Nucleocytoplasmic Shuttling of Smad1 Conferred by Its Nuclear Localization and Nuclear Export Signals*

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Smad1 mediates signaling by bone morphogenetic proteins (BMPs). In the resting state, Smad1 is found in both the nucleus and cytosol. BMP addition triggers Smad1 serine phosphorylation, binding of Smad4, and its accumulation in the nucleus. Mutations in the Smad1 N-terminal basic nuclear localization signal (NLS)-like motif, conserved among all Smad proteins, eliminated its ligand-induced nuclear translocation without affecting its other functions, including DNA binding and complex formation with Smad4. Addition of leptomycin B, an inhibitor of nuclear export, induced rapid nuclear accumulation of Smad1, whereas overexpression of CRM1, the receptor for nuclear export, resulted in Smad1 re-localization to the cytoplasm and inhibition of BMP-induced nuclear accumulation. Thus, in addition to the NLS, Smad1 also contains a functional nuclear export signal (NES). We identified a leucine-rich NES motif in the C terminus of Smad1; its disruption led to constitutive Smad1 nuclear distribution. Reporter gene activation assays demonstrated that both the NLS and NES are required for optimal transcriptional activation by Smad1. Despite its constitutive nuclear accumulation, a Smad1 NES mutant did not display higher basal reporter gene activity. We conclude that Smad1 is under constant nucleocytoplasmic shuttling conferred by its NLS and NES; nuclear accumulation after ligand-induced phosphorylation represents a change in the balance of the activities of these opposing signals and is essential for transcriptional activation.

The transforming growth factor-β (TGF-β)1 superfamily of cytokines regulates a diverse array of important biological and developmental processes, including cell differentiation, adhesion, migration, inhibition of proliferation, and cell death. The superfamily members include TGF-β, activins, and bone morphogenetic proteins (BMPs), all of which signal through heteromeric complexes of cell-surface type I and II protein serine/threonine kinase receptors. Upon ligand binding, the type II receptor phosphorylates the type I receptor, thereby activating its kinase activity for phosphorylation of Smad proteins, the key intracellular mediators of TGF-β signaling pathways (1).

Smad proteins contain two highly conserved domains: MH1, which is located at the N terminus and mediates DNA binding, and MH2, which is located at the C terminus and is capable of transcriptional activation and complex formation among different Smad proteins. These are joined in the middle by a more variable proline-rich linker region. According to their functional characteristics, Smad proteins are divided into three classes: receptor-regulated Smad (R-Smad) proteins, which are direct substrates of activated type I receptors (including Smad1 and Smad5 of the BMP signaling pathway and Smad2 and Smad3 of the TGF-β/activin pathway); common Smad (Co-Smad) proteins, which are not direct substrates of receptor kinases, but form complexes with phosphorylated R-Smad proteins; and inhibitory or antagonistic Smad (I-Smad) proteins, which counteract the functions of R-Smad proteins (1, 2). In mammalian cells, the only member of the Co-Smad class is Smad4, which forms signaling complexes with Smad1 or Smad5 upon BMP stimulation or with Smad2 or Smad3 upon TGF-β/activin addition. Following complex formation, heterooligomers between R-Smad and Co-Smad proteins translocate into the nucleus to effect transcriptional regulation of target genes (1, 2).

Signal-directed nuclear import and export are two ways to regulate the availability of transcription factors inside the nucleus and are widely used by cells to modulate signal transduction pathways. Nuclear import is usually conferred by a nuclear localization signal (NLS), a basic sequence sufficient and necessary for nuclear import of the protein. There are two major types of NLSs: (i) a single stretch of five to six basic amino acids, exemplified by the SV40 large T-antigen NLS; and (ii) a bipartite NLS composed of two basic amino acids, a spacer region of 10–12 amino acids, and a basic cluster in which three of five amino acids must be basic, typified by nucleoplasmin. In NLS-mediated nuclear import, the NLS first associates with a signal recognition particle (SRP)–SRP receptor complex, which binds at the nuclear envelope and delivers the import substrate to the nuclear pore. Following additional interactions, heterooligomers translocate into the nucleus to effect transcriptional regulation of target genes (3, 4).

