were assessed by scoring β-galactosidase ac-
Smurf2 Is a Smad2-Ubiquitin E3 Ligase

**RESULTS AND DISCUSSION**

**Identification of Human Smurf2**—In a search to identify factors for Smad ubiquitination and degradation, we identified a new ubiquitin E3 ligase of the HECT subclass. Smurf2 contains 748 amino acids and is 83% identical to Smurf1 (see Supplemental Material). Like Smurf1, Smurf2 has a phospholipid/calcium-binding C2 domain (aa 17–42), WW domains, and a HECT ubiquitin ligase catalytic domain (aa 645–748) (Fig. 1A). Interestingly, Smurf2 has three WW domains. Besides two WW domains aligned with Smurf1, there is an insert sequence (aa 159–188) when compared with Smurf1, and this insert also contains a typical WW domain (Fig. 1A). The presence of these WW domains may determine the substrate specificity for Smurf2-mediated ubiquitination.

**WW2/3 Domains of Smurf2 Are Responsible for Its Interaction with R-Smads**—To determine which Smads are potential targets for Smurf2-mediated ubiquitination, we first tested the interaction of Smurf2 with various Smads in a yeast two-hybrid assay. As shown in Fig. 1B, all three R-Smads, Smad1, Smad2, and Smad3, displayed strong interaction with Smurf2.

**Degradation of Smad2**—To investigate whether Smurf2 induces degradation of Smad2, we transiently transfected HEK293 cells with His6-Smad2 and Flag-tagged Smurf2, together with HA-tagged ubiquitin (WT) or an ubiquitin mutant (as in aa 2–681) or deletion of N-terminal 243 amino acids (as in aa 244–748) still retained the ability to interact with R-Smads in a similar level. However, further deletion of the first N-terminal 328 amino acids abolished the ability of Smurf2 to interact with R-Smads, indicating that the Smurf2-Smad interaction requires the aa 244–328 region. In fact, the aa 244–328 region overlaps with the WW2/WW3 domain of Smurf2. These data suggest that Smurf2 interacts with R-Smads in a similar level. Furthermore, removal of WW2 abolished Smad2-Smad2 interaction. In conclusion, interaction domain mapping suggests that the region overlapping the WW2/WW3 domain but not the C2 or HECT domain mediates the Smurf2 interaction with R-Smads.

**Smurf2 Reduces the Steady-state Level of Smad2**—We next investigated whether Smurf2-induced degradation of these R-Smads. As shown in Fig. 2, expression of Smurf2 in 293 cells significantly decreased the steady-state levels of TGF-β-responsive Smad2. Smurf2 also moderately promoted degradation of BMP-responsive Smad1. Interestingly, Smurf2 expression had little effect on the level of TGF-β-responsive Smad3 or Smad4, a co-Smad for TGF-β signaling, did not interact with Smurf2. These data suggest that Smurf2 interacts with R-Smads but not Smad4. This observation was further evaluated with a GST fusion in vitro binding assay. As shown in Fig. 1C, GST-Smad1, GST-Smad2, and GST-Smad3 fusions bound to in vitro translated 35S-labeled Smurf2. Smurf2 had higher affinity to Smad1 and Smad2 than to Smad3. In contrast, no association was observed between Smurf2 and GST-Smad4. These results support the conclusion that Smurf2 specifically and directly interacts with R-Smads.

**Immunoprecipitations**—HEK293 cells were transiently transfected with cDNAs for N-terminally HA-tagged Smads (35) and Flag-tagged Smurf2 using LipofectAMINE (Life Technologies, Inc.) (36). Anti-Flag (M2, Sigma) or anti-HA antibodies (12CA5, Roche) were used to immunoprecipitate Smurf2 or Smad2 proteins from transfected cell lysates. To detect Smurf2-bound Smad2 or Smad2-bound Smurf2, the immunoprecipitated proteins were separated by SDS-PAGE, analyzed by Western blotting with primary antibody, and finally detected by horseradish peroxidase-conjugated goat anti-mouse secondary antibodies and visualized by chemiluminescence (Pierce).

