Smad4 Protein Stability Is Regulated by Ubiquitin Ligase SCFβ-TrCP1*

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Smad4 is a key intracellular mediator for the transforming growth factor-β (TGF-β) superfamily of growth factors and is also an important tumor suppressor. The receptor-regulated Smad (R-Smad) proteins are regulated by ubiquitin-mediated degradation, yet the precise control of Smad4 protein stability is unclear. We have identified SCFβ-TrCP1, a ubiquitin (E3) ligase, as a critical determinant for the protein degradation of Smad4 protein. F-box protein β-TrCP1 in this E3 ligase interacts with Smad4 both in yeast and in mammalian cells, but has no interaction with Smad2 and has weak interaction with Smad3. The β-TrCP1/Smad3 interaction was abolished by Smad4 gene silencing, indicating the interaction is indirect and is through Smad4. Ecotropic expression of SCF complex containing β-TrCP1 is sufficient to induce the ubiquitination and degradation of Smad4. Furthermore, small interfering RNA-triggered endogenous β-TrCP1 suppression increases the expression of Smad4 protein. Consistent with these results, cells that overexpress the SCF complex display an inhibited TGF-β-dependent transcriptional activity and an impaired cell cycle arrest function. Thus, SCFβ-TrCP1 abrogates TGF-β function in vivo by decreasing Smad4 stability.

Transforming growth factor-β (TGF-β) and related polypeptides constitute the largest cytokine family and regulate many aspects of cellular processes such as proliferation, differentiation, adhesion, and apoptosis. Signaling responses to TGF-β-like factors are mediated by two types of transmembrane receptors, TGF-β type I receptor and type II receptor, and their intracellular substrates, the Smad proteins (1). Following ligand binding, the activated receptors directly phosphorylate regulatory Smads (R-Smads). Phosphorylated R-Smads form complexes with Smad4 and accumulate in the nucleus to regulate transcription of a variety of genes. Thus, Smad4 is the common signaling molecule shared by the entire TGF-β superfamily, and it is critical for transcriptional activation. Proper TGF-β signaling requires precise control of Smad functions. It is reported that Smads are regulated by ubiquitin-mediated degradation. Specifically, Smurf1, a Hect-domain-containing E3 ligase triggers the degradation of Smad1 and Smad5 and inhibits bone morphogenetic protein signaling (2). Smurf2, a Smurf1-related ligase, can target Smad1 and sometimes Smad2 for degradation in a TGF-β-dependent manner (3, 4). It remains to be determined whether and how the ubiquitin–proteasome pathway regulates Smad4 degradation.

Protein degradation by the ubiquitin–proteasome pathway plays a vital role in monitoring the abundance of many regulatory proteins. A polyubiquitin chain is built onto one or multiple lysine residues of a substrate to target it for capture and degradation by the 26 S proteasome. Ubiquitination of proteins requires a multi-enzyme system. A key component of ubiquitination pathways, the ubiquitin (E2) ligases, controls both the specificity and timing of substrate ubiquitination. A class of ubiquitinating ligase is the SCF complexes formed by Skp1, cullin1, Roc1, and a variable F-box protein (5), which serves as the substrate recognition subunit. SCF complex that contains β-TrCP as the F-box protein (SCFβ-TrCP) recognizes phosphorylated IκB (6), β-catenin (7), NF-κb p105 (8), ATF4 (9), and Cdc25A (10) and mediates their protein degradation.

We previously demonstrated that Jun activation domain binding protein 1 (Jab1) interacts with Smad4 and induces its degradation (11). Jab1 is one of the eight subunits of the COP9 signalosome (CSN) that also induces the degradation of p53 (12) and p27 (13). In this study, we sought to identify a Jab1-correlated E3 ligase that mediates Smad4 degradation. We found an E3 ligase complex SCFβ-TrCP that is responsible for Smad4 ubiquitination and degradation. F-box protein β-TrCP1 in this complex associates with Smad4 and SCFβ-TrCP overexpressing cells display increased ubiquitination and degradation of Smad4. Consequently, SCFβ-TrCP reduced the transcriptional activity and cellular function of TGF-β signaling.