The nuclear export signal (NES), a short leucine-rich motif, has been identified as a transport signal that is necessary and sufficient to mediate nuclear export of large proteins (4). The consensus sequence is defined as a set of critically spaced hydrophobic residues, usually leucines (LXXLXXLX, where X indicates any residue) (5), although certain variations are allowed. Many proteins owe their extranuclear distributions to an NES, including human immunodeficiency virus Rev and Rex, protein kinase A inhibitor, Ran-binding protein 1, and

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1 The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; R-Smad, receptor-regulated Smad; Co-Smad, common Smad; I-Smad, inhibitory Smad; NLS, nuclear localization signal; NES, nuclear export signal; MAPK, mitogen-activated protein kinase; LMB, leptomycin B; GFP, green fluorescent protein; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate; WT, wild-type.
The Smad3 NLS is conserved in Smad1 and other R-Smad proteins, suggesting that it is responsible for ligand-induced nuclear import of all R-Smad proteins. In contrast, the Smad4 NES is not conserved in R-Smad proteins; and therefore, we do not know whether the cytoplasmic distribution of R-Smad proteins in the resting state is due to active nuclear export. Here we demonstrate that leptomycin B (LMB) or BMP treatment induced rapid nuclear accumulation of endogenous Smad1 as well as transfected Smad1. Ligand-induced nuclear import is dependent on the N-terminal basic NLS motif since mutations or deletions of this segment, although unable to interfere with normal ligand-dependent DNA binding of Smad1 and its complex formation with Smad4, abrogated nuclear accumulation of Smad1 in response to BMP-4. Additionally, we show that Smad1 contains a C-terminal leucine-rich NES that mediates its constant nuclear export by interacting with CRM1. Furthermore, this NES motif directed nuclear exclusion of a fused heterologous protein, and disruption of the NES resulted in nuclear retention of Smad1 regardless of ligand stimulation. Finally, disruption of either the NLS or NES resulted in decreased reporter gene activities, indicating that both signals are required for optimal transcriptional activation by Smad1.

We conclude that Smad1 is under constant nucleocytoplasmic shuttling conferred by its NLS and NES and that its accumulation in the nucleus after ligand-induced phosphorylation represents a change in the balance of activities between these opposing signals.

### EXPERIMENTAL PROCEDURES

**Constructs and Cell Lines**—Green fluorescent protein (GFP)-tagged Smad1 was constructed with the CLONTECH pEGFP-C1 vector that allows an in-frame fusion of human Smad1 to the C terminus of enhanced GFP. COS-7, C2C12, and BOSC cells were transiently transfected with Fugene 6 reagent (Roche Molecular Biochemicals) or Lipofectamine 2000 (Invitrogen), encoding enhanced GFP. COS-7 cells were transiently transfected with Fugene 6 reagent (Roche Molecular Biochemicals) or Lipofectamine Plus reagent (Life Technologies, Inc.).

Site-directed Mutagenesis—Mutations of the Smad1 NLS and NES were created using the QuikChange mutagenesis Kit (Stratagene). For Smad1 NLS mutant 1 (NLSmut1), K38R/LKKKK45 was changed to N38Q/LKKKK45; for Smad1 NLS mutant 2 (NLSmut2), K38Q/LKKKK45 was changed to K38Q/K45L (deletion of K38KKKK45); and for the Smad1 NES mutant (NESmut), L406TK409CTIRM was changed to A406TKA409CTIRM (first two large hydrophobic residues changed to alanines).

