Chromosomal Region Maintenance 1 (CRM1)-dependent Nuclear Export of Smad Ubiquitin Regulatory Factor 1 (Smurf1) Is Essential for Negative Regulation of Transforming Growth Factor-β Signaling by Smad7*

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Smad ubiquitin regulatory factor 1 (Smurf1), a HECT type E3 ubiquitin ligase, interacts with inhibitory Smad7 and induces translocation of Smad7 to the cytoplasm. Smurf1 then associates with the transforming growth factor (TGF)-β type I receptor, TβR-I, enhancing turnover. However, the mechanism of nuclear export of Smad7 by Smurf1 has not been elucidated. Here we identified a functional nuclear export signal (NES) in a C-terminal region of Smurf1. In transfected cells, the Smurf1-Smad7 complex was accumulated in the cytoplasm by the nuclear export receptor, CRM1; this action was prevented by treatment with leptomycin B, a specific inactivator of CRM1 function. A green fluorescence protein fusion protein containing the C-terminal NES motif of Smurf1, located in the cytoplasm, accumulated in the nucleus following treatment with leptomycin B. Moreover, Smurf1 was shown to bind physically to CRM1 through NES, and nuclear export of the Smurf1-Smad7 complex was prevented by mutations of Smurf1 within the NES. Finally, the Smurf1 NES mutant reduced inhibition by Smad7 of the transcriptional activity induced by TGF-β. These results thus suggest that CRM1-dependent nuclear export of Smad7 by Smurf1 is essential for the negative regulation of TGF-β signaling by Smad7.

Members of the transforming growth factor-β (TGF-β)1 superfamily are multifunctional cytokines that regulate growth, differentiation, apoptosis, and morphogenesis (1). TGF-β superfamily proteins, including TGF-β, activins, and bone morphogenetic proteins, initiate cellular responses by binding to type I and type II serine/threonine kinase receptors. 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 mainly through Smad proteins. Eight different Smad proteins have been identified in mammals and 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 are direct substrates of the type I receptors. They are phosphorylated by type I receptors and form heteromeric complexes with Co-Smad, Smad4. The Smad complexes then translocate into the nucleus, where they regulate transcription of various target genes together with transcriptional factors and co-activators (3). Among R-Smads, Smad2 and Smad3 act in the TGF-β, activin, and nodal pathways, whereas Smad1, Smad5, and Smad8 function in the bone morphogenetic protein and anti-Müllerian hormone pathways.

In contrast to R-Smads and Co-Smad, I-Smads, including Smad6 and Smad7, bind to type I receptors and compete with R-Smads for activation by the receptors, resulting in inhibition of TGF-β superfamily signaling (6–8). Interestingly, however, Smad7 has been reported to be located in the nucleus in many types of cells (9), although it is observed in the cytoplasm in certain cells (10). Recently, HECT type E3 ubiquitin ligases, Smad ubiquitin regulatory factor 1 (Smurf1) and Smurf2, have been reported to interact with Smad7 in the nucleus and to induce translocation of Smad7 to the cytoplasm. The Smurf-Smad7 complexes then associate with TGF-β type I receptor (TβR-I) enhancing its turnover (11, 12). However, the mechanism of nuclear export of the Smurf-Smad7 complex has not been elucidated.

The transport of many large proteins from the nucleus to the cytoplasm is mediated by a short leucine-rich motif, known as the nuclear export signal (NES) sequence. The consensus sequence is defined as a set of critically spaced large hydrophobic residues, usually leucines LXX/XXX/XXXLXX/XXX/LX, where X is phenylalanine, PI, 4,6-diamidino-2-phenylindole; GFP, green fluorescence protein; CRM1, chromosomal region maintenance 1.

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indicates any residue) (13), although some variations exist. The NESs of human immunodeficiency virus-1 Rev, cyclic AMP-dependent protein kinase inhibitor, and MAPK kinase have been well characterized. Mutations of leucines in NES disrupt the ability of the protein to locate in the cytoplasm. CRM1, which belongs to the family of importin-β-related nuclear transport receptors, directly and specifically associates with NES and mediates nuclear export of the protein containing NES (14, 15).

