Smurf1 Regulates the Inhibitory Activity of Smad7 by Targeting Smad7 to the Plasma Membrane

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Smad ubiquitin regulatory factor 1 (Smurf1), a HECT-type E3 ubiquitin ligase, interacts with inhibitory Smad7 and induces cytoplasmic localization of Smad7. Smurf1 then associates with transforming growth factor-β type I receptor (TβR-I) and enhances the turnover of this receptor. However, the mechanisms of the nuclear export and plasma membrane localization of the Smurf1-Smad7 complex have not been elucidated. We show here that Smurf1 targets Smad7 to the plasma membrane through its N-terminal conserved 2 (C2) domain. Both wild-type Smurf1 (Smurf1(WT)) and Smurf1 lacking the C2 domain (Smurf1(ΔC2)) bound to Smad7 and translocated nuclear Smad7 to the cytoplasm. However, unlike Smurf1(WT), Smurf1(ΔC2) did not move to the plasma membrane and failed to recruit Smad7 to the cell surface TβR-II-TβR-I complex. Moreover, although Smurf1(ΔC2) induced ubiquitination of Smad7, it failed to induce the ubiquitination and degradation of TβR-I and did not enhance the inhibitory activity of Smad7. Thus, these results suggest that the plasma membrane localization of Smad7 by Smurf1 requires the C2 domain of Smurf1 and is essential for the inhibitory effect of Smad7 in the transforming growth factor-β signaling pathway.

Members of the transforming growth factor-β (TGF-β) superfamily are multifunctional proteins that regulate a wide spectrum of cellular responses including growth, differentiation, apoptosis, and morphogenesis (1). TGF-β and related proteins initiate cellular responses 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, Smad4, positively regulate signaling by the TGF-β superfamily (3). Among R-Smads, Smad2 and Smad3 act in the TGF-β/activin pathway, whereas Smad1, Smad5, and Smad8 function in bone morphogenetic protein (BMP) and anti-Müllerian hormone pathways. In contrast to R-Smads and Co-Smads, I-Smads including Smad6 and Smad7 bind to type I receptors and compete with R-Smads for activation by the type I receptors, resulting in the inhibition of TGF-β superfamily signaling (6–8). Smad6 also inhibits BMP signaling by forming a complex with Smad1 and by interfering with complex formation between Smad1 and Smad4 (9).

Ubiquitin-dependent protein degradation plays key roles in various biological processes including signal transduction, cell cycle progression, and transcriptional regulation (10, 11). Ubiquitination of proteins is induced by an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases. In the ubiquitin-proteasome pathway, E3 ligases play a crucial role in the recognition of target proteins and subsequent protein degradation by 26 S proteasomes (12). Of the E3 ubiquitin ligases, the RING-type and HECT-type ligases have been well characterized in mammals. Many proteins containing RING finger domains have been found to function as E3 ligases, and some of those are involved in signaling pathways (12). For example, ROC1-Skp1-Cullin1-F-box protein complex containing Fbw1a (also termed βTrCP1) is an E3 ligase for IκB and β-catenin, which participate in the NFκB- and Wnt-signaling pathways, respectively. We have recently shown that the ROC1-Skp1-Cullin1-F-box protein complex containing Fbw1a induces ubiquitination and degradation of activated Smad3 (13). Neuronal precursor cell-expressed developmentally down-regulated 4 (Nedd4) and Smad ubiquitin regulatory factor (Smurf) family proteins represent the HECT-type subclass of E3 ligases; however, the number of HECT-type E3 ligases is less than that of RING-type E3 ligases.

Smurf1 was originally identified as an E3 ligase in which ligand independently induces the ubiquitination and degradation of BMP-specific Smads1 and 5 (14). Smurf2, a Smurf1-related E3 ubiquitin ligase, also interacts with Smad1/5/8 as well as with activated Smad2 (15, 16). In addition, Smurf2 interacts with a transcriptional co-repressor SnoN and thereby targets SnoN for ubiquitin-mediated degradation by proteasomes (17). In addition, Smurf1 and Smurf2 interact with Smad7 in the nucleus and induce translocation of Smad7 to the cytoplasm.
The Smurfs-Smad7 complexes then associate with TGF-β-type I receptor (TβR-I) and enhance its turnover (18, 19). However, the mechanism of the nuclear export and the plasma membrane localization of the Smurf1-Smad7 complex have not been elucidated.

