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Smad7 Binds to Smurf2 to Form an E3 Ubiquitin Ligase that Targets the TGFβ Receptor for Degradation

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Summary

Ubiquitin-mediated proteolysis regulates the activity of diverse receptor systems. Here, we identify Smurf2, a C2-WW-HECT domain ubiquitin ligase and show that Smurf2 associates constitutively with Smad7. Smurf2 is nuclear, but binding to Smad7 induces export and recruitment to the activated TGFß receptor, where it causes degradation of receptors and Smad7 via proteasomal and lysosomal pathways. IFNγ, which stimulates expression of Smad7, induces Smad7–Smurf2 complex formation and increases TGFß receptor turnover, which is stabilized by blocking Smad7 or Smurf2 expression. Furthermore, Smad7 mutants that interfere with recruitment of Smurf2 to the receptors are compromised in their inhibitory activity. These studies thus define Smad7 as an adaptor in an E3 ubiquitin–ligase complex that targets the TGFß receptor for degradation.

Introduction

Transforming growth factor-β (TGFβ) superfamily members signal through heteromeric complexes of type II and type I transmembrane Ser/Thr kinase receptors. Within this receptor complex the activated type I receptor propagates signals to the Smad signal transduction pathway (Derynck et al., 1998; Massagué and Chen, 2000; Wrana, 2000). One class of Smads, the receptor-regulated Smads (R-Smads), is directly phosphorylated by distinct type I receptors. This induces binding of the R-Smad to the common Smad, Smad4. The R-Smad/Smad4 complex then accumulates in the nucleus where it modulates transcription by interacting with a variety of DNA binding partners. A third class of Smads, the inhibitory Smads (I-Smads) represented by Smad6 and Smad7, negatively regulates TGFβ signaling by interacting with the activated type I receptor (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997). In addition, Smad6 may interfere with Smad1/Smad4 association (Hata et al., 1998). Expression of both Smad6 and Smad7 is regulated by TGFβs, BMPs, IFNγ, and other growth factors and cytokines, thereby providing for negative feedback regulation of the Smad signaling pathway (Nakao et al., 1997; Afrakhte et al., 1998; Ulloa et al., 1999; Bitzer et al., 2000).

The ubiquitin proteasome system is a common pathway for the degradation of a wide range of cellular proteins (Hershko and Ciechanover, 1998). Ubiquitination is controlled by a multienzyme cascade that involves E1 (ubiquitin-activating enzymes), E2 (ubiquitin-conjugating enzymes), and E3 (ubiquitin–protein ligases) activities (Hershko and Ciechanover, 1998). The E3 activity recruits substrates to the ubiquitination machinery and plays a critical role in specifying which proteins are targeted for degradation. The ubiquitin proteasome pathway has been implicated in the degradation of Smads. Smurf1, a C2-WW-HECT domain E3 ubiquitin–protein ligase, targets the BMP-regulated R-Smads, Smad1, and Smad5 for degradation through specific interactions between its WW domains and a PPXY motif located in the Smad linker region (Zhu et al., 1999). Activated Smad2 is also targeted in the nucleus for ubiquitin-dependent degradation, thus providing a mechanism to turn off the activity of nuclear Smad complexes (Lo and Massagué, 1999).

Regulation of cell surface receptors by ubiquitin-dependent pathways is emerging as a potent means to control the activity of signaling pathways. Conjugation of ubiquitin to receptors is used in diverse systems to control endocytosis and signaling, as well as receptor steady-state levels by both proteasome- and lysosome-mediated degradation (Bonifacino and Weissman, 1998; Hicke, 1999). Direct ubiquitination of membrane receptors has been characterized in a number of systems, although in some cases ubiquitin-dependent regulation does not appear to involve direct conjugation of ubiquitin to the receptor (van Kerkhof et al., 2000). Although many cell surface receptors are regulated by ubiquitin-dependent pathways, only a few E3 ubiquitin ligases that bind to membrane proteins, such as Nedd4 (Staub et al., 1997) and c-Cbl (Joazeiro et al., 1999; Levkowitz et al., 1999), have been defined. In these cases, ubiquitination involves direct interactions between the E3 ligase and the target protein. Whether adaptor proteins might also function to recruit E3 ligases to specific receptor complexes is unknown.

The fate of activated TGFβ receptor complexes has been largely unexplored. Ligand-dependent downregulation of receptor complexes has been observed in a number of cell types (Centrella et al., 1996; Koli and Arteaga, 1997; Wells et al., 1997; Anders et al., 1998; Zwaagstra and O'Connor-McCourt, 1999) and appears to be dependent on activation of the type I receptor by type II (Anders et al., 1998). However, the molecular mechanisms that regulate Ser/Thr kinase receptor turnover are unknown. Here, we identify Smurf2, a C2-WW-HECT domain E3 ubiquitin ligase, and show that Smad7

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functions as an adaptor protein that recruits Smurf2 to the TGFβ receptor complex to promote its degradation.

Results

Smurf1, first identified in *Xenopus*, is a HECT domain E3 ubiquitin ligase that can target the BMP-regulated R-Smads for degradation (Zhu et al., 1999). In a search for Smurf1-related proteins in the EST database, we identified a novel protein we call Smurf2. It contains a C2 domain at the amino terminus, followed by three WW domains and a HECT ubiquitin ligase domain (GenBank accession #AF310676). Smurf2 was expressed throughout early development in most adult tissues and in a variety of cell lines that include P19, HepG2, 293T, and U4A/Jak1 (data not shown).

Smurf2 Does Not Regulate Smad Steady-State Levels

Smurf1 binds to Smad1 and Smad5 and induces their degradation through the ubiquitin proteasome pathway (Zhu et al., 1999). Thus, we sought to determine whether Smurf2 might regulate Smad steady-state levels. Surprisingly, Smurf2 expression did not alter the steady-state levels of Smads 1, 2, 4, or 7 (Figure 1A). To determine whether Smurf2 might bind any of these Smads, the cell lysates were subjected to immunoprecipitation of Smurf2 and associated Smads examined by immunoblotting. Under these conditions in unstimulated cells, Smad1, 2, or 4 did not coprecipitate with Smurf2 (Figure 1A). In contrast, an interaction between Smurf2 and Smad7 was detected, which is likely to be direct since bacterially produced Smad7 and Smurf2 formed complexes in vitro (data not shown). Next, we examined by pulse–chase analysis whether Smurf2 might alter Smad7 stability. In the presence of Smurf2 there was a small enhancement in Smad7 turnover (Figure 1B) that was not sufficient to significantly change Smad7 steady-state levels. These data indicate that Smad7 is not a major target for Smurf2 in the absence of TGFβ signaling.

To characterize endogenous Smad7–Smurf2 association, we generated an antibody that recognizes Smurf2 but not Smurf1 (Figure 1C). In most cells, the basal level of Smad7 is low, but its expression can be induced by various stimuli. In particular, IFNγ induces a sustained increase in Smad7 expression in U4A/Jak1 cells (Ulloa et al., 1999), which also express Smurf2 protein (Figure 1C). Therefore, we determined whether IFNγ induces the assembly of endogenous Smad7–Smurf2 complexes in U4A/Jak1 cells. In the absence of IFNγ, no Smurf2 was found to coprecipitate with Smad7, consistent with the lack of Smad7 protein in unstimulated cells (Figure 1C, right panel; Ulloa et al., 1999). However, upon stimulation with IFNγ, we observed that Smurf2 coprecipitated with Smad7, concomitant with increased Smad7 protein levels. In these experiments, we observed that Smurf2 bound to Smad7 appeared as a doublet. The upper band may represent Smurf2 that is conjugated to ubiquitin or ubiquitin-like moieties, as observed for other ubiquitin ligases such as Mdm2 (Buschmann et al., 2000). These results demonstrate that Smad7 and Smurf2 associate at endogenous levels of expression.