Leptomycin B (LMB) directly binds to CRM1 and disrupts the interaction between CRM1 and NES, resulting in the inhibition of the effects of CRM1 (16).

Smad1 and Smad3 contain a lysine-rich nuclear localization signal in their MH1 domains that is required for ligand-induced nuclear translocation. In Smad3, phosphorylation of the C-terminal SSXS motif results in conformational changes that expose the nuclear localization signal, so that importin-β1 can
bind Smad3 and mediate its nuclear import (17–19). Smad1 contains a C-terminal leucine-rich NES that mediates its constant nuclear export by interacting with CRM1 (20). Smad1 is therefore continuously shuttling between the nucleus and the cytoplasm. Smad4 has an NES at the linker region, which is not conserved in R-Smads. Smad4 does not accumulate in the nucleus by itself, because of the presence of the NES. Only upon complex formation with R-Smads is the NES masked, enabling Smad4 to accumulate inside the nucleus (21, 22). In I-Smads, neither the NES in Smad4 nor that in Smad1 is conserved, and the putative NES of I-Smads has not been identified.

In the present study, we have shown that Smurf1 has a functional NES in the C-terminal region. The Smurf1 NES mutant did not transport Smad7 from the nucleus to the cytoplasm, and it reduced inhibition by Smad7 of TGF-β transcriptional activity, suggesting that CRM1-dependent nuclear export of Smurf1 is essential for negative regulation of the TGF-β pathway by Smad7.

MATERIALS AND METHODS

DNA Construction and Transfection—The original constructs of the constitutively active form of TjR1 (c.a.TjR1), Smurf1(WT), Smurf1(CA), and Smad7 were generated as described previously (6, 12, 23). Construction of the Smurf1 NES mutant (Smurf1(NES-mut)) and CRM1 were performed by a polymerase chain reaction (PCR)-based approach. Smurf1(NES-mut) does not contain the CA inactivation mutation. COS7 cells, 293T cells, HeLa cells, and I mutant mink lung epithelial (Mv1Lu) cells were transiently transfected using FuGENE6 (Roche Molecular Biochemicals) as described (24).

Immunofluorescence Labeling—Immunohistochemical staining of FLAG-tagged Smad7, 6Myctagged Smurf1(CA), or 6Myctagged Smurf1(NES-mut) in transfected cells was performed using mouse anti FLAG antibody or mouse anti-Myc antibody followed by incubation with fluorescein isothiocyanate-labeled goat anti-mouse IgG or rhodamine isothiocyanate-labeled goat anti-rabbit IgG, respectively. For double staining of Smad7 and Smurf1, immunohistochemical staining of 6Myctagged Smurf1 and CRM1-HA was performed using mouse anti-Myc 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. Cell nuclei were stained by 4,6-diamidino-2-phenylindole (PI). Intraacellular localization was determined by confocal laser scanning microscopy. For LMB treatment, cells were incubated with 20 ng/ml LMB for 1 h just before fixation.

In Vitro Protein Binding Assay—GST fusion proteins were prepared by insertion of a cassette of two annealed oligonucleotides into EcoRI-Xhol-cut pEGFP-C1 (Clontech) in which the multicloning site was changed. The six NES-like sequences with three upstream residues from Smurf1, GWE-V34RSTVSGRIYT356, VKQ-L356, KVLRHELS, LSY-W466HFVGRIMALV474, END-I521TPVLDHTFCV531, and ELI606GGILDKIDL614, were fused to the C terminus of Smurf1, 6Myc-Smurf1, and Smad7 were generated as described previously (6, 12, 23). COS7 cells were transiently transfected using FuGENE6 transfection kit. GFP and transfected into cells using the FuGENE6 transfection kit. GFP images were recorded 24 h after transfection. For LMB treatment, cells were incubated with 20 ng/ml LMB for 1 h just before image recording.