**Microinjection, Immunofluorescence, and GFP Fluorescence Microscopy**—Peptides corresponding to WT or mutant Smad1 NES motif were fused in frame to the C terminus of glutathione S-transferase (GST) and expressed in BL21 cells. Various GST-NES fusion proteins were purified at 0.5–1.0 mg/ml in injection buffer (20 mM Hepes-KOH (pH 7.4) and 200 mM KCl), combined 1:1 with fluorescein isothiocyanate (FITC)-dextran (15 mg/ml at 4 mol of FITC/mol of dextran (Mr, 70,000); Molecular Probes, Inc.), and injected into the nuclei of 35% confluent 3T3 fibroblasts using an Eppendorf Injectman system at the Keck Microscope Facility of the Whitehead Institute for Biomedical Research. After a 30-min incubation at 37 °C, injected cells were fixed with 4% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.1% Triton X-100. GST-NES fusion proteins were detected with goat anti-GST antibody (Amersham Pharmacia Biotech) followed by Texas Red-conjugated donkey anti-goat secondary antibody (Molecular Probes, Inc.).

For endogenous Smad1 immunofluorescence, goat polyclonal anti-Smad1 antibody (N18, Santa Cruz Biotechnology) was used. This antibody also cross-reacts with Smad5 and Smad8, close homologs of Smad1. COS-7 cells were grown to 50% confluence on coverslips in six-well plates. Before or after BMP-4 or LMB treatment, the cells were fixed with 4% paraformaldehyde for 10–15 min, permeabilized with 0.15% Triton X-100 for 10 min, blocked with 4% normal donkey serum for 30 min, and then stained with a 1:75 dilution of the goat anti-Smad1 antibody (in 4% donkey normal serum) overnight at 4 °C. After extensive washing, FITC-conjugated donkey anti-goat secondary antibody was added at a 1:500 dilution for 1 h.

To detect endogenous Smad3 in Mv1Lu cells, rabbit anti-Smad3 antibody (sc-8332, Santa Cruz Biotechnology) was used at a 1:50 dilution. This antibody also recognizes Smad2. Immunofluorescence was performed essentially as described (14).

**GFP fluorescence in transfected or infected cells (live or fixed) and GST immunofluorescence were visualized with a Nikon TE300 inverted microscope equipped for epifluorescence using a 488-nm excitation filter and a 522/535-nm emission filter. Images were recorded with a Hamamatsu Orca CCD camera and analyzed with OpenLab software at the Keck Microscope Facility of the Whitehead Institute for Biomedical Research.

**Gel Mobility Shift Assay**—Gel mobility shift assays were performed essentially as described (15). Whole cell extracts from COS-7 cells transfected with an appropriate combination of Smad1 and Smad4 expression plasmids were used. Prior to cell lysis, cells were treated or not with 100 ng/ml BMP-4 for 1 h to achieve Smad1 activation. Probe 9xGCCG was purified and labeled as described previously (15). 3 μg of cell lysates were added to premix solution containing poly(dI-dC) and 5 × 105 cpm of the labeled probe. Supershifts were performed with anti-Smad1 or anti-Smad4 antibodies. Complexes were resolved on a 4.5% polyacrylamide gel and analyzed by autoradiography.

**Luciferase Assays**—24 h before transfection, Mv1Lu cells were seeded in triplicate at 2 × 105 cells/well in a six-well plate. 1 μg each of 3GC2-luciferase, constitutively active ALK3(Q204D), and the indicated Smad1 vectors were cotransfected per well using Fugene 6. 0.5 μg of pSVβ (CLONTECH), encoding β-galactosidase, were included in each sample to control for transfection efficiency. 24 h after transfection, luciferase and β-galactosidase activities were measured as described previously (9).

### RESULTS

Smad1 Contains an N-terminal Basic NLS-like Motif That Is Required for Its Ligand-induced Nuclear Import—Smad1 contains an N-terminal basic NLS-like motif that is well conserved among all R-Smad proteins (Fig. 1). In Smad3, this motif acts as an NLS since mutations or deletions in this segment specifically disrupt ligand-induced nuclear import of Smad3 without affecting its ability to bind RNA or form complexes with Smad4 (9). Interestingly, in Smad1 several close homologs Smad3 and Smad8, the NES motif contains two more lysines than does Smad3 (KKLKKKG for Smad3 and KKLKKKK for Smad1, Smad5, and Smad8), thus more closely mimicking the classical NLS of large T-antigen. Interestingly, most of the basic residues in this motif are also present in the I-Smad proteins Smad6 and Smad7, implying that this sequence may be respon-
sible for the observed nuclear localization of Smad7 in the resting state (16).