We also analyzed the determinants on Smad7 and Smurf2 that mediate their interaction. Smad7 possesses a PPXY sequence (PY motif) in its linker region. This motif can mediate interaction with WW domains such as those found in Smurf2 (Chen and Sudol, 1995). Analysis of Smurf2 binding to Smad7 PY motif mutants revealed that interaction with Smurf2 was reduced but not entirely abolished (Figure 1D). This suggests that the PY motif is important for Smad7–Smurf2 interaction, but is not the sole determinant. We also made mutants of Smurf2 in which each of the three WW domains was deleted. Deletion of the first WW domain did not interfere with Smurf2–Smad7 interaction; however, deletion of either WW2 or WW3 abolished complex formation (Figure 1E). Thus, the WW2 and WW3 domains in Smurf2 are both required to mediate binding to Smad7.

Smad7 Recruits Smurf2 into a Complex with the TGFβ Receptors

Smad7 was shown previously to bind heteromeric complexes of TGFβ type II (TβRII) and type I (TβRI) receptors through interactions with the activated type I receptor subunit (Hayashi et al., 1997; Nakao et al., 1997). The constitutive association between Smad7 and Smurf2 thus raised the interesting possibility that Smad7 might function to recruit Smurf2 to the TGFβ receptor complex. To test this, we expressed TGFβ receptors in COS-1 cells in the presence and absence of Smad7 and Smurf2 and examined [^{125}I]TGFβ-labeled receptor complexes that coprecipitated with Smurf2. In the absence or presence of Smad7, few or no TGFβ receptor complexes were found to coprecipitate with wild-type Smurf2 (Figure 2). Previously, we found that the interaction of the Smad1 substrate with Smurf1 was stabilized by a catalytic mutant of the HECT ubiquitin ligase domain (Zhu et al., 1999). Consequently, we constructed a comparable mutation in Smurf2 (Smurf2[C716A]). When Smurf2 (C716A) was expressed alone, we detected a slight interaction with the TGFβ receptors (Figure 2) that was dramatically enhanced in the presence of Smad7. Thus, Smad7 mediates the interaction of Smurf2 with the TGFβ receptors.

In these experiments, we also examined Smad7 bound to the receptors. In the presence of wild-type Smurf2, we observed a strong decrease in the amount of TGFβ receptor complexes that coprecipitated with Smad7 (Figure 2), correlating with a decrease in total type I receptor. Since Smad7 binds to receptors via activated receptor I, these results suggest that Smurf2 decreases the level of Smad7-bound receptor complexes. Consistent with this notion, when Smad7 was coexpressed with Smurf2(C716A), there was no decrease in Smad7-bound receptor levels (Figure 2). These results indicate that the catalytic activity of Smurf2 mediates the downregulation of TGFβ receptors that are bound to Smad7.

Smad7 Controls the Subcellular Localization of Smurf2

Previous studies have shown that Smad7 resides in the nucleus, but that cytoplasmic accumulation can occur in response to various stimuli including TGFβ signaling (Itoh et al., 1998). To investigate whether Smad7 can recruit Smurf2 to the receptors in intact cells, we determined whether Smad7 might regulate Smurf2 localiza-

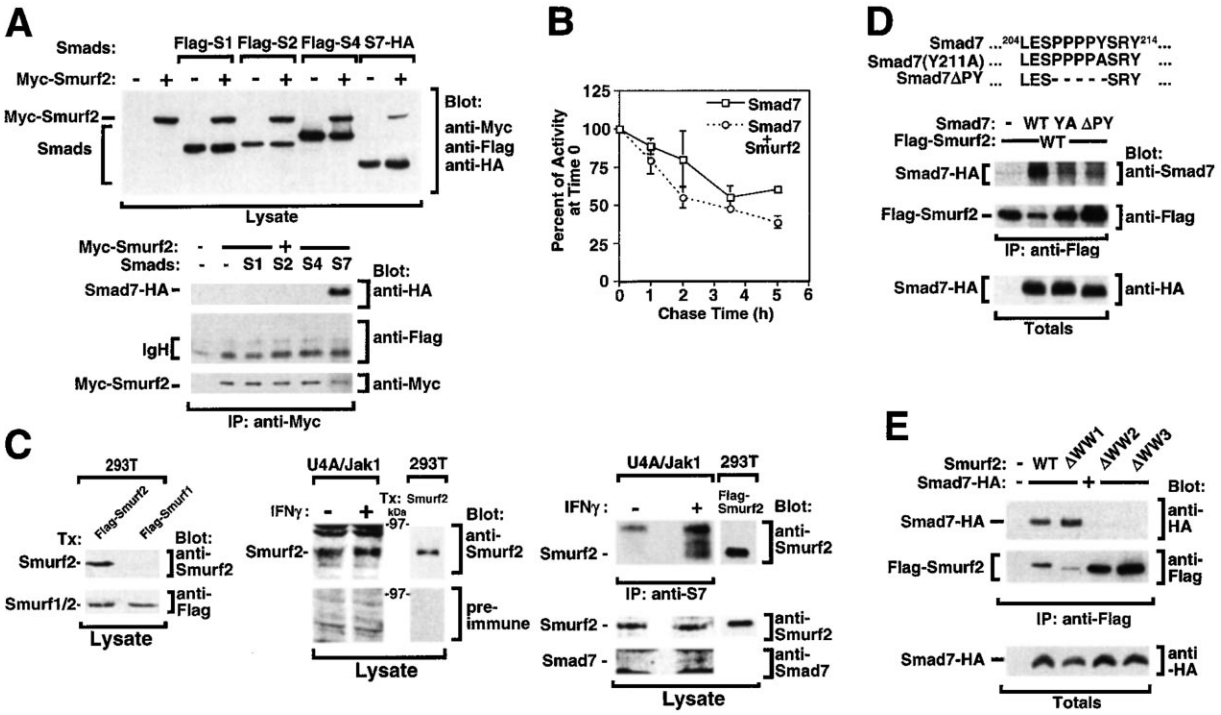

Figure 1. Smurf2 Interacts with Smad7

(A) Expression of Smurf2 does not decrease steady-state levels of the Smads. 293T cells were transfected with Flag- or HA-tagged Smads either alone or together with Myc-tagged Smurf2. Aliquots of total cell lysates were immunoblotted to detect expression of Smurf2 and the Smads (upper panel), or were subjected to immunoprecipitation with anti-Myc antibody followed by anti-Flag or anti-HA immunoblotting to detect Smads (lower panel). The migration of the anti-Myc heavy chain (IgH) is marked.

(B) Expression of Smurf2 does not alter Smad7 turnover. COS-1 cells, transfected with either Smad7-HA alone or together with Flag-Smurf2, were pulse labeled with [^{35}S]methionine and then chased for the indicated times in media containing unlabeled methionine. ^{35}S-labeled Smad7-HA in anti-HA immunoprecipitates was quantified by phosphorimaging, and the levels in control cells (squares) and Smurf2-expressing cells (circles) were plotted relative to the amount present at time 0. Data represents the average of two experiments ± SD.

(C) Endogenous interaction between Smad7 and Smurf2. Left panel, Smurf2 antibodies recognize Smurf2 and not Smurf1. Lysates from 293T cells transfected with Flag-Smurf2 or Flag-Smurf1 were subjected to immunoblotting with Smurf2 polyclonal antiserum. Smurf protein expression was confirmed by anti-Flag immunoblotting. Middle panel, U4A/Jak1 cells express Smurf2. U4A/Jak1 cells treated with or without IFNγ were lysed and immunoblotted with anti-Smurf2 or preimmune serum. Cell lysates from Smurf2-transfected 293T cells were immunoblotted in parallel. Right panel, endogenous interaction between Smad7 and Smurf2. Cell lysates from U4A/Jak1 cells were subjected to immunoprecipitation with an affinity-purified anti-Smad7 antibody followed by immunoblotting with Smurf2 antiserum. To confirm expression of Smad7 and Smurf2, cell lysates were immunoblotted with affinity-purified Smad7 and Smurf2 antibodies.