RESULTS

Smurf1 Has a Functional NES—To test whether Smad7 or Smurf1 contains an NES, we examined the subcellular local-
As indicated, the sera were subjected to FLAG immunoprecipitation followed by HA together with FLAG-Smurf1(CA) or FLAG-Smurf1(NES-mut). Cell lysates were then incubated at the indicated temperatures, and the precipitate was collected and analyzed by Western blotting.

Input: a control, cell lysate was directly subjected to SDS-PAGE (Fig. 1D, top row, and Fig. 1E), and LMB induced nuclear accumulation of Smurf1(CA) (Fig. 1D, bottom row, and Fig. 1E), suggesting that Smurf1 contains a functional NES.

CRM1, the cellular export receptor for NESs, has been reported to dramatically induce nuclear export of NES-containing proteins (14, 15). We found that overexpression of CRM1 did not affect the subcellular localization of nuclear Smad7 (data not shown). However, it induced an almost exclusively cytoplasmic distribution of Smurf1(CA), and the effect of CRM1 was prevented by LMB (Fig. 1F). Although the experiments have been performed using HeLa cells in Fig. 1, similar results were obtained using COS7 cells (data not shown).

Identification of a Functional NES in Smurf1—To define the NES in Smurf1 that is responsible for nuclear export of the Smurf1-Smad7 complex, we scanned the amino acid sequence of Smurf1 and identified six putative leucine-rich NES-like motifs: amino acids 33–42, 288–298, 356–365, 463–474, 521–531, and 606–614 (Fig. 2A, left panel). To identify a functional NES in Smurf1, we generated six GFP fusion proteins containing NES-like peptides with three upstream amino acid residues flanking each NES: GFP-NES-(33–42) (FRR-L33PDFFAKIVV42), GFP-NES-(288–298) (GWE-V288RSTVSGRIYV298), GFP-NES-(356–365) (VQK-L356KVLRHELSL365), GFP-NES-(463–474) (LSY-F463HFVGRIMGLAV474), GFP-NES-(521–531) (END-I521TPVLDHTFCV531), and GFP-NES-(606–614) (ELII606GGLDKIDL614) (Fig. 2A, right panel). In conformity with the classical Rev NES, each motif contains four properly spaced large hydrophobic residues. In transfected HeLa cells, although GFP alone and five other hybrids were distributed diffusely in both the cytoplasm and nucleus, GFP-NES-(606–614) was localized in both the nucleus and cytoplasm without LMB (Fig. 1F, top panel), as indicated. Mutation in NES abolishes interaction of Smurf1 with CRM1 in transfected cells. 293T cells were transfected with CRM1-HA together with FLAG-Smurfl(CA) or FLAG-Smurfl(NES-mut). Cell lysates were subjected to FLAG immunoprecipitation followed by HA immunoblotting. The top panel shows the interaction, and the lower two panels show the expression of each protein as indicated. D, there is a cold-sensitive interaction between Smurf1 and CRM1 in vitro. GST-Smurfl(CA) or GST-Smurfl(NES-mut) was mixed with cell lysates in which CRM1-HA had been transfected. The samples were then incubated at the indicated temperatures, and the precipitates were subjected to SDS-PAGE followed by immunoblotting using anti-HA antibody or anti-GST antibody to detect GST-Smurfl(CA). As a control, cell lysate was directly subjected to SDS-PAGE (Input).

Fig. 3. Smurf1 binds to CRM1 through C-terminal NES. A, schematic representation of the functional NES motif in Smurf1. Isoleucine-612 and leucine-614 were each mutated to alanine to generate the Smurf1 NES mutant (Smurf1(Mut)). B, the interaction between Smad7 and Smurf1(CA) or Smurf1(NES-mut) was examined. Transfected COS7 cells were subjected to FLAG immunoprecipitation (IP) followed by Myc immunoblotting (Blot). The top panel shows the interaction, and the lower two panels show the expression of each protein as indicated. C, mutation in NES abolishes interaction of Smurf1 with CRM1 in transfected cells. 293T cells were transfected with CRM1-HA together with FLAG-Smurfl(CA) or FLAG-Smurfl(NES-mut). Cell lysates were subjected to FLAG immunoprecipitation followed by HA immunoblotting. The top panel shows the interaction, and the lower two panels show the expression of each protein as indicated. D, there is a cold-sensitive interaction between Smurf1 and CRM1 in vitro. GST-Smurfl(CA) or GST-Smurfl(NES-mut) was mixed with cell lysates in which CRM1-HA had been transfected. The samples were then incubated at the indicated temperatures, and the precipitates were subjected to SDS-PAGE followed by immunoblotting using anti-HA antibody or anti-GST antibody to detect GST-Smurfl(CA). As a control, cell lysate was directly subjected to SDS-PAGE (Input).
examined at different temperatures, Smurf1(CA), but not Smurf1(NES-mut), bound to CRM1 at 37°C but not at 4°C (Fig. 3D). These findings suggest that Smurf1 binds to CRM1 through the NES and that the binding of the two proteins is cold-sensitive.