Smurf1 is composed of a protein kinase C conserved 2 (C2) domain at the N terminus with WW domains in the middle and HECT domain at the C terminus. The WW domains bind to a PY motif in the linker regions of Smads, and the HECT domain is responsible for its ubiquitin protein ligase activity. However, the role of the C2 domain of Smurf1 has not been elucidated. The C2 domain was first identified as the Ca²⁺ binding site in conventional protein kinase C and has been found in various proteins including those involved in signal transduction and membrane trafficking (20, 21). The C2 domain of Ned44, which is structurally similar to Smurf1, was found to bind phospholipids and to be responsible for the localization of Ned44 at the plasma membrane (22). However, Snyder et al. (23) reported that the C2 domain of human Ned44 is not required for inhibition of the epithelial Na⁺ channel. Thus, the functional role of the C2 domain of Ned44 is still unclear. In this study, we examined the role of the C2 domain of Smurf1 in its subcellular localization and biological function in the TGF-β signaling pathway.

**EXPERIMENTAL PROCEDURES**

**DNA Construction and Transfection**—Construction of Smurf1 lacking the C2 domain (Smurf1(ΔC2)) was performed by deleting amino acids 22–37 by a PCR-based approach. The catalytically inactive form of Smurf1(ΔC2-CA) was also generated by a PCR-based approach. COS7 cells, 293T cells, HeLa cells, and R-mutant mink lung epithelial cells were transiently transfected using FuGENE6 (Roche Molecular Biochemicals) as described previously (24).

**Plasma Membrane Localization of Smad7 by Smurf1**

**Fig. 1. Smurf1 induces plasma membrane association of Smad7 via its C2 domain.** A, schematic representation of Smurf1(WT), Smurf1(WT) with cysteine 699 to alanine mutation (Smurf1(WT-CA)), Smurf1(ΔC2) lacking the C2 domain (amino acids 22–37), and Smurf1(ΔC2) with cysteine 699 to alanine mutation (Smurf1(ΔC2-CA)). Smurf1(WT-CA) and Smurf1(ΔC2-CA) have mutations in the HECT domain and do not have ubiquitin ligase activity. B, intracellular localization of Smurf1(WT) and Smurf1(ΔC2). Smurf1(ΔC2) was analyzed in transfected HeLa cells. Anti-FLAG staining for Smurf1(WT) (green) or Smurf1(ΔC2) (green) and nuclear staining by 4,6-diamidino-2-phenylindole (PI) (red) were performed. C and D, the effect of Smurf1(ΔC2) on subcellular localization of Smad7. Subcellular localization of Smad7 in the presence or absence of Smurf1(WT) and Smurf1(ΔC2) was analyzed in transfected HeLa cells. Cells were stained for anti-FLAG staining for Smad7 (green) and nuclear staining (red) (C) or anti-HA staining for TβR-I (red) (D).

**Immunofluorescence Labeling**—Immunohistochemical staining of FLAG-tagged Smurf1(WT) or FLAG-tagged Smurf1(ΔC2) in transfected HeLa cells was performed using mouse anti-FLAG antibody followed by incubation with fluorescein isothiocyanate-labeled goat anti-mouse IgG as described previously (19). For the double staining of Smad7 and TβR-I, the immunohistochemical staining of FLAG-tagged Smad7 and hemagglutinin (HA)-tagged constitutively active TβR-I (TβR-I(TD)) was performed using mouse anti-FLAG or rabbit anti-HA antibody followed by incubation with fluorescein isothiocyanate-labeled goat anti-mouse IgG or rhodamine isothiocyanate-labeled goat anti-rabbit IgG, respectively. The nuclei of the cells were stained by 4,6-diamidino-2-phenylindole. Intracellular localization was determined by confocal laser scanning microscopy. For ionophore treatment, cells were washed twice with Ca²⁺/Mg²⁺-free medium containing 5 mM EGTA and incubated with the same medium or with the Ca²⁺/Mg²⁺-free medium containing 1 mM EGTA, 0.1–1.0 mM Ca²⁺, and 100 μM maitotoxin (Wako Chemicals) for 20 min followed by observation by confocal microscopy.

**Immunoprecipitation and Immunoblotting**—Cells were lysed with Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40). Immunoprecipitation and immunoblotting were performed as described previously (25). For inhibition of proteasomal degradation, cells were incubated with 2.5 μM lactacystin (Calbiochem) for 24 h.

**Affinity Cross-linking and Immunoprecipitation**—Recombinant TGF-β1 (R&D Systems) was iodinated using the chloramine-T method as described previously (26). The immunoprecipitation of the cross-linked complex and analysis by SDS-PAGE were performed as described previously (25).