To achieve live cell imaging, we constructed a GFP-Smad1 fusion protein and transiently expressed it in BMP-responsive COS-7 cells. GFP fusion does not interfere with the normal functionality of Smad1 (see below). In resting (non-BMP-stimulated) COS-7 cells, Smad1 was distributed throughout the cytoplasm and nucleus (Fig. 2, panel 1). We do not know whether this partial nuclear localization is due to weak autocrine stimulation by endogenously produced BMP. To elucidate the role the NLS-like motif may play in Smad1 nuclear translocation, we created substitution or deletion mutants in this segment and studied their localization with or without BMP stimulation. After a 1-h treatment with BMP-4, the GFP-wild type (WT) Smad1 fusion protein completely accumulated inside the nucleus. In contrast, the substitution mutant (NLSmut1, K39N/K40Q) showed much weaker nuclear accumulation, and the deletion mutant (NLSmut2, ΔK38K45) failed to display any nuclear enrichment. This indicates that the N-terminal basic motif of Smad1 acts as its NLS and is essential for BMP-induced nuclear accumulation. Transient expression of these proteins in other BMP-responsive cell lines such as C2C12 yielded similar results (data not shown).

We confirmed these observations using FLAG-tagged Smad1 constructs, which were visualized by immunofluorescence after cell fixation. Again, WT Smad1 showed rapid nuclear import after BMP-4 stimulation. In contrast, NLSmut1 and NLSmut2 both were defective in this response, and the latter was largely excluded from the nucleus both in the basal state and after BMP-4 stimulation (data not shown).

Smad1 Is Subject to CRM1-mediated Nuclear Export—Smad4 has previously been shown to contain an NES in the N-terminal part of its linker region that is unique to Co-Smad proteins and that is not conserved in R-Smad proteins (12, 13). To investigate the possibility that R-Smad proteins may contain a hitherto undefined NES, we treated C2C12 cells transiently expressing GFP-Smad1 with LMB, a specific inhibitor of the CRM1-mediated nuclear export pathway. Despite the fact that GFP-Smad1 is partially nuclear in the resting state, LMB induced significant nuclear accumulation within 1 h of treatment (Fig. 3A), suggesting that in the basal state, Smad1 is subject to constant NES-mediated nuclear export. This was confirmed by a similar experiment in a different cell line (Fig. 3B). Similar to overexpression in COS-7 or C2C12 cells, in unstimulated BOSC cells, GFP-Smad1 was also dispersed throughout the cells. It underwent dramatic nuclear accumulation within 30 min of LMB treatment (Fig. 3B, panels 1 and 2).

We further confirmed that Smad1 contains an NES by overexpressing CRM1, the cellular export receptor for NESs. CRM1 overexpression can induce dramatic nuclear exclusions of NES-containing proteins, especially ones with a weak NES. This assay has been used to prove the existence of NES in various proteins (17). Indeed, overexpression of CRM1 forced Smad1 into an almost exclusive cytoplasmic distribution (Fig. 3B, panel 3), proving that Smad1 contains a functional NES and that CRM1 is the physiological export receptor for Smad1.