**C-terminal NES of Smurf1 Is Responsible for Nuclear Export of the Smad7-Smurf1 Complex**—We next examined the effect of the Smurf1 NES on subcellular localization of the Smad7-Smurf1 complex. Smurf1(CA) alone localized in both the nucleus and cytoplasm (data not shown; see Fig. 1, D and E), whereas Smurf1(NES-mut) localized mainly in the nucleus (Fig. 4, A and B, top rows), confirming the findings shown in Figs. 2 and 3. We next examined the effect of CRM1 on subcellular localization of Smurf1(NES-mut). Overexpressed CRM1 accumulated Smurf1(CA) in the cytoplasm (data not shown; see Fig. 1F) but did not affect the localization of nuclear Smurf1(NES-mut) (Fig. 4, A and B, bottom rows). Moreover, when Smad7 was co-transfected, Smurf1(CA), but not Smurf1(NES-mut), induced nuclear export of Smad7 (Fig. 4, C and D).

**Smurf1 NES Mutant Prevents Inhibition by Smad7 of TGF-β Transcriptional Activity**—To examine the effect of Smurf1(NES-mut) on the inhibitory activity of Smad7, we performed a reporter assay using a TGF-β-responsive promoter-reporter construct, p3TP-lux. Smurf1(WT), but not Smurf1(CA), reduced transcriptional activity induced by c.a.TβR-I in the presence of Smad7 (Fig. 5). These findings thus confirm the involvement of Smurf1 in the negative regulation of TGF-β signaling by Smad7.

**DISCUSSION**

Smad7 inhibits TGF-β signaling by binding to TβR-I and preventing activation of R-Smads. Because Smad7 is located predominantly in the nucleus in many transfected mammalian cells (9, 12), nuclear export of Smad7 may play an important role in the negative feedback regulation of TGF-β signaling. We recently reported that Smurf1 binds to Smad7 and induces its nuclear export (12). Because endogenous expression of Smurf1 was negligible in the transfection experiments (data not shown), Smad7 was exported to the cytoplasm only in cells

![Fig. 4. C-terminal NES in Smurf1 induces nuclear export of the Smad7-Smurf1 complex. A and B, subcellular localization of Smurf1(NES-mut) in the absence (top rows) or presence of CRM1 (bottom rows) was examined in transfected HeLa cells. Anti-Myc staining for Smurf1 (green in A and B) and nuclear staining by PI (red in A) or HA staining for CRM1 (red in B) were performed. FITC, fluorescein isothiocyanate; RITC, rhodamine isothiocyanate. C and D, mutation in NES abolishes cytoplasmic localization of the Smad7-Smurf1 complex. HeLa cells were transfected with FLAG-Smad7 together with 6Myc-Smurf1(CA) (top rows) or 6Myc-Smurf1(NES-mut) (bottom rows). Anti-FLAG staining for Smad7 (green in C and D) and nuclear staining by PI (red in C) or anti-Myc staining for Smurf1(CA) or Smurf1(NES-mut) (red in D) were performed. Similar results were obtained in COS7 cells (data not shown).](http://www.jbc.org/Downloadedfrom)