**Biotinylation of Cell Surface Receptors**—Biotinylation of cell surface receptors was performed as described previously (27). Cells were incubated with 1 mg/ml of sulfo-LC-biotin (Pierce) in phosphate-buffered saline for 1 h at 4 °C. Cells were then lysed and subjected to immunoprecipitation followed by SDS-PAGE. Cell surface receptors labeled with NHS-LC-biotin were detected with streptavidin conjugated to horseradish peroxidase using ECL system.

**Pulse-Chase Analysis**—Cells were labeled for 10 min at 37 °C with 50
RESULTS

Smurf1(ΔC2) Does Not Target Smad7 to the Plasma Membrane—Smurf1 has a C2 domain at the N terminus that was first described as a regulatory domain in protein kinase C (20). We generated Smurf1(ΔC2) (Fig. 1A) and examined its subcellular localization in transfected HeLa cells. Although both Smurf1(WT) and Smurf1(ΔC2) were predominantly located in the cytoplasm, they had distinct expression profiles. Smurf1(WT) was observed as a diffuse pattern with staining of plasma membrane. In contrast, Smurf1(ΔC2) was observed as an intracellular dotted pattern but failed to be located to the plasma membrane (Fig. 1B).

We next examined the effect of Smurf1(ΔC2) on the subcellular localization of Smad7 in HeLa cells (Fig. 1, C and D). In the absence of Smurf1, Smad7 was predominantly located in the nucleus in both the presence and absence of the TβR-I(TD). In the presence of Smurf1(WT), Smad7 was predominantly detected in the cytoplasm with clear staining of the plasma membrane. Smurf1(ΔC2) also induced the nuclear export of Smad7, but in contrast to Smurf1(WT), it did not efficiently target Smad7 to the plasma membrane. These results suggest that the C2 domain of Smurf1 plays an important role in the localization of the Smurf1-Smad7 complex to the plasma membrane.

Smurf1(ΔC2) Interacts with Smad7 But Not with the TβR-II/TβR-I Complex—To determine whether the lack of the C2 domain affects the function of Smurf1, we examined the binding of Smurf1(ΔC2) to Smad7 in vitro. An enzymatically inactive form of Smurf1, Smurf1(WT-CA) or Smurf1(ΔC2-CA), was used in this experiment to avoid degradation of Smad7 by Smurf1. Similar to Smurf1(WT), Smurf1(ΔC2) efficiently interacted with Smad7 in transfected COS7 cells (Fig. 2A).

We next examined the interaction of Smurf1 with TGF-β receptor complex in the presence and absence of Smad7 by an affinity cross-linking of the TGF-β receptors followed by immunoprecipitation of FLAG-Smurfl or FLAG-Smad7. When FLAG-Smurfl(WT) was immunoprecipitated, the TGF-β receptor complex was more efficiently co-immunoprecipitated in the presence than in the absence of Smad7 (Fig. 2B, lane 3 and 4 from the left) as reported by Ebisawa et al. (19). In contrast, the TGF-β receptor complex was co-immunoprecipitated only weakly by immunoprecipitation of FLAG-Smurfl(ΔC2) (Fig. 2B, lanes 5 and 6 from the left), suggesting that Smurf1(ΔC2) did not interact with the receptor complex, even in the presence of Smad7. Interestingly, when FLAG-Smad7 was immunoprecipitated, the TGF-β receptor complex was more efficiently co-precipitated by Smad7 in the presence than in the absence of Smurf1(WT) (Fig. 2B, lanes 7 and 8 from the left), suggesting that Smurf1(WT) enhanced the binding of Smad7 to the TGF-β receptors. In contrast, Smurf1(ΔC2) failed to enhance the association of Smad7 with the receptors (Fig. 2B, lane 9 from the left). These results indicate that the C2 domain of Smurf1 is not required for interaction between Smurf1 and Smad7 but that it is essential for the association of Smurf1-Smad7 complex with TGF-β receptors at the plasma membrane.

Smurf1(ΔC2) Induces Ubiquitination of Smad7 But Not of TβR-I in Vivo—To determine the role of the C2 domain of Smurf1 in protein ubiquitination, we investigated the ubiquitination of Smad7 and TβR-I by Smurf1 in vivo. Smad7 was transfected into 293T cells together with Smurf1(WT) or Smurf1(ΔC2) and HA-tagged ubiquitin. Ubiquitination of Smad7 was observed in the presence of Smurf1(ΔC2) as well as in the presence of Smurf1(WT) (Fig. 3A), consistent with the physical interaction of Smurf1(ΔC2) with Smad7. Interestingly, Smad7 was more efficiently ubiquitinated by Smurf1(ΔC2) than by Smurf1(WT).