Endogenous Smad1 Is Subject to Constitutive Nucleocytoplasmic Shuttling—The previous experiments utilized living cells expressing a transfected GFP-Smad1 fusion protein. To confirm that our conclusions also apply to endogenous Smad1, we studied the subcellular distributions of native Smad1 in different BMP-responsive cell lines through immunostaining with a specific Smad1 antibody (Fig. 4). Under resting conditions, endogenous Smad1 was distributed throughout C2C12 cells with a preferential cytoplasmic localization (Fig. 4A, left panel). BMP-4 treatment induced its rapid nuclear accumulation within 30 min (Fig. 4A, right panel), confirming the involvement of Smad1 in BMP signal transduction. Treatment of C2C12 cells with LMB for 1–2 h caused a dramatic accumulation of Smad1 within the nucleus (Fig. 4A, middle panel), indicating that it is normally subject to both nuclear import and nuclear export. In the resting state, nuclear export must be relatively stronger than the import to maintain the preferential cytoplasmic profile of Smad1. When export is suppressed by LMB, Smad1 accumulates inside the nucleus due to import signaled by the N-terminal NLS motif. However, since BMP-4-induced nuclear enrichment of Smad1 seems more complete than LMB-induced nuclear accumulation (Fig. 4A, compare middle and right panels), we speculate that BMP-4 suppresses nuclear export of Smad1 as well as enhances its nuclear import function. To our knowledge, this study represents the first analysis of the subcellular distribution of endogenous (i.e., untransfected) Smad1.

A second study of endogenous Smad1 in COS-7 cells confirmed and extended the above results obtained with C2C12 cells (Fig. 4B). Similar to C2C12 cells, in the resting state, Smad1 in COS-7 cells was more cytoplasmic than nuclear (Fig. 4B, panel 1) and became rapidly enriched in the nucleus within 30 min of BMP-4 treatment (panel 2). LMB addition also resulted in significant nuclear accumulation of Smad1 (Fig. 4B, panel 7), indicating that nucleocytoplasmic shuttling of endogenous Smad1 occurs in both cell lines. More interestingly, when the BMP treatment was allowed to proceed for 3–4 h, Smad1
re-localized back to the cytoplasm (Fig. 4B, panel 5), suggesting that prolonged ligand treatment may lead to desensitization of the signaling pathway by cytoplasmic retention of Smad1. Since Smad1 undergoes constant nuclear export and since the export could be enhanced by increased CRM1 levels (Fig. 3B), we studied whether CRM1 overexpression could reverse...
the BMP-dependent nuclear accumulation of endogenous Smad1. CRM1 and GFP (as transfection marker) were transiently transfected into COS-7 cells, and the nuclear import of endogenous Smad1 was analyzed after 30 min of treatment with BMP-4 (Fig. 4C, part 1). Cells were stained for Smad1 and GFP (the latter used to mark the cells overexpressing CRM1). GFP-positive cells (arrowheads) showed essentially no nuclear accumulation of Smad1, whereas neighboring GFP-negative cells displayed normal Smad1 nuclear translocation. Transfection of GFP alone did not interfere with Smad1 nuclear translocation (data not shown). This experiment establishes that BMP-4 stimulates Smad1 nuclear enrichment mainly through suppression of its nuclear export.

To ascertain whether TGF-β pathway R-Smad proteins such as Smad3 are also subject to CRM1-mediated nuclear export, we also overexpressed CRM1 in Mv1Lu cells in an attempt to inhibit the TGF-β-induced nuclear translocation of Smad3 (Fig. 4C, part 2). Again, GFP was cotransfected as a CRM1 expression marker. Without TGF-β, Smad3 was distributed throughout the cells, and this profile was not affected by CRM1 expression (upper panels, arrowheads). After TGF-β stimulation, Smad3 was completely localized inside the nucleus, and this nuclear accumulation was not disrupted by CRM1 overexpression either (lower panels, arrowheads). Additionally, we did not observe any LMB-induced Smad3 nuclear accumulation in Mv1Lu cells (data not shown). Since the anti-Smad3 antibody used for the immunostaining also recognizes Smad2, the above observations apply to Smad2. This suggests that, among the R-Smad proteins, only the BMP pathway Smad proteins are subject to nucleocytoplasmic shuttling.