(D) The PY motif in Smad7 is important for mediating interaction with Smurf2. 293T cells were transfected with Flag-Smurf2 either alone or together with wild-type (WT) or mutant Y211A (YA) or ΔPY versions of Smad7-HA. Cell lysates were subjected to anti-Flag immunoprecipitation, and coprecipitating Smad7 proteins were detected by immunoblotting with Smad7 antiserum. Smad7 expression was confirmed by immunoblotting aliquots of total cell lysates (bottom panel).

(E) The WW domains of Smurf2 are necessary for binding to Smad7. 293T cells were transfected with Smad7-HA and either wild-type (WT) or mutant (ΔWW1, ΔWW2, or ΔWW3) versions of Flag-Smurf2. Cell lysates were subjected to anti-Flag immunoprecipitation, and coprecipitating Smad7 was detected by immunoblotting with an anti-HA antibody. Smad7 expression was confirmed by immunoblotting aliquots of total cell lysates (bottom panel).

tion. For this, we expressed TβRII, TβRI, and Smurf2 (C716A) in the absence or presence of Smad7 and examined the subcellular distribution of the appropriate protein by immunofluorescence microscopy. Like Smad7, Smurf2 was found predominantly in the nucleus (Figure 3A, i). This localization was not altered in the presence of TGFβ receptor complexes (Figure 3A, ii), which were found predominantly in a punctate pattern as described previously by us and others (Henis et al., 1994; Tsukazaki et al., 1998). However, when Smad7 was coexpressed, Smurf2 was now found predominantly outside the nucleus and was extensively colocalized with the TGFβ receptors (Figure 3A, iii). These results suggest that Smad7 expression leads to the export of Smurf2 from the nucleus and recruitment to the TGFβ receptor complex.

To explore in more detail how the subcellular distribution of Smad7 and Smurf2 is controlled, we examined how TGFβ treatment of cells regulates Smad7 and Smurf2 localization. Similar to Smurf2, Smad7 was predominantly in the nucleus when overexpressed alone (Figure 3B, i). Surprisingly, we found that simply coexpressing Smurf2 with Smad7 caused redistribution of both proteins into the cytosol in the absence of TGFβ signaling (Figure 3B, ii). Furthermore, upon TGFβ treatment a substantial proportion of Smad7 and Smurf2 redistributed to the plasma membrane region of the cell, presumably due to Smad7-dependent binding to acti-

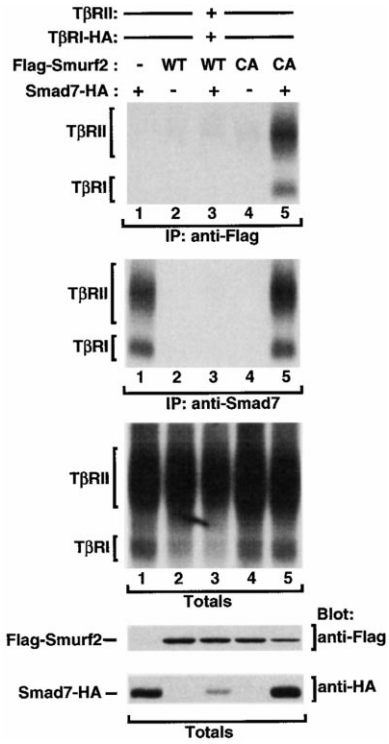

Figure 2. Smad7 Recruits Smurf2 to the TGFβ Receptor Complex

COS-1 cells were transfected with combinations of TβRII, TβRI-HA, Smad7-HA, and wild-type (WT) or mutant (C716A) Flag-Smurf2 as indicated. Cells were affinity labeled with [^{125}I]TGFβ, and lysates immunoprecipitated with the anti-Flag antibody or Smad7 antiserum; coprecipitating receptor complexes were visualized by autoradiography. Total cell lysates were analyzed by autoradiography for total receptor levels, and for Smurf2 and Smad7 levels by immunoblotting.

vated TGFβ receptors (Figure 3B, iii). We also tested Smad7(Y211A) and found that both mutant Smad7 and Smurf2 remained in the nucleus, even in the presence of TGFβ signaling (Figure 3B, iv). Together, these data indicate that the cytosolic accumulation of the Smad7–Smurf2 complex is not directly regulated by TGFβ, but is mediated by their physical association. To investigate this further, we examined Smurf2 localization in IFNγ-treated U4A/Jak1 cells. In 96% of the transfected cells, Smurf2 was localized throughout the cell (Figure 3C, i). However, treatment with IFNγ caused Smurf2 to accumulate predominantly in the cytosol in ~40% of the cells (Figure 3C, ii). Moreover, treatment of the cells with an antisense oligonucleotide to Smad7, which blocks expression of Smad7 protein (Ulloa et al., 1999), significantly inhibited IFNγ-dependent Smurf2 export, whereas sense oligonucleotides had no effect (Figure 3C, iii, and data not shown). Thus, induction of Smad7 expression can mediate cytosolic accumulation of Smad7–Smurf2 complexes independently of TGFβ signaling.

Smurf2 Induces Degradation of TGFβ Receptors and Smad7

The strong decrease in Smad7-associated receptors in the presence of wild-type but not mutant Smurf2 suggested the possibility that Smurf2 might catalyze degradation of Smad7-bound receptor complexes. To analyze Smurf2-dependent turnover of TGFβ receptor complexes, we coexpressed TβRII and TβRI in 293T cells. This causes assembly of heteromeric receptor complexes, thus allowing us to investigate the turnover of the entire receptor pool. Smurf2 had minimal effects on the steady-state levels of type II or type I receptors when the receptors were expressed either alone or together (Figure 4A). However, in the presence of wild-type Smad7, increasing Smurf2 expression led to a strong decrease in the steady-state levels of the type I receptor (Figure 4B). In contrast, Smurf2(C716A) had no effect. We also tested a constitutively active version of the type I receptor, TβRI(T204D), which signals in the absence of the type II receptor and also binds Smad7 (Hayashi et al., 1997). Similar to receptor complexes, wild-type Smurf2, but not Smurf2(C716A), caused a decrease in the steady-state levels of the activated type I receptor (Figure 4B). To confirm these findings, we analyzed by pulse–chase the half-life of TβRII and TβRI (Figure 4C). Similar to previous studies, the type II receptor had a half-life of ~1 hr, whereas TβRI was more stable, with a half-life of ~4–6 hr (Koli and Arteaga, 1997; Wells et al., 1997). Furthermore, the half-life of the type I receptor was unchanged when either Smad7 or Smurf2 was expressed individually with the receptors. However, when Smurf2 and Smad7 were coexpressed with the receptor complex, the half-life of the type I receptor was decreased to ~1 hr. Thus, Smad7 and Smurf2 enhance the turnover of the type I receptor.

Ubiquitin-dependent proteolysis of membrane receptors can be mediated by both the proteasome and lysosome (Hicke, 1999). To test whether Smurf2-dependent degradation of the TGFβ receptor occurred through these pathways, we assessed the turnover of receptors in the presence and absence of lactacystin and chloroquine, which inhibit protein degradation by the proteasome and lysosome, respectively. Pulse–chase analysis of receptors revealed that each inhibitor on its own caused stabilization of a subset of the total TGFβ receptor pool (Figure 4D), suggesting that both the proteasome and the lysosome contribute to the enhanced turnover of the receptors that is mediated by Smad7 and Smurf2.