We next examined the ubiquitination of TβR-I by Smurf1. As shown in Fig. 3B, ubiquitination of TβR-I was observed in the presence of Smurf1(WT) but not in the presence of Smurf1(ΔC2). Thus, the C2 domain is essential not only for the interaction of Smurf1-Smad7 with TGF-β receptors but also for ubiquitination of TβR-I.

Degradation of TβR-I Is Enhanced by Smurf1(WT) But Not by Smurf1(ΔC2)—To investigate whether Smurf1(ΔC2) regulates degradation of Smad7 and TβR-I, we examined steady-state levels of Smad7 in transfected COS7 cells co-expressing 6Myc-Smurfl(WT) or 6Myc-Smurfl(ΔC2) (Fig. 4A). Both Smurf1(WT) and Smurf1(ΔC2) enhanced the degradation of Smad7 in the presence of Smurf1(ΔC2) as well as in the presence of Smurf1(WT) (Fig. 4B), consistent with the physical interaction of Smurf1(ΔC2) with Smad7. Interestingly, Smad7 was more efficiently ubiquitinated by Smurf1(ΔC2) than by Smurf1(WT).

Smurf1(ΔC2) interacts with Smad7 but not with the TβR-II/TβR-I complex via Smad7. A binding of Smurf1(WT) and Smurf1(ΔC2) to Smad7 was analyzed in vivo. COST7 cells were transfected with the indicated plasmids and were subjected to FLAG immunoprecipitation (IP) followed by Myc immunoblotting (Biot). The top panel shows the interaction of Smurf1(WT) and Smurf1(ΔC2) with Smad7. In the lower two panels, aliquots of cell lysates were directly subjected to immunoblotting to confirm the expression of each protein as indicated. B, the binding of Smurf1(WT) and Smurf1(ΔC2) to the TβR-II/TβR-I complex was examined by immunoprecipitation of affinity cross-linked TGF-β receptor complexes. COST7 cells were transfected with the indicated plasmids. Cells were affinity-labeled with [35S]methionine and cysteine (Amersham Biosciences) in methionine- and cysteine-free Dulbecco’s modified Eagle’s medium and chased in Dulbecco’s modified Eagle’s medium supplemented with 0.2% fetal bovine serum and unlabeled methionine and cysteine for the time periods indicated as previously described (19). Cells were then lysed and subjected to immunoprecipitation followed by SDS-PAGE.

Luciferase Assay—R-mutant mink lung epithelial cells were transiently transfected with an appropriate combination of p3TP-lux promoter-reporter construct, expression plasmids, and pcDNA3 (28). Total amounts of transfected DNAs were the same in each experiment, and values were normalized using Renilla luciferase activity.
Smad7. Smurf1(C2-CA), which lacks the ubiquitin ligase activity, did not enhance the degradation of Smad7 (data not shown) (see supplementary Fig. 1), confirming that the weak bands of Smad7 in the presence of Smurf1 in Fig. 4A is not because of interference of protein expression of the transfected proteins in COS7 cells.

We next analyzed TβR-I levels in transfected COS7 cells. As shown in Fig. 4B, top panel, examined by FLAG immunoprecipitation followed by FLAG immunoblotting, FLAG-tagged TβR-I proteins were observed as two types of differentially migrating bands. Because membrane receptors are post-translationally modified by the addition of N-linked oligosaccharides, we thought that the slowly migrating bands represent a mature form of TβR-I, whereas the fast migrating bands represent its immature form. To explore this possibility, we tried to detect the mature form of TβR-I by cell surface protein labeling by NHS-LC-biotin. By FLAG immunoprecipitation and Biotin blotting (second panel from the top), we detected only slowly migrating bands, strongly suggesting that the slowly migrating bands are the mature form of TβR-I. In both FLAG immunoblotting and Biotin blotting, Smurf1(WT) but not Smurf1(C2) enhanced the degradation of the mature form of TβR-I (Fig. 4B, top two panels).

We also examined TβR-I turnover by pulse-chase analysis (Fig. 4, C and D). The TβR-I protein was observed as a fast migrating band representing the immature form at 0 h. At later periods, slowly migrating bands that represent the mature form of TβR-I were detected. In the absence of Smad7 and Smurf1, slowly migrating bands increased with chase time. Smurf1(WT) induced the degradation of TβR-I, which was enhanced in the presence of Smad7 (Fig. 4C); thus, the slowly migrating bands were not detected at later periods. In contrast, Smurf1(C2) did not alter the turnover of TβR-I, and both fast and slowly migrating bands were observed, similar to the absence of Smurf1 and Smad7 (Fig. 4D). These findings suggest that the degradation of TβR-I induced by Smurf1 requires the C2 domain of Smurf1.