*Smad1 Contains a C-terminal NES*—To define the NES responsible for Smad1 nuclear export, we scanned the Smad1 sequence and noticed a leucine-rich NES-like motif near its C terminus: LTKMCTIRM (amino acids 406–414). In conformity to the classical Rev NES, this motif contains four properly spaced large hydrophobic residues (underlined). Sequence alignment showed that it is fairly conserved among R-Smad proteins as well as Co-Smad proteins (Fig. 5A). Interestingly, this motif is poorly conserved in the I-Smad proteins such as Smad6 and Smad7, which is consistent with the preferential localization of Smad7 within the nucleus in the resting state (16).

To establish the nuclear export functions of this motif, we fused it to GST and microinjected the purified fusion protein into the nuclei of NIH 3T3 cells (Fig. 5B). As a control, we also injected GST-Rev NES (GST fused to the NES from the human immunodeficiency virus Rev protein). As expected, Rev NES induced complete nuclear exclusion of GST (Fig. 5B, panels 1 and 2). The putative Smad1 NES caused a similar nuclear exclusion of GST, indicating that in this assay, it was as functional as the Rev NES (Fig. 5B, panels 3 and 4). In contrast, mutations of the first two large hydrophobic residues to ala-nines (L406TKM409 → A406TKA409), shown previously to abrogate the export abilities of NESs (8), eliminated its ability to exclude GST from the nucleus (Fig. 5B, panels 5 and 6).

To prove that the identified NES is responsible for Smad1 nuclear export observed in *vivo*, we expressed the Smad1 NES mutant (L406TKM409 → A406TKA409) in BOSC cells and monitored its distribution (Fig. 5C). Impressively, the NES mutation converted Smad1 into a mostly nuclear protein even in the absence of ligand stimulation, confirming that the identified NES also mediates Smad1 nuclear exclusion in *vivo*.

Both the NLS and NES of Smad1 Are Required for Optimal Reporter Gene Transcriptional Activation—To investigate the roles of the NLS and NES in Smad1 transcriptional activation, we studied reporter gene activation profiles of the Smad1 NLS and NES mutants (Fig. 6). We transfected various Smad1 constructs together with a Smad1/Smad5-specific 3GC2-luciferase reporter (18) into Mv1Lu cells. Consistent with previous results, expression of FLAG-WT Smad1 induced a 3-fold increase in reporter gene activity over the control without ligand and a 7-fold increase with BMP activation. GFP-Smad1 expression conferred similar increases, suggesting that a fusion of GFP with Smad1 does not grossly interfere with its normal functionality. Introduction of mutations into the NLS caused moderate (NLSmut1) to severe (NLSmut2) reductions in Smad1 transcriptional activation, consistent with the moderate to severe defects these mutants displayed in ligand-induced nuclear import (Fig. 2). The deletion mutant (NLSmut2) showed essentially background levels of transcription. In comparison, disruption of the Smad1 NES (NESmut) lowered both basal and ligand-induced reporter activities by ~40–60%. Thus, despite the fact that NESmut was largely localized in the nucleus in the absence of BMP stimulation (Fig. 5C) and that

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**Fig. 5. Smad1 contains a C-terminal NES that is functional both *in vivo* and *in vitro*.** A, the C-terminal regions encompassing the presumptive NES motifs from vertebrate Smad proteins or their invertebrate homologs were aligned with ClustalX and displayed by SeqYu. The NES motif is indicated, and the four large hydrophobic residues that are critical for NES function are marked with asterisks. B, the Smad1 NES confers nuclear exclusion on GST. The nine-residue NES from Smad1 was fused to the C terminus of GST and co-injected into the nuclei of 3T3L1 cells together with FITC-labeled dextran as an injection site marker. A GST fusion protein with the Smad1 NES mutant (L406TKM409 → A406TKA409) was also injected. GST fused to the Rev NES was used as a positive control. After a 30-min incubation at 37 °C, cells were fixed and stained with anti-GST antibody. Shown are the FITC stain as the injected nucleus marker and the GST signal indicating the fusion protein localization. C, the NES mutation causes Smad1 to be constitutively nuclear. BOSC cells expressing GFP-WT Smad1 or GFP-NESmut (L406TKM409 → A406TKA409) were imaged for GFP fluorescence.
we could not detect additional nuclear translocation after BMP addition (data not shown), we infer that a small pool of NES-mut must either pre-exist in the cytoplasm or move from the nucleus to the cytoplasm to respond to the BMP stimulation and become phosphorylated by activated BMP receptor. These results indicate that both the NLS and NES of Smad1 are required for optimal transcriptional activation, although mutating the NLS motif is apparently more disruptive.