![Fig. 5. Smurf1 NES mutant reduces inhibition by Smad7 of TGF-β transcriptional activity. Effects of Smurf1(WT), Smurf1(CA), and Smurf1(NES-mut) on the transcriptional activity of c.a.TβR-I in the presence of Smad7 were examined using a p3TP-lux assay. R mutant Mv1Lu cells were co-transfected with the p3TP-lux luciferase construct and various combinations of c.a.TβR-I, Smad7(WT), Smad7(CA), and Smurf1(NES-mut) cDNAs. + and ++ are 0.2 and 0.4 μg of Smurf1(WT) DNA, 0.4 and 0.8 μg of Smurf1(CA) DNA, and 0.7 and 1.4 μg of Smurf1(NES-mut) DNA, respectively, transfected into R mutant Mv1Lu cells. This experiment was performed five times with essentially the same results. Similar results were obtained using COS7 cells and HeLa cells (data not shown).](http://www.jbc.org/Downloadedfrom)
co-transfected with Smurf1 in the present study. Generally, the nuclear export of many large proteins is mediated by NESs, through which CRM1 binds to such proteins in an LMB-sensitive manner. In this study, we showed that nuclear export of Smad7 by Smurf1 is enhanced by co-expression of CRM1 in an LMB-sensitive fashion, suggesting that Smurf1 and/or Smad7 have functional NESs. Furthermore, we found that CRM1 does not affect localization of nuclear Smad7, whereas Smurf1 accumulates in the cytoplasm in the presence of CRM1, and the effect of CRM1 on Smurf1 is prevented by treatment with LMB. These findings strongly suggest that Smurf1, but not Smad7, has a functional NES.

To provide insights into the molecular mechanisms of nuclear export of the Smurf1-Smad7 complex, we generated GFP fusion proteins containing putative NES-like sequences of Smurf1. Interestingly, only the most C-terminal NES-like sequence was found to function as NES in Smurf1. The mutant Smurf1 containing alanine substitutions in the C-terminal NES-like sequence, Smurf1(NES-mut), was able to interact with Smad7 but failed to bind efficiently to CRM1. Also, nuclear export of the Smurf1-Smad7 complex was not observed in Smurf1(NES-mut), suggesting that the C-terminal NES of Smurf1 plays a critical role in nuclear export of Smad7 by Smurf1. Furthermore, we demonstrated that Smurf1(NES-mut) is more potent than Smurf1(CA) in reducing the inhibitory activity of Smad7. These results suggest that CRM1-dependent nuclear export of Smurf1 is required for the negative feedback regulation of TGF-β signaling.

Although we have demonstrated CRM1-dependent nuclear export of Smurf1 in this study, the molecular mechanisms of nuclear import of Smurf1 that are required for Smurf1 to bind to nuclear Smad7 are still unclear. It is interesting to note that, although Smurf1 is too large to pass through the nuclear pore by diffusion, Smurf1(NES-mut) accumulated in the nucleus following treatment with LMB. Moreover, in our preliminary study, the C-terminal deletion mutant of Smurf1 localized predominantly in the cytoplasm and failed to accumulate in the nucleus in the presence of LMB. These results suggest that not only NES but also the nuclear localization signal may exist in the C-terminal region of Smurf1. The distribution of Smurf1 between the nucleus and cytoplasm might therefore be driven by a dynamic balance between nuclear import and nuclear export.

Our present study suggested that nucleocytoplasmic shuttling of Smurf1 is necessary for the function of Smurf7. Recently, I-Smads have been reported to have certain biological functions in the nucleus. For example, Smads 6 and 7 have been demonstrated to act as transcriptional co-repressors in the nucleus (27), and nuclear Smad7 has been shown to induce apoptosis in prostatic cancer cells (28). Thus, Smurf1 may regulate the nuclear and cytoplasmic functions of I-Smads by altering their subcellular distribution.

In conclusion, we have demonstrated CRM1-dependent nuclear export of the Smurf1-Smad7 complex and identified a functional NES in Smurf1. Nuclear export of the Smurf1-Smad7 complex appears to be crucial for Smad7 function in the negative feedback regulation of TGF-β signaling. It will be important to determine how the nuclear import and export of Smurf1 are regulated under physiological and pathological conditions.

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