**DISCUSSION**

Smurf1 was originally identified as an E3 ubiquitin ligase that specifically degrades the BMP-specific R-Smads, Smad1 and Smad5 (14). Smurf2 is highly related structurally to Smurf1 and induces the degradation of Smad1 as well as that of a TGF-β-specific R-Smad, Smad2 (15, 16). In addition,
Smurf1 and Smurf2 have been shown to physically interact with I-Smads, Smad6 and Smad7. Smurfs induce nuclear export of Smad7, interact with the TGF-β receptor complex, and facilitate the degradation of Smad7 as well as that of the TGF-β receptor complex (18, 19). Thus, Smurfs negatively regulate the Smad signaling pathways through interaction with both R-Smads and I-Smads.

Smad signaling pathways are conserved in vertebrates and in Drosophila (30). MAD serves as an R-Smad that specifically transmits signals for decapentaplegia. MEDEA acts as a Co-Smad, and DAD is an I-Smad in Drosophila. DSmurf was recently identified as a Drosophila ortholog of vertebrate Smurf1 and 2 (31). The function of DSmurf is restricted to the decapentaplegic pathway during development, suggesting critical roles for Smurf ubiquitin ligases in TGF-β superfamily signaling. Similar to Smurf1 and Smurf2, DSmurf appears to control the intracellular pool of MAD and to down-regulate the accumulation of phosphorylated MAD by receptor turnover, possibly through interaction with DAD.

In this study, we have shown that the C2 domain of Smurf1 is responsible for the association with the plasma membrane. Although Smurf1 may exhibit activity by degrading R-Smads in the cytoplasm or in the nucleus, membrane localization of Smurf1 appears to be essential for inhibition of TGF-β signaling by the Smurf1-Smad7 complex. Intriguingly, Smurf1(C2) ubiquitinylates Smad7 more efficiently than Smurf1(WT), sug-
suggesting that Smurf1 degrades Smad7 and possibly other Smads if it is not targeted to the membrane. Several HECT-type E3 ligases have been identified in mammals including Nedd4 and related Nedd4-like proteins (32). Nedd4 interacts with epithelial Na+ channel and some other proteins (33). Nedd4 is observed throughout the cytoplasm in cells, and its interaction with the plasma membrane is mediated by cytosolic Ca2+ (22).

In contrast, membrane localization of Smurf1 was not regulated by cytosolic Ca2+, suggesting that subcellular localization of Smurf1 is controlled by certain other mechanisms. There are many non-Ca2+-binding C2 domains, and some of them including those of PTEN and protein kinase Cε still have abilities to bind to the membrane (34, 35). Thus, it will be important to determine how the association of the C2 domain of Smurf1 with the plasma membrane is mediated under physiological conditions.

Levels of expression of I-Smads are regulated by various stimuli including TGF-β, BMPs, growth factors, interferon-γ, NFκB signaling, and shear stress (36). Smad3 and Smad4 and BMP-specific R-Smads bind to the promoter regions of Smad7 and Smad6, respectively (37, 38). In contrast, Smurf1 and Smurf2 appear to be ubiquitously expressed in various tissues, and the regulation of their levels of expression has not been reported. Smurfs may be constitutively expressed in cells once I-Smads are induced by certain stimuli and Smurfs capture I-Smads, induce their nuclear export, and associate with the TGF-β receptor complex.

For determination of the ubiquitination of TβR-I by Smurfs, a constitutively active form of TβR-I was used in this study as well as by others (18, 19). Because TβR-I and TβR-II spontaneously form a complex without ligand stimulation in transfected cells (39), it has not been determined whether ubiquitination of TβR-I by the Smurf1-Smad7 complex is induced only upon ligand stimulation. However, the ubiquitination of TβR-I by Smurf1-Smad7 may be dependent on the activation status of TβR-I by the TβR-II kinase, because Smad7 fails to efficiently associate with TβR-I in the presence of kinase-inactive form of TβR-II (data not shown) (7).

It is important to note that Smurf1 enhanced the interaction of Smad7 with the TGF-β receptor complex through targeting of Smad7 to the plasma membrane (see Fig. 2B). This finding suggests that Smurf1 brings Smad7 to the TGF-β receptor complex and allows Smad7 to compete with R-Smads for receptor activation before it begins to degrade the receptor complex (Fig. 7A). After several hours, Smurf1 begins to induce the degradation of Smad7 and the TGF-β receptor complex, leading...
to further inhibition of TGF-β signaling (Fig. 7B). Thus, the C2 domain of Smurf1 plays important roles in both the early and late phases of inhibition of TGF-β signaling by the Smurf1-Smad7 complex.

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