Smad proteins are intracellular mediators of transforming growth factor-β (TGF-β) and related cytokines. Although ligand-induced nuclear translocation of Smad proteins is clearly established, the pathway mediating this import is yet to be determined. We previously identified a nuclear localization signal (NLS) in the N-terminal region of Smad 3, the major Smad protein involved in TGF-β signal transduction. This basic motif (Lys40-Lys-Leu-Lys-Lys44), conserved among all the pathway-specific Smad proteins, is required for Smad 3 nuclear import in response to ligand. Here we studied the nuclear import pathway of Smad 3 mediated by this NLS. We demonstrate that the isolated Smad 3 MH1 domain displays significant specific binding to importin β, which is diminished or eliminated by mutations in the NLS. Full-size Smad 3 exhibits weak but specific binding to importin β, which is enhanced after phosphorylation by the type I TGF-β receptor. In contrast, no interaction was observed between importin α and Smad 3 or its MH1 domain, indicating that nuclear translocation of Smad proteins may occur through direct binding to importin β. We propose that activation of all of the pathway-specific Smad proteins (Smads 1, 2, 3, 5, 8, and 9) exposes the conserved NLS motif, which then binds directly to importin β and triggers nuclear translocation.

Smad proteins are a family of intracellular mediators of TGF-β family ligands. Upon ligand binding to their respective type I receptor, the corresponding type I receptor is phosphorylated and hence activated. The active type I receptor phosphorylates the C-terminal serine residues of Smad proteins, inducing their nuclear translocation. Once inside the nucleus, Smad proteins act as transcription factors to regulate the expressions of a host of target genes (1–5).

Smad proteins are classified into three general categories: 1) receptor-regulated or pathway-specific Smads, which directly interact with an activated type I receptor kinase and become phosphorylated at the C termini. These include Smad 1 and Smad 5 of the bone morphogenetic proteins pathway and Smad 2 and Smad 3 of the activin/TGF-β pathway. 2) Co-Smad or common-mediator Smad. The only mammalian member of this class is Smad 4. While not a receptor substrate, it forms a complex with activated pathway-specific Smads to effect transcriptional activation. 3) Antagonistic or inhibitory Smads such as Smad 6 and 7, which counteract the effects of the other two types of Smads (2).

Although much has been learned about the interactions between Smad proteins and receptor kinases and about transcriptional regulation by Smad proteins once inside the nucleus, we know very little about how Smads translocate into the nucleus. We recently showed that a N-terminal basic motif in Smad 3 (Lys40-Lys-Leu-Lys-Lys44), conserved among all the pathway-specific Smads, is not only responsible for the constitutive nuclear localization of the isolated Smad 3 MH1 domain, but is also required for Smad 3 nuclear import in response to ligand. Mutations in this motif completely abolish TGF-β-induced nuclear translocation of Smad 3 (6). In contrast, an isolated Smad 4 MH1 domain, which has a critical Lys → Glu change at the last position of this motif (Lys45-Lys-Leu-Lys-Glu46), does not localize to the nucleus. However, mutation of the Glu to Lys allows the Smad 4 MH1 domain to accumulate in the nucleus. These results explain why Smad 4 normally requires binding to Smad 3 or another pathway-specific Smad to be transported into the nucleus (5).

Depending on the size of the protein, nuclear import through the nuclear pore complex (NPC) can occur either through passive diffusion (for small molecules of less than 40–50 kDa), or by an active process facilitated by the NLS present in nuclear proteins (7, 8). Two types of NLSs have been defined: a single stretch of 5–6 basic amino acids, exemplified by the SV40 large T antigen NLS; and a bipartite NLS composed of two basic amino acids, a spacer region of 10–12 amino acids, and a cluster of 3–5 basic residues, as typified by nucleoplasm (7, 8). NLSs are usually recognized by the heterodimeric receptor proteins importin α and β. Importin α contains the NLS-binding site and importin β is responsible for the docking of the importin-substrate complex to the cytoplasmic side of the NPC and its subsequent translocation through the pore. Once inside the nucleus, Ran-GTP binding to import β causes the dissociation of the import complex and release of the cargo. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of Ran between the cytoplasm and the nucleus, with the GTP form predominant in the nucleus (7, 8).