**Smad1 NLS and NES Mutants Maintain Normal DNA-binding Ability and Form Ligand-dependent Complexes with Smad4**—To ascertain whether the lowered transcriptional activities of the Smad1 NLS and NES mutants are due to defective complex formation with Smad4 or abnormal DNA-binding abilities, we carried out Smad1 gel mobility shift assays with the 9xGCCG probe (Fig. 7), shown previously to be a direct DNA-binding target of the Smad1-Smad4 complex (15). We used whole cell lysates from COS-7 cells cotransfected with Smad4 and Smad1 that were treated with BMP-4 prior to cell lysis. Smad1 alone (Fig. 7, lane 1) induced only background levels of the gel shifting Smad-DNA complex, similar to the control lysate from untransfected COS cells (data not shown), indicating that complex formation with Smad4 is necessary for efficient DNA binding. In combination with Smad4, both WT Smad1 and GFP-Smad1 generated similar gel shift profiles (Fig. 7, lanes 2 and 3), again demonstrating that GFP-Smad1 is functionally equivalent to WT Smad1. Supershifting with anti-Smad1 and anti-Smad4 antibodies caused retardation of the shifted bands, confirming the presence of both Smad1 and Smad4 in the complex (Fig. 7, lanes 4 and 5). Both the NLS deletion mutant (NLSmut2) and the NES mutant produced shifting and supershifting DNA binding profiles that were indistinguishable from those of WT Smad1 (Fig. 7, lanes 6–11), demonstrating that their lowered transcriptional activities are not due to an inability to form ligand-dependent DNA-binding complexes with Smad4.

**DISCUSSION**

**Smad1 Contains Both an NLS and an NES**—The major result of this study is that Smad1 contains an N-terminal NLS and a C-terminal NES. In the absence of ligand, Smad1 was found in both the cytoplasm and nucleus and rapidly accumulated in the nucleus after BMP stimulation. Addition of LMB, an inhibitor of nuclear export, induced nuclear accumulation of Smad1; and overexpression of CRM1, the receptor for nuclear export, resulted in re-localization of Smad1 to the cytoplasm. Mutations in the NLS disrupted ligand-induced nuclear accumulation of Smad1, whereas the NES mutation caused Smad1 to be localized in the nucleus even under resting conditions. Reporter gene activation assays demonstrated that both the NLS and NES were required for optimal transcriptional activation by Smad1. Strikingly, constitutive nuclear accumulation of the Smad1 NES mutant did not translate into higher basal transcriptional activity, suggesting that nuclear targeting per se is not sufficient for Smad1 activation. We conclude that Smad1 is under constant nucleocytoplasmic shuttling conferred by its NLS and NES and that its accumulation in the nucleus after ligand-induced phosphorylation represents an inhibition of nuclear export as well as enhancement of import.

The basic NLS is highly conserved among all R-Smad proteins and has been shown previously to mediate TGF-β-induced nuclear import of Smad3 (9). Additionally, since the Smad1 NLS contains more lysine residues compared with the Smad3 NLS (KKLKKKK versus KKLKKGQ), it more closely resembles the classical T-antigen NLS. This implies that BMP pathway Smad proteins may possess a stronger NLS than those in the TGF-β pathway.