EXPERIMENTAL PROCEDURES

Immunoprecipitation and Western Blotting Analysis—Cells were lysed in 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholic acid, 50 mM Tris buffer, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin. For immunoprecipitation, lysed cells were incubated with different antibodies as indicated in the figures and protein G plus agarose beads (Amersham Biosciences) were added. Immunocomplexes were washed and were separated on SDS-polyacrylamide gel and blotted to nitrocellulose. All blots were developed by the ECL technique (Amersham Biosciences).

Pulse-Chase Assays—To measure the degradation rate of Smad4, cells were incubated at 48 h after transfection with Dulbecco’s modified Eagle’s medium without methionine and cysteine for 30 min and were pulse-labeled with 100 µCi of [35S]methionine-cysteine protein labeling mix per ml for 40 min. The cells were then washed and incubated in complete Dulbecco’s modified Eagle’s medium for various periods of time. The cells were lysed in radioimmunoprecipitation assay buffer, and the protein extract was immunoprecipitated with anti-FLAG M2-agarose affinity gel (Sigma). The precipitates were separated on a 10% polyacrylamide gel before autoradiography.

In Vivo Ubiquitination Assay—Forty hours after transfection, cell lysates were immunoprecipitated using antibody against FLAG, boiled, and then reprecipitated prior to immunoblotting. To detect ubiquitina-
**RESULTS**

We had previously demonstrated that Jab1 interacts with Smad4 and induces its degradation (11). Jab1, integrated in COP9 complex, has intense association with SCF E3 ligases (14). The SCF E3 ligases consist of four subunits including Cullin, Roc1, Skp1, and a variable F-box protein, which function as the substrate recognition subunit. To determine whether SCF complex ligase is responsible for Smad4 ubiquitination and degradation, we first tested whether F-box proteins in SCF E3 ligase interact with Smad4. β-TrCP1 and Skp2 are two well-characterized F-box proteins in mammalian cells. Both F-box proteins were examined for their possible interaction with Smad4 in a yeast two-hybrid assay. Liquid β-galactosidase assay results indicate that Smad4 interacts with β-TrCP1 but not with Skp2. None of the other Smad proteins interacted with either β-TrCP1 or Skp2 (Fig. 1A), suggesting that Smad4 specifically associates with SCFβ-TrCP1. To confirm the Smad4/β-TrCP1 interaction in mammalian cells, we overexpressed the epitope-tagged versions of these proteins in 293T cells and assessed their ability to coimmunoprecipitate. The results demonstrated that FLAG-Smad4 communiprecipitated with Myc-β-TrCP1 antiserum (Fig. 1B, lane 4) when both proteins were overexpressed. Potential interactions of β-TrCP1 with Smad2 or Smad3 were also examined. β-TrCP1 has non-detectable interaction with Smad2 and weak interaction with Smad3 (Fig. 1B, lanes 2 and 3). To further investigate whether the β-TrCP1/Smad3 interaction is via Smad4, immunoprecipitation assays were then performed in endogenous Smad4 silenced cells using small interfering RNA (siRNA) from DNA templates in vivo. The β-TrCP1/Smad3 interaction was abolished by cotransfection with higher dose of BS/U6/Smad4 plasmid but not with an irrelevant control plasmid, BS/U6/GFP (Fig. 1C), indicating that this interaction is via Smad4. Furthermore, endogenous Smad4 also communiprecipitated with endogenous β-TrCP1, and Jab1 or TGF-β treatment significantly enhances the interaction (Fig. 1D). Taken together, these results indicate that Smad4 specifically interacts with F-box protein β-TrCP1.