Since the Smad 3 NLS bears close resemblance to that of the large T antigen, we investigated whether Smad 3 import also involves the importin α/β pathway. Consistent with its exclusive nuclear localization (6), here we show that the isolated Smad 3 MH1 domain displays significant specific binding to importin β, which is diminished or eliminated by mutations in the NLS motif. In contrast, neither the Smad 3 MH2 domain nor the Smad 4 MH1 or MH2 domains bind to importin β. More
importantly, full-size Smad 3 shows a much weaker binding to importin β, which is enhanced after phosphorylation by the Type I TGF-β receptor. Neither Smad 3 nor its MH1 domain binds to importin α, indicating that nuclear localizations of Smad proteins probably occur through direct binding to imp-β. Since this NLS motif is conserved in all of the pathway-specific Smad proteins (Smads 1, 2, 3, 5, 8, and 9), it is likely that phosphorylation by a type I receptor exposes this basic motif which then binds directly to importin β and triggers nuclear translocation. This could represent a general mechanism governing the nuclear import of Smads.

EXPERIMENTAL PROCEDURES

Constructs—GFP-tagged Smad 3 and Smad 4 were made using the CLONTECH pEGFP vector (C1 version). To generate a retroviral vector encoding a GFP-Smad fusion, the pEGFP-Smad vector was digested with AgeI and SfiI and then ligated into a similarly restricted pMX vector (9) to create the pMX-GFP-Smad. These constructs have all been described previously (6).

Cell Lines and Transfections—For transient expression BOSC cells were transfected with LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. Construction of stable cell lines expressing various GFP-Smad fusion proteins has been described elsewhere (6). Briefly, BOSC cells were transfected with pMX-GFP-Smad constructs. Two days after transfection, cell supernatant containing the retroviruses was collected and used to infect L20 cells, a Mv1Lu cell line expressing the mouse ecotropic viral receptor (9).

Site-directed Mutagenesis—Specific amino acids in Smad 3 NLS were mutated or deleted using the QuikChange Mutagenesis kit (Stratagene). These mutations have been described previously (6).

Importin α and β Binding Assays—DNA constructs encoding GST fusions with human importin α (NPI-1/hSRP-1), importin β, and the importin α IBB domain (as a 6 × histidine-tagged protein) were kindly provided by Dr. M. Malim (University of Pennsylvania); GST-importin α (Rch-1/hSRPα1) was kindly provided by Dr. Yoneda (Osaka University, Suita, Japan); His-tagged importin β (in pQE80) was a gift from Dr. R. Guschlbauer (University of Heidelberg, Germany); and GST-importin b3 was generously provided by Dr. X. Hua (Whitehead Institute). Plasmids encoding the cell cycle inhibitor p27 and a p27 mutant with a C-terminal amino acid 160–197 deletion that eliminates its nuclear localization signal were kindly provided by Dr. Y. Sun (Whitehead Institute). The fusion proteins were purified on glutathione beads or Ni2+-NTA resin according to the manufacturer’s suggestions and published procedures (10–12). The importin proteins were not eluted and remained on the beads. BOSC cells were lysed in buffer C (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 50 mM NaF, 50 mM glycerophosphate, 2 mM EDTA, 10% glycerol plus 1 mM EDTA, 10% glycerol plus 1 mM EDTA, 10% glycerol plus 1 mM EDTA) and the mixture was then centrifuged at 100,000 × g for 15 min, and the lysate was diluted with buffer C without NaCl. Lysates from 2–3 × 107 BOSC cells that contain overexpressed Smad proteins were pre-absorbed with glutathione beads and then added to beads containing 2–3 μg of prebound importin α or β. After 1–2 h binding at 4 °C, the beads were washed three times in buffer C with 200 mM NaCl; the bound fraction was eluted by 15 mM glutathione and analyzed by SDS-PAGE.