In the course of these analyses, we also evaluated Smad7 protein levels. In the absence of TGFβ receptors, Smad7 steady-state level and turnover was slightly affected by Smurf2 (see Figures 1A and 1B). However, in the presence of TGFβ receptor complexes, Smad7 steady-state levels and half-life were decreased by Smurf2 (Figures 4B and 4C, respectively). Furthermore, this decrease in Smad7 was dependent on the catalytic activity of the Smurf2 HECT domain, since expression of Smurf2(C716A) did not alter Smad7 levels. Smad7 turnover was also stabilized by lactacystin and chloroquine, suggesting that like the receptor complex, Smad7 is degraded by both proteasomal and lysosomal pathways. Thus, in the presence of TGFβ signaling, Smurf2 induces degradation of Smad7, possibly by targeting the entire receptor–Smad7 complex.

To investigate ubiquitination of the receptors and Smad7, we expressed an HA epitope–tagged version of ubiquitin and evaluated ubiquitin conjugates of TβRII, TβRI, or Smad7 by immunoprecipitation followed by im-

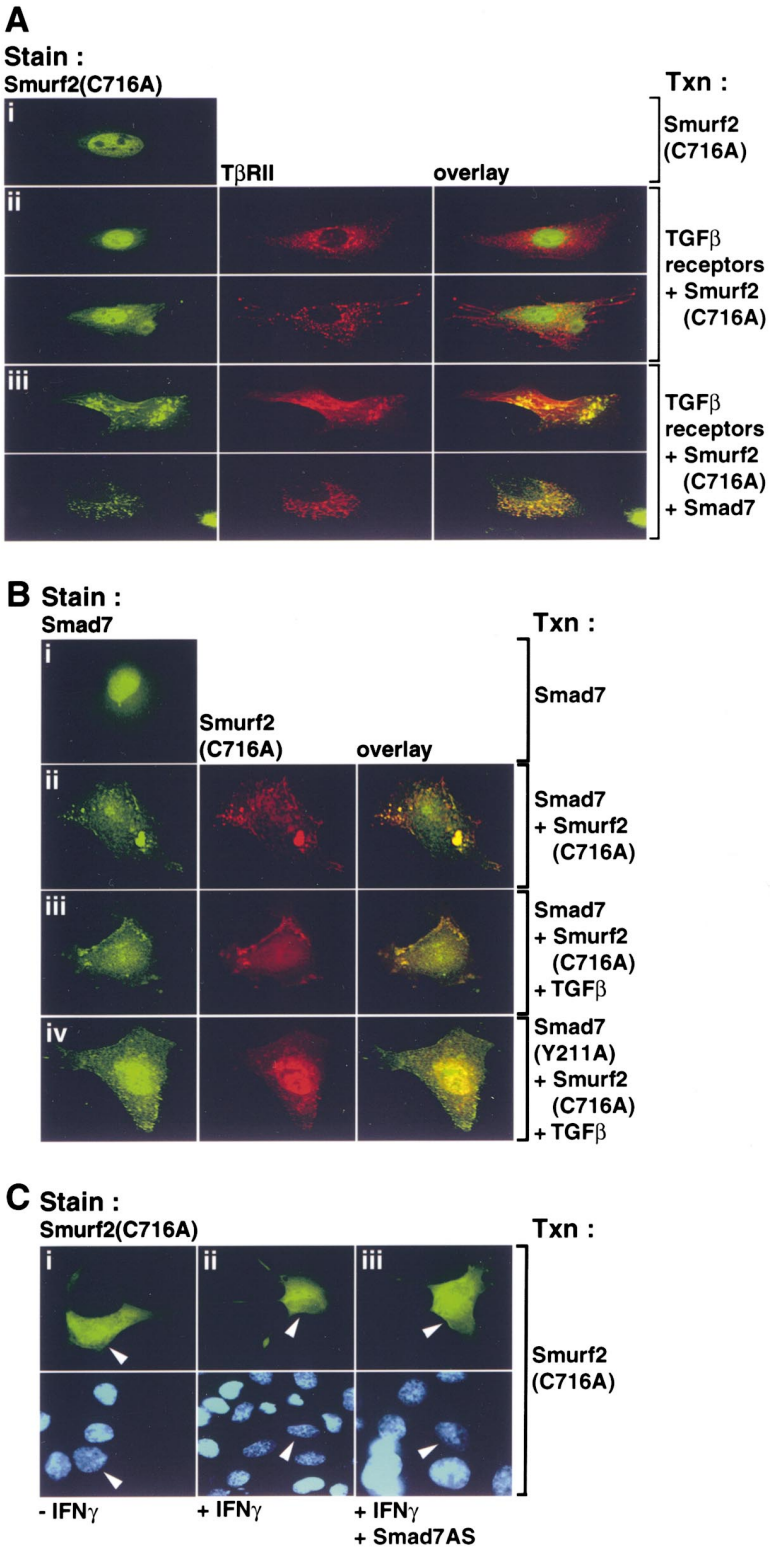

Figure 3. Smad7 Controls the Subcellular Localization of Smurf2

The subcellular localization of expressed proteins was visualized by immunofluorescence and deconvolution microscopy.

(A) Mv1Lu cells were transiently transfected with Flag-Smurf2(C716A) alone (i), Flag-Smurf2 (C716A) with TβRII-HA and TβRI-His (ii), and Flag-Smurf2(C716A) with Smad7, TβRII-HA, and TβRI-His (iii). Smurf2(C716A) was visualized with an anti-Flag monoclonal antibody followed by FITC-conjugated goat anti-mouse IgG (green). TβRII was visualized with the polyclonal anti-HA and Texas red–conjugated goat anti-rabbit IgG (red). Colocalization of Smurf2 and TβRII (overlay) appears as yellow.

(B) Mv1Lu cells were transiently transfected with Smad7-HA alone (i), Smad7-HA with Flag-Smurf2(C716A) (ii and iii), and Smad7 (Y211A)-HA with Flag-Smurf2(C716A) (iv). Cells were either unstimulated (i and ii) or stimulated with 100 pM TGFβ for 1 hr (iii and iv), and the subcellular localization of Smad7 was detected with a polyclonal anti-HA and FITC-conjugated goat anti-rabbit IgG (green). Smurf2 was visualized using monoclonal anti-Flag and Texas red–conjugated goat anti-mouse IgG (red). Colocalization of Smurf2 and Smad7 (overlay) appears as yellow.

(C) U4A/Jak1 cells were transiently transfected with Flag-Smurf2(C716A) alone (i and ii) or together with 8 μg/ml Smad7 antisense oligonucleotide (iii). Cells were either unstimulated (i) or stimulated with 500 U/ml of IFNγ (ii and iii). Smurf2(C716A) was visualized as in (A), and nuclei were detected using 4′, 6-diamidino-2-phenylindole staining (blue). Arrows indicate the location of the nucleus in stained cells. A representative result from three separate experiments is shown.

munoblotting. Analysis of Smad7 ubiquitination revealed that in the presence of Smurf2 a slight amount of ubiquitin–Smad7 conjugate was observed (Figure 4E), consistent with our observation that Smurf2 had a slight effect on Smad7 turnover (Figure 1B). However, when Smurf2 was coexpressed with Smad7 and the TGFβ receptors, we observed a strong increase in high–molecular weight ubiquitin conjugates of Smad7 that was not detected when the catalytic mutant of Smurf2 was used (Figure 4E). In contrast to Smad7, we were unable to detect significant Smurf2-dependent ubiquitination of the type I receptor (data not shown). The inabil-