**Ubiquitination and Proteasome-dependent Degradation Assays**—HEK293 cells were transfected with His6-Smad2 and HA-tagged ubiquitin, and Flag-tagged Smurf2 or its mutant C716A. Forty hours after transfection, cell lysates were subjected to precipitations using GST-Smurf2(244–434) (on beads) for another 2 h at 4 °C. Lysates were then incubated with 2 μg of GST-Smurf2(244–434) for another 2 h at 4 °C. Smurf2-Smad2 complex was then further washed twice with the lysis buffer containing 0.125% Triton X-100 and analyzed by Western blotting. Western analysis was essentially carried out as described for immunoprecipitation, except that anti-Smad2 (Zymed Laboratories Inc.) and anti-phospho-Smad2 (Zymed Laboratories Inc.) were used as primary antibodies.

**Gal4 Transactivation Assays**—HepG2 cells at 25–30% confluency were transfected with plasmids encoding Gal4-Smad2 (15, 16), together with Smurf2 and the reporter plasmid pFR-Luc (Stratagene). Transfected cells were treated for 24 h with or without 400 μM TGF-β. The ability of Gal4-Smad2 to transactivate the heterologous Gal4-binding promoter was quantitated by measuring the luciferase expression from the Gal4-binding promoter.

**Degradation of Smad2**—To map the Smad2-interacting domain of Smurf2, we created a series of deletion mutants of Smurf2 and detected their interaction with Smads in the yeast two-hybrid system (Fig. 1D). Smurf2 with a deletion of C-terminal 67 amino acid residues (as in aa 2–681) or deletion of N-terminal 243 amino acids (as in aa 244–748) retained the ability to interact with R-Smads in a similar level. However, further deletion of the first N-terminal 328 amino acids abolished the ability of Smurf2 to interact with R-Smads, indicating that the Smurf2-Smad interaction requires the aa 244–328 region. In fact, the aa 244–328 region overlaps with the WW2/WW3 domain but not the C2 or HECT domain mediates the Smurf2 interaction with R-Smads.
tion at aa 716, from Cys to Ala, in the HECT domain that abolished the formation of the thiol ester bond between ubiquitin and the Cys residue.

To determine whether Smurf2-mediated reduction of Smad2 protein depends on the proteasome pathway, we carried out degradation assays in the presence or absence of a proteasome inhibitor. HEK293 cells were transfected with HA-tagged Smad2 and Flag-Smurf2 and subsequently treated with MG132, a potent inhibitor of the 26 S proteasome. Western blotting analysis indicated that Smurf2, but not Smurf2(C716A), induced Smad2 degradation in the absence of MG132 (Fig. 3B, compare lanes 1 and 3). Addition of MG132 to the cells blocked the Smurf2-mediated Smad2 degradation (Fig. 3B, compare lanes 1 and 2). Our results suggest that Smurf2-induced Smad2 degradation is dependent on the HECT catalytic activity and through proteasome pathways.

Smurf2 Interacts with Activated Smad2 in Vivo—Based on the observation that addition of proteasome inhibitor MG132 or use of catalytically inactive Smurf2 stabilized Smad2 (Fig. 3B), we sought to determine the in vivo interaction between Smad2 and Smurf2 in the presence of MG132 or using Smurf2(C716A) mutant. The same lysates from transfected cells as shown in B were subjected to immunoprecipitation with anti-Flag antibodies. C, Smad2 interacts with Smurf2 in vivo. The same lysates from transfected cells as shown in B were subjected to immunoprecipitation with anti-Flag antibodies, followed by anti-HA immunostaining to detect Smurf2-bound Smad2 or in a reverse antibody order to detect Smad2-bound Smurf2. Lanes 1–4 are identical to those in B.
alyzed for a Smad2-bound Smurf2 or Smurf2-bound Smad2 complex. As shown in Fig. 3C, in the presence of MG132, precipitation of Flag-tagged Smurf2 could bring down HA-tagged Smad2 (Fig. 3C, lane 2, upper panel) or in a reverse order Smad2 could precipitate Smurf2 (Fig. 3C, lane 2, lower panel). The mutual coprecipitation of Smurf2 and Smad2 was also observed when Smurf2 had a mutation in the HECT catalytic domain (C716A) (Fig. 3C, lane 3). Wild-type Smurf2 could not coprecipitate Smad2 without proteasome inhibitor MG132 because of the instability of Smad2 (compare lanes 1 and 2).