The Smad1 NES is fairly conserved among all R-Smad and Co-Smad proteins, which implies that it might also be functional in Smad2 and Smad3. However, no LMB-induced nuclear enrichment has been observed for endogenous Smad2 or Smad3 in HaCaT cells and MvILu cells (15). Consistently, CRM1 overexpression was able to override the ligand-induced nuclear import of only Smad1, but not of Smad2 and Smad3 (Fig. 4C), indicating that the NES may be active only in BMP-specific R-Smad proteins, but not in TGF-β-specific R-Smad proteins. Correspondingly, in the crystal structures of the Smad2 and Smad4 MH2 domains (19), the NES region has a high degree of hydrophobicity, suggesting restricted surface accessibility. We speculate that Smad1 contains an exposed NES motif and displays less structural conservation in this region with Smad2 and Smad4.

Interestingly, Smad1 contains an additional C-terminal se-

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2 Z. Xiao, N. Watson, C. Rodriguez, and H. F. Lodish, unpublished observation.
We propose that the same mechanism, bition of Smad1 nuclear export may be due to masking or nuclear translocation of Smad3 (10). On the other hand, inhibition of endogenous Smad1 in C2C12 cells than did LMB. However, since BMP-4 induced more complete nuclear accumulation of Smad1 even though the protein is constantly shuttling between the nucleus and cytoplasm. Upon BMP-induced phosphorylation, Smad1 undergoes a conformational change and forms a complex with Smad4; somehow, this masks the Smad1 C-terminal NES while fully activating the NLS, thus leading to complete nuclear accumulation of the Smad1-Smad4 complex. The eventual dissolution of the Smad1-Smad4 complex inside the nucleus, with or without Smad1 dephosphorylation, will reset the pathway to the basal state by exposing the Smad1 NES and causing its nuclear export.

Ligand-dependent Enhancement of the Smad1 NLS and Suppression of the Smad1 NES—BMP treatment induced rapid and complete nuclear accumulation of Smad1. This could be achieved through either enhanced NLS function or repressed NES function. Since the Smad1 NES is not to be active even in the absence of BMP stimulation as revealed by LMB-induced nuclear enrichment of Smad1, we favor the latter scenario. However, since BMP-4 induced more complete nuclear accumulation of endogenous Smad1 in C2C12 cells than did LMB (Fig. 4A), ligand stimulation may lead to both activation of nuclear import and suppression of nuclear export.

Enhancement of nuclear import may stem from increased exposure of the Smad1 NES in response to BMP activation. A similar mechanism has been proposed for the ligand-induced nuclear translocation of Smad3 (10). On the other hand, inhibition of Smad1 nuclear export may be due to masking or sequestration of its NES. It has been previously shown that Smad4 achieves nuclear translocation by masking of its central linker NES through complex formation with R-Smad proteins (12). We propose that the same mechanism, i.e. ligand-induced interaction between Smad1 and Smad4, could also mask the Smad1 NES, which is in the MH2 domain. This is consistent with the notion that oligomerization between R-Smad and Co-Smad proteins occurs through their homologous MH2 domains (21). However, it is equally conceivable that ligand-induced phosphorylation of Smad1, which targets its C-terminal serine residues, may somehow directly alter the conformation of the NES due to physical proximity and cause its sequestration. This scenario seems more likely in light of the fact that even overexpressed Smad1 achieves quantitative nuclear import upon ligand activation in the absence of Smad4 coexpression.

Elevated Reporter Gene Activation—The Smad1 mutant with a nonfunctional NES is nuclear in both the presence and absence of ligand stimulation. Surprisingly, such nuclear localization not only failed to lead to higher basal transcriptional activity, but also caused a reduction in its ability to drive reporter gene activation both in the basal state and after BMP stimulation. This indicates that simple nuclear targeting of Smad1, without proper C-terminal phosphorylation and possibly other ligand-induced modifications, does not translate into higher transcriptional activity. This is similar to the case of disrupting the Smad4 NES sequence, which also causes constitutive nuclear accumulation, but fails to induce higher reporter gene activity (12).

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Interplay between the Smad1 NLS and NES