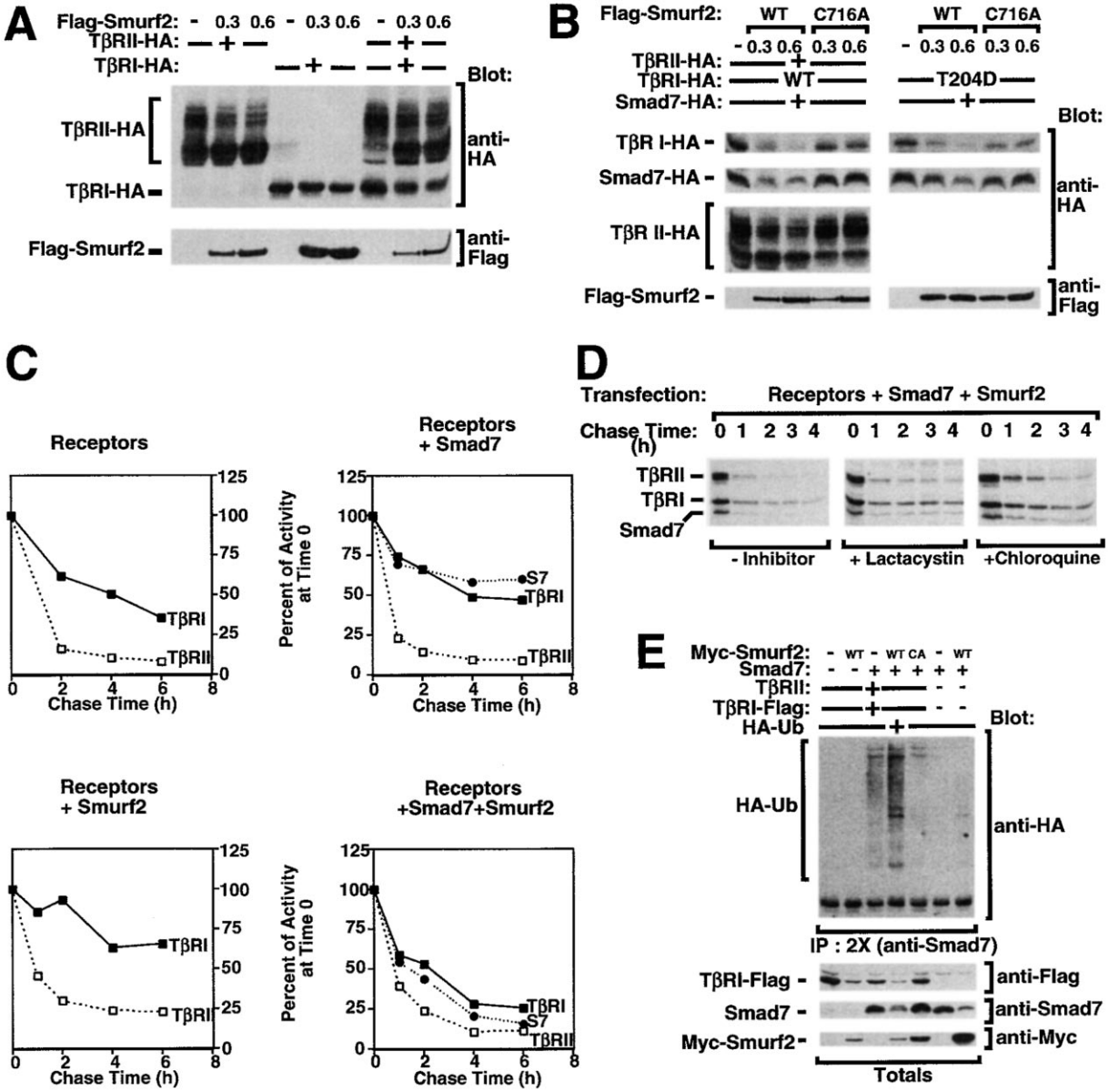

Figure 4. Smurf2 Induces Degradation of TGFβ Receptors and Smad7

(A) Smurf2 expression in the absence of Smad7 does not decrease receptor steady-state levels. 293T cells were transfected with combinations of TβRII-HA, TβRI-HA, and varying amounts of Flag-Smurf2 (plasmid DNA in micrograms). Expression levels of proteins were determined by immunoblotting aliquots of total cell lysates.

(B) Smurf2 in the presence of Smad7 causes a decrease in steady-state receptor levels. 293T cells were transfected with Smad7-HA, either TβRII-HA and TβRI-HA (left panels), or with a constitutively active type I receptor, TβRI-HA (T204D) (right panels), together with increasing amounts of wild-type (WT) or mutant Flag-Smurf2(C716A). Steady-state levels of the receptors, Smad7 and Smurf2, were determined by immunoblotting.

(C) Smurf2 increases the turnover rate of the receptor complex. COS-1 cells transfected with TGFβ receptors (TβRII-HA and TβRI-HA) alone or together with Smad7-HA, Flag-Smurf2, or both were analyzed by pulse–chase as in (1B). The amount of labeled receptors and Smad7 was quantified by phosphorimaging and is plotted relative to the amount present at time 0.

(D) Proteasome and lysosome inhibitors block Smurf2-induced degradation of the receptor complex. COS-1 cells transfected with TGFβ receptors (TβRII-HA and TβRI-HA), Smad7-HA, and Flag-Smurf2 were analyzed by pulse–chase with or without 30 μM lactacystin or 0.4 mM chloroquine. Cell lysates were subjected to anti-HA immunoprecipitation, and receptor and Smad7 levels were visualized by autoradiography.

(E) Smurf2 induces the ubiquitination of Smad7 in the presence of the receptors. 293T cells were transfected with HA-tagged ubiquitin together with combinations of Smad7, TβRII, TβRI-Flag, and wild-type (WT) or mutant (C716A) Myc-Smurf2 as indicated. Cell lysates were subjected to immunoprecipitation with Smad7 antiserum, boiled in SDS, and then reprecipitated prior to immunoblotting. Protein expression was confirmed by immunoblotting total cell lysates.

ity to detect ubiquitin-conjugated receptors may reflect rapid degradation of the receptors. Alternatively, ubiquitination of Smad7 may serve as the signal that targets the entire receptor–Smad7 complex to the proteasome. Together, these results show that Smad7-dependent recruitment of Smurf2 to the TGFβ receptor leads to proteasome- and lysosome-mediated degradation of TGFβ receptor complexes and Smad7.

Our studies in overexpression systems suggested that TGFβ receptors are degraded by the proteasome and lysosome pathways. To determine whether endogenous receptors are similarly turned over and whether TGFβ receptors can be regulated by endogenous Smad7–Smurf2 complexes, we examined the turnover of affinity-labeled receptors in Mv1Lu and U4A/Jak1 cells. In Mv1Lu cells, TGFβ receptors turned over rapidly at 37°C but were extremely stable at 4°C (Figure 5A). Treatment of the cells with either lactacystin or chloroquine stabilized the receptors, and treatment with both drugs slightly enhanced this effect (Figure 5B). These results show that like overexpressed receptors, endogenous TGFβ receptors are degraded via the proteasome and lysosome pathways.

To examine whether endogenous Smad7 and Smurf2 can regulate receptor turnover, we turned our attention to U4A/Jak1 cells, in which IFNγ induces assembly of Smad7–Smurf2 complexes (Figure 1C). In these cells, the kinetics of receptor turnover were slower than that observed in Mv1Lu cells; however, treatment of the cells with IFNγ caused some enhancement of the receptor turnover rate (Figure 5C). To determine if endogenous Smurf2 might contribute to TGFβ receptor turnover, we examined the effect of antisense oligonucleotides to human Smurf2. Treatment of U4A/Jak1 cells with the antisense oligonucleotide blocked expression of transiently expressed Smurf2, whereas the control sense oligonucleotide had no effect (Figure 5D). Next, we tested TGFβ receptor turnover in IFNγ-stimulated cells. Whereas the sense oligonucleotide to Smurf2 had no effect on receptor turnover, the antisense oligonucleotide caused substantial stabilization of the receptor complex. Furthermore, an antisense oligonucleotide to Smad7 caused a similar degree of stabilization of receptors. These results demonstrate that TGFβ receptor turnover is controlled by endogenous Smad7–Smurf2 complexes.