Smurf2 became phosphorylated and nuclear imported upon TGF-β stimulation. We next tested whether Smurf2 interacted with the TGF-β-activated Smad2. The Smad-interacting domain (aa 244–434) of Smurf2 was used to pull down Smad2 from lysates of HaCaT cells, transfected with Smad2 or an empty vector control. As shown in Fig. 4, in vector-transfected cells, Smurf2 interacted with endogenous Smad2 only in the presence of TGF-β (compare lanes 1 and 2, upper panel). In Smad2-transfected cells, although Smurf2 had affinity to overexpressed Smad2 in the absence of TGF-β, Smurf2-Smad2 interaction was dramatically stimulated by TGF-β (compare lanes 3 and 4, upper panel). Using an anti-phospho-Smad2 antibody, a similar pattern of Smurf2-Smad2 interaction was observed as Smurf2 bound to activated Smad2 upon TGF-β stimulation (Fig. 4, third panel). Therefore, Smurf2 preferably interacted with TGF-β-phosphorylated Smad2, strongly suggesting that Smurf2 is an ubiquitin E3 ligase for targeting nuclear Smad2 for degradation.

**Smurf2 Decreases Smad2-dependent Transcription**—Since Smurf2 selectively promoted the degradation of Smad2, we next determined whether Smurf2 would reduce the transcriptional effects of Smad2 and Smad3 in TGF-β signaling. We transiently transfected into HepG2 cells Smurf2 or its mutant C716A with Gal4-Smad2 or Gal4-Smad3. The effect of Smurf2 on the transcription activity of Smads was assessed by luciferase reporter assay. As shown in Fig. 5, TGF-β treatment increased the activity of both Gal4-Smad2 and Gal4-Smad3 by 4- and 6-fold, respectively. Although expression of Smurf2 decreased the activity of both Gal4-Smad2 and Gal4-Smad3, much more dramatic reduction was observed for Gal4-Smad2 than Gal4-Smad3. In the presence of Smurf2, TGF-β-induced activity of Gal4-Smad2 was reduced by nearly 70%, whereas the TGF-β-induced activity of Gal4-Smad3 was reduced by 15%. Smurf2(C716A) mutant was unable to affect Smad2 transcriptional activity.

In conclusion, we have identified Smurf2, a novel HECT class ubiquitin E3 ligase. Unlike previously identified Smurf1 that specifically targets BMP signaling (e.g., Smad1, -5, and -8) (28), Smurf2 targets both Smad2 and Smad1 but with preference toward Smad2. Smad2 and Smad3 are nearly identical (92% identity) and both are mediators for TGF-β signaling. Interestingly, Smurf2 promoted the degradation of Smad2 and potently reduced the transcriptional activity of Smad2 in TGF-β signaling. Smurf2 exhibited little effect on Smad3 degradation or signaling, although it can similarly interact with Smad2 and Smad3 in vitro. This raises an interesting possibility that Smurf2 selectively inactivates a subset of signaling responses mediated by Smad2 but not Smad3. A previous study indicated that Smad2 is targeted for ubiquitin-dependent degradation upon its nuclear entry (29). In this study, we demonstrated that Smurf2 physically associated with TGF-β-activated Smad2, strongly suggesting that Smurf2 is a ubiquitin E3 ligase targeting nuclear Smad2 for proteasome-dependent degradation. However, it remains to be determined whether Smurf2 is localized in the nucleus or it travels into the nucleus together with activated Smad2. We anticipate that a tight regulation of Smurf2 and related proteins will contribute to the proper control of cellular responses induced by TGF-β or related factors in both normal and cancer cells. Further investigation of Smurf2 in developmental processes, such as using the Xenopus early embryo system, will also help in understanding the physiological roles of Smurf2 in TGF-β and BMP signaling.

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