We then examined whether the association of SCFβ-TrCP1 with Smad4 regulates endogenous Smad4 steady-state level in cells. FLAG-tagged Smad4 was co-transfected into 293T cells with either a single component of the SCF complex (Roc1, Cul1, or β-TrCP1) or in various combinations of the components. While individual component of the SCF complex did not affect the expression of Smad4 (Fig. 2A, lanes 2–4), Cul1 with Roc1 or β-TrCP1 (lanes 6 and 8) significantly reduces Smad4 expression. Smad4 expression was further decreased in the presence of all three components (lane 9). We attempted to determine whether the suppression of endogenous β-TrCP1 by siRNA would increase Smad4 expression. 293T cells were transfected with either BS/U6/GFP or BS/U6/β-TrCP1 plasmid. BS/U6/β-TrCP1 dose-dependently reduced endogenous β-TrCP1 expression (Fig. 2B, lanes 4 and 5 in the first panel). In contrast to the effect of SCFβ-TrCP1, the expression level of endogenous Smad4 increased in the β-TrCP1 siRNA-transfected cells (second panel). BS/U6/GFP, an irrelevant siRNA control, did not change the expression level of both β-TrCP1 and Smad4 (Fig. 2B, lanes 2 and 3). The observed low Smad4 expression levels in the presence of SCF complex suggested that the SCF complex might have led to increased protein turnover of Smad4. To investigate this possibility, SCF components were co-transfected with FLAG-tagged Smad4 plasmids into 293T cells, and the protein degradation rate was determined in 293T cells by pulse-chase assays. The 35S-labeled Smad4 protein in the presence of SCF E3 ligase rapidly decreased and disappeared at 16 h (Fig. 2C, lower panel). In contrast, 35S-labeled Smad4 without SCF E3 ligase overexpression was considerably more stable and was still present at 16 h (upper panel). The half-life of Smad4 in the absence of SCF was ~16 h, whereas the half-life of Smad4 in the presence of SCF was ~7 h (Fig. 2D). We then examined whether SCFβ-TrCP1 degraded R-Smads. The SCF complex (Roc1, Cul1, and β-TrCP1) was cotransfected with epitope-tagged version of Smad2, Smad3, or Smad4 in 293T cells in the presence of TGF-β. SCF complex down-regulated Smad4 expression (Fig. 2E, lanes 6), modestly decreased Smad3 expression (lane 4), and has no effect on Smad2 expression (lane 2) in the presence of TGF-β stimulation.

Potential Smad4 ubiquitination mediated by SCFβ-TrCP1 was examined by in vivo ubiquitination assays. Overexpression of SCF components (Roc-1, Cul1, β-TrCP1) in 293T cells promotes the ubiquitination of ectopically expressed FLAG-tagged Smad4 (Fig. 2F, lanes 2 and 3). The effect of TGF-β on SCFβ-TrCP1-induced Smad4 ubiquitination was examined by adding TGF-β in cell culture medium 24 h after gene transfection. The results demonstrated that TGF-β further enhanced the ubiquitination of Smad4 (Fig. 2G, lane 4). Collectively, these data strongly indicate that SCFβ-TrCP1 is a specific E3 ligase that mediates Smad4 degradation and TGF-β enhances the degradation.

Smad4 is the only common partner of all the R-Smads and is also known as an activator in R-Smad-induced gene transcription. Conventional views would predict that, because it targets

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**Fig. 1. Interaction of Smad4 with SCF complex.** A, interactions of Smad4 with F-box proteins in yeast. Intact human β-TrCP1 or human SKP2 cDNA was fused with the Gal4 DNA binding domain and transformed into yeast with indicated Smads fused to the Gal4 transactivation domain. The interactions were quantified by a liquid β-galactosidase assay. B, overexpressed Smad3 and Smad4 interact with β-TrCP1 in mammalian cells. 293T cells were transfected with the indicated vectors. Immunoprecipitation assays were performed using either anti-FLAG antibody, and the immunocomplex was detected by Western blotting using anti-Myc antibody. C, the interaction of Smad3 with β-TrCP1 was abolished by Smad4 siRNA. GFP siRNA (BS/U6/GFP) or Smad4 siRNA (BS/U6/Smad4) plasmids were cotransfected with FLAG-Smad3 and Myc-β-TrCP1 into 293T cells. Immunoprecipitation assays were performed using anti-FLAG antibody, and the immunocomplex was detected by Western blotting using anti-Myc antibody. D, Jab1 or TGF-β enhances the interaction of endogenous Smad4 with β-TrCP1. 293T cells were transfected with empty vector or HA-tagged Jab1 as indicated. TGF-β (2 ng/ml) was added into the medium of the indicated cells 12 h before lysis. Immunoprecipitation assays were performed using anti-β-TrCP1 antibody, and the immunocomplex was detected by Western blotting using anti-Smad antibody.