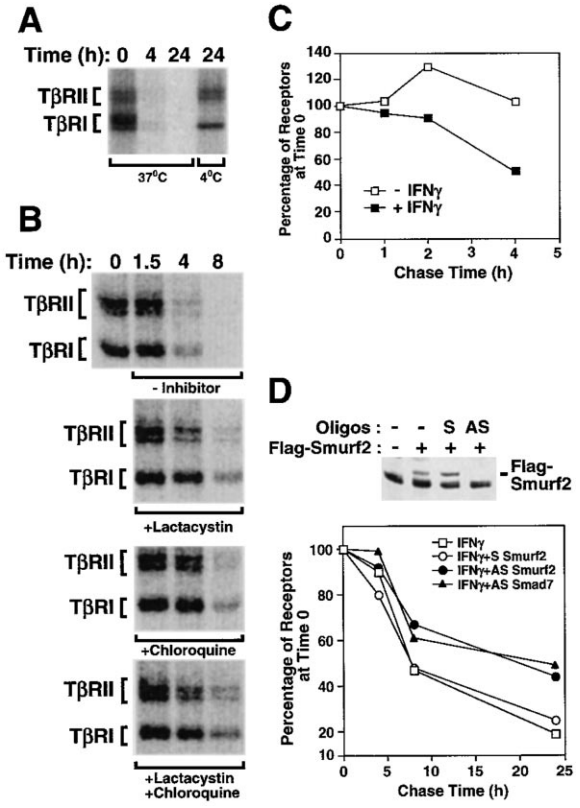

Figure 5. TGFβ Receptor Downregulation

(A) Endogenous TGFβ receptors are downregulated. Mv1Lu cells were affinity labeled with [^{125}I]TGFβ and incubated at either 4°C or 37°C for the indicated times. Total receptor expression was analyzed by autoradiography.

(B) Endogenous TGFβ receptors are degraded through both the proteasome and lysosome pathway. Mv1Lu cells were labeled with [^{125}I]TGFβ and incubated with or without lactacystin or chloroquine for the indicated times. Total receptor expression was visualized by phosphorimaging.

(C) IFNγ increases the rate of receptor downregulation. U4A/Jak1 cells were treated with or without IFNγ, and receptors were affinity labeled with [^{125}I]TGFβ. Cells were lysed at the indicated times and receptor levels visualized and quantitated by phosphorimaging.

(D) Endogenous Smurf2 and Smad7 participate in TGFβ receptor downregulation. Top panel, U4A/Jak1 cells were transfected with Flag-tagged Smurf2 either in the presence or absence of sense or antisense oligonucleotides to Smurf2. Smurf2 protein was assessed by immunoblotting whole-cell lysates. Bottom panel, U4A/Jak1 cells were transfected with either Smurf2 sense, antisense, or Smad7 antisense oligonucleotides, treated with IFNγ, and receptors labeled with [^{125}I]TGFβ. Cells were lysed at the indicated times and the receptor levels quantitated by phosphorimaging.

Association of Smurf2 Enhances the Inhibitory Activity of Smad7

Our studies indicate that Smad7 recruits Smurf2 to the TGFβ receptor complex and suggest that ubiquitin-mediated degradation of the receptor may contribute to Smad7 inhibitory activity. To test this, we first investigated whether Smad7(Y211A), which interacts poorly with Smurf2 (see Figure 1D), can recruit Smurf2 to the TGFβ receptor. The interaction of Smad7(Y211A) with TGFβ receptor complexes was comparable to wild-type Smad7 (Figure 6A). However, Smurf2(C716A) association with TGFβ receptors was substantially reduced in the presence of mutant Smad7. Next, we investigated whether Smad7(Y211A) had altered inhibitory activity in HepG2 cells, which express endogenous Smurf2. As described previously (Hayashi et al., 1997; Nakao et al., 1997), wild-type Smad7 strongly reduced TGFβ-dependent induction of the 3TP-lux reporter construct (Figure 6B). In contrast, the Smad7(Y211A) mutant had substantially reduced inhibitory activity, despite its efficient interaction with TGFβ receptors. Previous work showed that Smad7 can prevent access of Smad2 to the TGFβ receptors (Hayashi et al., 1997; Nakao et al., 1997). Therefore, we examined whether the mutant might retain inhibitory activity at higher levels of expression. For this, we compared the inhibitory activity of Smad7 versus Smad7(Y211A) by varying the amount of Smad7 expres-

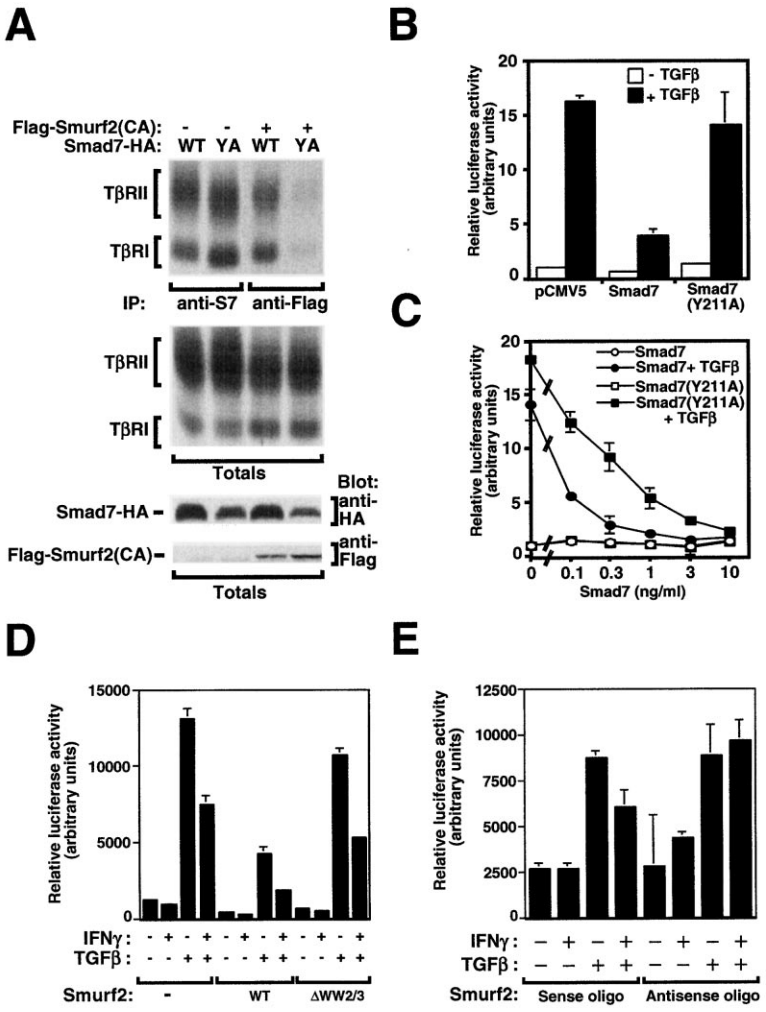

Figure 6. Association of Smurf2 with Smad7 Enhances Smad7 Inhibitory Activity

(A) Smad7(Y211A) binds to TGFβ receptors but has a reduced ability to recruit Smurf2 to the receptor complex. COS-1 cells were transfected with TGFβ receptors (TβRII and TβRI-HA) and either wild-type (WT) or Smad7 (Y211A)-HA in the absence or presence of Flag-Smurf2(C716A). Cells were affinity labeled with [^{125}I]TGFβ, and lysates immunoprecipitated with Smad7 antiserum or anti-Flag antibodies; coprecipitating receptor complexes were visualized by autoradiography. Receptor expression in total cell lysates was determined by autoradiography and Smad7 and Smurf2 protein levels by immunoblotting.

(B and C) Smad7(Y211A) is not as effective as wild-type Smad7 in inhibiting TGFβ-dependent activation of transcription. HepG2 cells were transfected with the 3TP-Lux reporter and varying concentrations of wild-type (WT) or Smad7(Y211A)-HA. In (B), 0.3 ng/ml of each Smad7 plasmid was used. Cells were incubated in the presence or absence of TGFβ, and luciferase activity was normalized to β-galactosidase activity and is plotted as the mean ± SD of triplicates from a representative experiment.

(D) Smurf2 increases IFNγ inhibitory effect. U4A/Jak1 cells were transfected with the 3TP-lux reporter and wild-type or mutant (ΔWW2/3) Smurf2. Cells were incubated in the presence or absence of IFNγ and/or TGFβ, and luciferase activity was determined as in (B).

(E) Endogenous Smurf2 is important for IFNγ-mediated inhibition. U4A/Jak1 cells were transfected with the 3TP-lux reporter and either Smurf2 sense or antisense oligonucleotides. Cells were incubated with IFNγ and/or TGFβ, and luciferase activity was determined as in (B) from duplicate samples.

sion. Wild-type Smad7 potently inhibited TGFβ signaling at the lowest doses tested, whereas Smad7(Y211A) was much less efficient (Figure 6C). However, at the highest dose tested, Smad7(Y211A) was capable of inhibiting TGFβ signaling. These results indicate that Smad7 (Y211A) retains some inhibitory activity, probably by preventing access of Smad2 or Smad3 to the TGFβ receptor. Together, these data suggest that Smurf2 enhances the inhibitory activity of Smad7.

To examine the cooperativity between Smurf2 and Smad7, we tested whether Smurf2 expression might modulate IFNγ-dependent inhibition in U4A/Jak1 cells. As previously reported, IFNγ inhibited TGFβ signaling in these cells (Figure 6D; Ulloa et al., 1999). Expression of Smurf2 alone also resulted in inhibition of TGFβ signaling and together with IFNγ reduced the TGFβ response close to basal levels. In contrast, a mutant of Smurf2 in which WW domains 2 and 3 were deleted had no effect, suggesting that the inhibitory activity of Smurf2 is dependent on its interaction with Smad7. Since TGFβ itself induces Smad7, the inhibition we observed by expressing Smurf2 alone likely reflects enhancement of this negative feedback loop. Consistent with this, the Smurf2 mutant had no effect. To further explore the requirement for Smurf2 in IFNγ inhibition, we examined the effect of Smurf2 antisense oligonucleotide in these assays. Treatment of U4A/Jak1 cells with the antisense oligonucleotide to Smurf2 reversed IFNγ inhibition of TGFβ signaling, whereas the sense oligonucleotide had no effect (Figure 6E). Together, these data indicate that Smurf2 binding to Smad7 plays an important role in mediating the inhibitory function of Smad7 at endogenous levels of expression.

Discussion

Type II and type I TGFβ receptors mediate TGFβ signaling by activating the Smad signaling pathway (Derynck et al., 1998; Massagué and Chen, 2000; Wrana, 2000). How active TGFβ receptor complexes are turned off is ill defined. TGFβ receptors can be downregulated in a variety of cell lines (Centrella et al., 1996; Koli and Arteaga, 1997; Wells et al., 1997; Anders et al., 1998; Zwaagstra and O'Connor-McCourt, 1999), and activation of the type I receptor by the type II receptor is critical for this (Anders et al., 1998). The inhibitory Smads, such as Smad7, can also turn off TGFβ and BMP signaling, and are suggested to function by binding to the activated receptor complex to prevent access and phosphorylation of the respective R-Smad (Hayashi et al.,

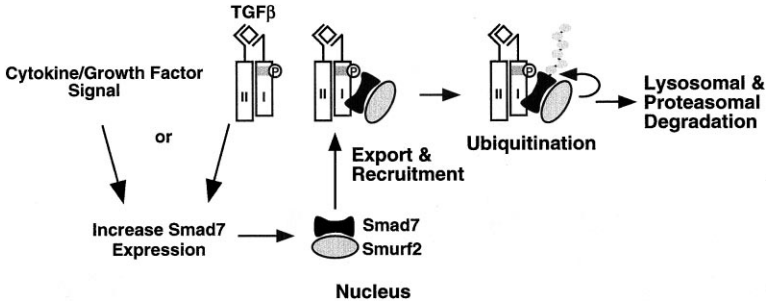

Figure 7. A Model for Smad7- and Smurf2-Mediated Degradation of the TGFβ Receptor Complex

Smad7 binds directly to Smurf2 and associates with the TGFβ receptor complex. Thus, Smad7 functions as an adaptor protein that mediates degradation of the TGFβ receptor complex.

1997; Imamura et al., 1997; Nakao et al., 1997). Here, we report on a novel ubiquitin ligase we call Smurf2 and show that Smurf2 functions in partnership with Smad7 to target the TGFβ receptor for degradation. Furthermore, we show that Smad7–Smurf2 complexes are induced by IFNγ and play an important role in mediating receptor turnover and IFNγ-dependent inhibition of TGFβ signaling.

Smad7 Functions as an Adaptor to Recruit Smurf2 to the TGFβ Receptor Complex

Smad7 binds TGFβ and BMP receptor complexes through interactions with the activated type I receptor subunit (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997). Smurf2, on the other hand, requires Smad7 for efficient interaction with the TGFβ receptor and to mediate degradation. Since Smad7 binds directly to Smurf2 and associates with the receptor complex, these results demonstrate that Smad7 functions as an adaptor protein to recruit Smurf2 to the activated receptor complex (Figure 7). Consistent with this, mutations in the Smad7 PY motif that disrupt binding to Smurf2 also interfere with Smad7-dependent association of Smurf2 with the receptor. This mutant of Smad7 was also compromised in its ability to block TGFβ signaling, indicating that Smurf2 plays a role in mediating Smad7 inhibitory function. Since Smad7 competes with R-Smads for binding to the activated TGFβ receptor, this cooperation may be particularly important when Smad7 is expressed transiently or at low levels (Nakao et al., 1997; Afrakhte et al., 1998; Ulloa et al., 1999; Bitzer et al., 2000). In agreement with this, we found that IFNγ inhibition of TGFβ signaling, which involves induction of Smad7 expression, was also dependent on expression of Smurf2 protein. Smad7 is also directly induced by TGFβ, and Smad7–Smurf2 complexes may also function in an autofeedback loop to mediate the rapid degradation of occupied TGFβ receptors. Thus, Smurf2 can provide a mechanism for removal of the Smad7-bound receptor complex, thereby resetting the Smad pathway for interpretation of subsequent TGFβ signals.

Our analysis of Smurf2 localization showed that the assembly of Smad7–Smurf2 complexes is sufficient to cause both proteins to accumulate in the cytosol, independent of TGFβ signaling. Consistent with this, IFNγ-induced accumulation of Smurf2 in the cytosol is dependent on Smad7 protein expression. Thus, the relative levels of Smad7 and Smurf2 expression are critical in determining where the proteins reside. Since Smad7 expression is under dynamic control, Smad7–Smurf2 complexes can accumulate in the cytosol in response to a variety of non-TGFβ signaling pathways. This mechanism allows Smad7–Smurf2 complexes immediate access to occupied TGFβ receptors and is likely critical for these pathways to inhibit TGFβ signaling. Indeed, blocking Smurf2 or Smad7 expression in U4A/Jak1 cells significantly stabilizes TGFβ receptors and reverses IFNγ-mediated inhibition of TGFβ signaling. Other WW domain and PY motif–containing proteins might also bind Smad7 and Smurf2, respectively, thus providing additional mechanisms to control the localization and biological function of these proteins.