Smad4 for destruction, SCFβ-TrCP1 should down-regulate Smad4-mediated transcriptional activity. Different TGF-β response reporter plasmids (SBE), 4-luc (four repeats of Smad binding element), and ARE-luc (Activin response element) were cotransfected with SCF components and/or Jab1 plasmids in Mv1Lu cells. Ectopic expression of SCF components to induce Smad4 gene silencing, and flow cytometry was performed. After 40 h, total cell extracts were analyzed by immunoblotting using antibodies against FLAG or β-actin. B, β-TrCP1 siRNA increases the expression of Smad4. Empty BS/U6 vector or different doses of GFP siRNA (BS/U6/GFP) or β-TrCP1 siRNA (BS/U6/β-TrCP1) plasmids were transfected into 293T cells. Cell extracts were assayed by Western blotting with antibodies specific for β-TrCP1 (upper row), Smad4 (middle row), or β-actin (lower row). C, protein degradation rate of Smad4 increased in the presence of ectopic SCF E3 ligase. 293T cells were transfected with indicated plasmids. Cells were pulse-labeled with [35S]methionine and chased with excess cold methionine. At the indicated times, cell extracts were collected, and [35S]-labeled Smad4 in anti-FLAG immunoprecipitates was detected by autoradiography of an SDS gel. D, the intensity of the bands was quantitated by phosphorimaging and plotted relative to the amount present at time 0. E, SCFβ-TrCP1 E3 ligase modestly down-regulates Smad3 in the presence of TGF-β. 293T cells were transfected with the indicated expression vectors. After 40 h, total cell extracts were analyzed by immunoblotting using antibodies against FLAG or β-actin. F, Smad4 is ubiquitinated by SCFβ-TrCP1. 293T cells were transfected with indicated plasmids, and MG132 was added 4 h before cell lysis. Cell lysates were immunoprecipitated with FLAG antibody and then reprecipitated prior to immunoblotting with anti-ubiquitin. G, TGF-β enhances Smad4 ubiquitination by SCFβ-TrCP1 E3 ligase. 293T cells were transfected with indicated plasmids, and TGF-β was added 24 h after transfection. Cell lysates were immunoprecipitated with FLAG antiserum and then reprecipitated prior to immunoblotting with anti-ubiquitin.

Fig. 2. SCFβ-TrCP1 mediates Smad4 degradation. A, SCFβ-TrCP1 E3 ligase down-regulates Smad4 protein expression. 293T cells were transfected with the indicated expression vectors. After 40 h, total cell extracts were analyzed by immunoblotting using antibodies against FLAG or β-actin. B, β-TrCP1 siRNA increases the expression of Smad4. Empty BS/U6 vector or different doses of GFP siRNA (BS/U6/GFP) or β-TrCP1 siRNA (BS/U6/β-TrCP1) plasmids were transfected into 293T cells. Cell extracts were assayed by Western blotting with antibodies specific for β-TrCP1 (upper row), Smad4 (middle row), or β-actin (lower row). C, protein degradation rate of Smad4 increased in the presence of ectopic SCF E3 ligase. 293T cells were transfected with indicated plasmids. Cells were pulse-labeled with [35S]methionine and chased with excess cold methionine. At the indicated times, cell extracts were collected, and [35S]-labeled Smad4 in anti-FLAG immunoprecipitates was detected by autoradiography of an SDS gel. D, the intensity of the bands was quantitated by phosphorimaging and plotted relative to the amount present at time 0. E, SCFβ-TrCP1 E3 ligase modestly down-regulates Smad3 in the presence of TGF-β. 293T cells were transfected with the indicated expression vectors. After 40 h, total cell extracts were analyzed by immunoblotting using antibodies against FLAG or β-actin. F, Smad4 is ubiquitinated by SCFβ-TrCP1. 293T cells were transfected with indicated plasmids, and MG132 was added 4 h before cell lysis. Cell lysates were immunoprecipitated with FLAG antibody and then reprecipitated prior to immunoblotting with anti-ubiquitin. G, TGF-β enhances Smad4 ubiquitination by SCFβ-TrCP1 E3 ligase. 293T cells were transfected with indicated plasmids, and TGF-β was added 24 h after transfection. Cell lysates were immunoprecipitated with FLAG antiserum and then reprecipitated prior to immunoblotting with anti-ubiquitin.