The Ubiquitin Pathway in Controlling Cell Surface Signaling Receptors

The ubiquitin pathway regulates endocytosis, trafficking, and downregulation of cell surface signaling receptors and transporters. However, little is known of the E3 ligases that target these diverse membrane proteins (Bonifacino and Weissman, 1998; Hicke, 1999). The RING finger protein c-Cbl is an E3 ubiquitin ligase that binds directly to the EGF receptor to mediate its ubiquitination and downregulation (Joazeiro et al., 1999; Levkowitz et al., 1999; Yokouchi et al., 1999). In contrast, Smurf2 is in the C2-WW-HECT class of ubiquitin ligases, whose members include Smurf1 and Nedd4, as well as the yeast proteins Rsp5 and Pub1. Smurf1 functions to degrade the BMP-regulated Smads (Zhu et al., 1999), whereas Nedd4 binds a PPXY motif in the amiloride-sensitive sodium channel to induce ubiquitination and downregulation of the channel (Staub et al., 1997). Our study now indicates that this class of ubiquitin ligases can also use adaptor proteins to interact with their targets. Interestingly, Rsp5 is implicated in regulating ubiquitination of the α factor receptor, Ste2, and the Gap1 and Fur4 permeases in *S. cerevisae*, but no direct interaction with these proteins has been found (Hein et al., 1995; Bonifacino and Weissman, 1998; Springael and Andre, 1998). Thus, the use of adaptors by C2-WW-HECT domain proteins may be generally applicable to a wide range of membrane targets. Other E3 ubiquitin ligase systems also use adaptors. In the nucleus, p53 is targeted to the HECT ubiquitin ligase E6-AP by the viral adaptor protein E6, while F box proteins can function as adaptors to recruit a variety of proteins to SCF ubiquitin ligases (Hershko and Ciechanover, 1998).

The mechanisms whereby ubiquitin controls cell surface protein function and turnover are still unclear. Direct ubiquitination of membrane proteins has been described for a number of systems. However, ubiquitin-

dependent downregulation of the growth hormone receptor does not involve direct ubiquitination (van Kerkhof et al., 2000), and in yeast, the Rsp5 ubiquitin protein ligase regulates internalization of non-ubiquitin-dependent proteins (Hicke, 1999). These observations have led to the suggestion that ubiquitination of adaptor proteins might control the downregulation of membrane proteins (Bonifacino and Weissman, 1998; Hicke, 1999). Our results are consistent with this, since we were unable to detect Smurf2-dependent ubiquitination of the TGFβ receptor, but readily detected ubiquitination of the Smad7 adaptor protein.

Smads as Receptor Components of E3 Ubiquitin Ligases

In addition to TGFβ receptors, BMP receptors are targeted by Smad6 and Smad7. Our preliminary evidence suggests that Smad6 also interacts with Smurf2 to mediate downregulation of BMP receptor complexes (R. R., P. K., and J. L. W., unpublished data). Although our studies have focused on the role of Smad7 as the receptor component of a ubiquitin ligase complex, Smad6 and the R-Smads all contain PY motifs in their linker regions and have the potential to stably assemble with other ubiquitin ligases. Smads could thus fulfill a more general function in regulating protein degradation in response to TGFβ signaling. Consistent with this, the transcriptional corepressor SnoN is degraded in response to TGFβ signaling through interaction with Smad2 and Smad3 (Stroschein et al., 1999; Sun et al., 1999). Thus, in addition to their role as transcriptional comodulators, Smads may function as receptor components of E3 ubiquitin ligases that target specific proteins for degradation in response to TGFβ signaling. It will be interesting to determine what role this activity may fulfill in mediating TGFβ biology.

Experimental Procedures

Isolation of Smurf2

Several overlapping human clones displaying similarity to Smurf1 were identified from the expressed sequence tag (EST) database, and a full-length version of Smurf2 was constructed by PCR using two overlapping EST clones (GenBank accession #AF310676).

Construction of Plasmids

Smurf2 was epitope tagged at the amino terminus by PCR. For Smurf2 WW domain deletions, amino acids 163–185 for ΔWW1, 257–279 for ΔWW2, 303–325 for ΔWW3, and 257–325 for ΔWW2/3 were deleted. To generate the catalytically inactive ubiquitin ligase mutant of Smurf2, cysteine 716 was replaced with alanine. To generate the Smad7 PY mutants, tyrosine 211 was replaced with alanine (Y211A), or the PPPPY sequence between amino acid residues 206–212 was deleted (ΔPY). For TβRI-Flag, a Flag tag was introduced at the carboxyl terminus of the receptor. All constructs were generated by PCR and confirmed by sequencing.

Immunoprecipitation, Immunoblotting, and Affinity Labeling

For studies in mammalian cells, 293T, COS-1, and U4A/Jak1 cells were transiently transfected using calcium phosphate precipitation, DEAE-dextran, or FuGENE transfection reagent (Roche), respectively. Immunoprecipitation and immunoblotting were carried out using anti-HA monoclonal (12CA5, Boehringer), anti-HA rabbit polyclonal (Santa Cruz), anti-Myc monoclonal (9E10 ascites, Developmental Studies Hybridoma Bank), anti-Flag M2 monoclonal (Sigma), anti-Smad7, or anti-Smurf2 rabbit polyclonal antibodies. For anti-Smad7 antibodies, rabbits were immunized with bacterially produced GST-Smad7-encoding amino acids 202–260, and the serum was affinity purified using standard methods. For anti-Smurf2 antibodies, rabbits were immunized with a synthetic peptide corresponding to residues 354–365 of human Smurf2, and antibodies were affinity purified using standard methods (Research Genetics, Huntsville, AL). Immunoprecipitates, immunoblotting, and affinity labeling were performed as described previously (Macías-Silva et al., 1996; Zhu et al., 1999). Where indicated, Mv1Lu cells were incubated in low serum with or without 30 μM lactacystin (obtained from E. J. Corey, Harvard University) or 0.1 mM chloroquine. For endogenous interactions, U4A-Jak1 cells were treated with lactacystin prior to treatment. For receptor turnover, endogenous receptors were affinity labeled at 4°C and receptors in total cell lysates analyzed by SDS-PAGE and autoradiography at the indicated times. In U4A/Jak1 cells, receptors transiently transfected with sense or antisense oligonucleotides were analyzed in the presence and absence of IFNγ (500 U/ml, Calbiochem) for the indicated times. All pulse–chase and ubiquitination assays were performed as described (Zhu et al., 1999).

Antisense Oligonucleotides

Phosphorothioate single-stranded oligonucleotides matching region 1531–1551 (5′-CAATTGCTTGGGAAGTCAATT-3′) of the human Smurf2 cDNA sequence or region 108–128 of human Smad7 cDNA (Ulloa et al., 1999) were synthesized in the sense and antisense orientations (GENSET, La Jolla, CA). Cells were transfected with 8 μg/ml oligonucleotide using FuGENE overnight.

Subcellular Localization by Immunofluorescence Deconvolution Microscopy

Mv1Lu and U4A/Jak1 cells, plated on gelatin-coated Permanox chamber slides (Nunc), were transfected with the indicated constructs. Fixation, permeabilization, and reaction with the primary and secondary antibodies were described previously (Tsukazaki et al., 1998). Images were obtained using the Olympus 1X70 inverted microscope equipped with fluorescence optics and Deltavision deconvolution microscopy software (Applied Precision).

Transcriptional Response Assay

HepG2 cells were transiently transfected using calcium phosphate DNA precipitation. The next day, cells were incubated overnight with or without 100 pM TGFβ. U4A/Jak1 cells were transiently transfected using FuGENE. The next day, cells were either incubated for 1 hr with or without IFNγ (500 U/ml) followed by an overnight incubation with 250 pM TGFβ (Figure 6D), or with 40 pM TGFβ for 4 hr (Figure 6E).

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