DISCUSSION

Oncogene Ras (15) and Jab1 (11) have been reported to mediate Smad4 degradation, but the precise mechanisms are not clear. Here we show that β-TrCP1, a F-box protein in the SCF E3 ligase complex, interacts with Smad4 and induces the degradation of Smad4. This defines a new role for E3 ligase SCFβ-TrCP1 in our yeast two-hybrid assays and has weak interaction in mammalian cells. Furthermore, β-TrCP1/Smad3 interaction was abolished by Smad4 gene silencing, suggesting that this interaction is Smad4-dependent. F-box protein in SCF E3 ligase is the substrate recognition subunit, and the binding between F-box subunit with its protein substrate is essential for protein ubiquitination and degradation. It is most likely that β-TrCP1/Smad, but not Roc1/Smad, interaction would
mediate Smad degradation. β-TrCP1 recognizes the phosphorylated DSGLXS motif (6–10). There is a similar motif in Smad4 protein “DLSGLTLOQ.” It is possible that this motif is a Smad4 phosphorylation site for β-TrCP1 recognition.

TGF-β stimulation increases both Smad4/β-TrCP1 association and Smad4 ubiquitylation by SCFβ-TrCP1. One possibility is that the activated R-Smads upon TGF-β stimulation may play some role in this event. Upon activation by the TGF-β signaling, R-Smad forms a heteromeric complex with Smad4 and translocates into the nucleus where it regulates transcription of target genes. The R-Smad/Smad4 heteromeric complex formation may also enhance the interaction of Smad4 with SCFβ-TrCP1 and trigger the ubiquitylation and degradation of this complex. Our data demonstrated that SCF complex induced modest Smad3 degradation in the presence of TGF-β, providing indirect evidence for this hypothesis. Jab1 also enhances the Smad4/β-TrCP1 association. It is possible that Jab1 regulates the formation of the E3 SCFβ-TrCP1 ligase complex by either serving as an adaptor protein between Smad4 and β-TrCP1 or by phosphorylating Smad4 and promoting substrate recognition by its F-box protein β-TrCP1. Jab1 is one of the eight subunits in the CSN complex whose function is at the interface between signal transduction and ubiquitin-dependent proteolysis. Recent work indicates that the CSN copurifies with kinases and possesses kinase activity (12, 17). Thus, Jab1/CSN might phosphorylate Smad4 and promote its association with F-box protein β-TrCP1.

Our data also indicate that SCFβ-TrCP1 efficiently impairs TGF-β-induced biological events in cells, such as cell cycle arrest. While SCFβ-TrCP1 has little effect on the cell cycle progression in those cells with Smad4 gene silencing, indicating the effects of SCFβ-TrCP1 are Smad4-dependent. Loss of Smad4 plays a pivotal role in TGF-β resistance in tumorigenesis and is associated with poor prognosis of cancer. Thus, the mechanism underlying the degradation of Smad4 demonstrated in this study may provide some useful information for further understanding the pathogenesis of cancer disease.

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