RESULTS

The N-terminal Basic Motif in Smad3 Functions as Its NLS—Our previous studies using GFP fusion constructs revealed that a NLS, K45KLKE49, in the N-terminal region of Smad 3 is not only required for the constitutive localization of the isolated Smad 3 MH1 domain, but is also critical for Smad 3 nuclear import in response to ligand (6). Table IA summarizes showing that mutation of this NLS motif in the isolated Smad 3 MH1 domain is disrupted or abolished by any of several mutations or deletions in this NLS motif, including K43N/K44Q, K44E, and K43K44. Similarly, the presence of any of these mutations in an intact Smad 3 protein abolished its ability to be translocated into the nucleus in response to TGF-β (Table IB). In contrast, the isolated Smad 4 MH1 domain is uniformly distributed between cytosol and nucleus, and we hypothesized that this is due to a change of lysine to glutamate in the last position of Smad 4’s NLS-homologous site (K45KLKE49, underlined) (6). Indeed, mutation of the deviant Glu to Lys, to mimic the Smad 3 motif, caused the mutant Smad 4 MH1 to exhibit a dramatic concentration in the nucleus (Table IA). Taken together, these results indicate that our identified NLS motif is a bona fide nuclear import signal for Smad 3.

Smad 3 and Its MH1 Domain Bind Specifically to Importin β But Not Importin α—To determine whether the nuclear translocation of Smad 3 involves the classical importin α/β pathway, we studied the interactions between Smad 3 and importins α and β using an in vitro GST-pull down assay. To this end fusion proteins of GST with importin α (NPI-1/hSRP1) and importin β were immobilized on glutathione beads. Lysates from BOSC cells overexpressing Smad 3 or its isolated domains, all as GFP fusions, were added to the beads; after extensive washing, the bound protein was eluted. Previous studies showed that GST fusions with importin α or β exhibited normal import functions and that normal nuclear translocation could be reconstituted by these GST fusion proteins in vitro (13). Both Smad 3 and its isolated MH1 domain displayed specific binding to GST-importin β but not to GST-importin α (Figs. 1, B and A, respectively). GST itself did not bind to either importin (data not shown). Furthermore, our GST-importin α fusion is active, since it binds to the cell cycle inhibitor p27, a known nuclear protein containing a classical NLS, but not to a mutant p27 in which the NLS was deleted (Fig. 1C). Identical results (not shown) were obtained when we used another major imp-α isoform, Rch-1/hSRPα1, for this binding assay.

To ascertain whether this observed importin β binding is mediated through the NLS motif, we tested importin binding of Smad 3 MH1 domains with NLS mutations resulting in disrupted nuclear localizations (Fig. 2A). All the constructs were expressed as GFP fusions. Mutant K43N/K44Q exhibited decreased binding to importin β (about 30–40% of the level of the wild-type protein, compare lanes 1 and 5), suggesting that the interaction of Smad 3 MH1 with imp-β involves its NLS motif. Another Smad 3 MH1 NLS mutation, K44E, essentially eliminated any importin β binding (compare lanes 6 and 7).

Neither the isolated Smad 4 MH2 domain nor the Smad 4 MH1 domain can bind to imp-β (Fig. 2A, lanes 3 and 4), consistent with the inability of these domains to undergo nuclear accumulation (6). We also found that, as expected, the Smad 4 MH2 domain does not bind to importin β (data not shown).

Since we have previously shown that the E49K mutation in the NLS-homologous segment (K45KLKE49) of the Smad 4
FIG. 2. Binding of Smad 3 and Smad4 MH1 domains to imp-β, A, the nuclear localization of an isolated Smad 3 MH1 domain corresponds to its ability to bind imp-β. Lysates from 3 × 10^6 BOSC cell expressing wild-type Smad 3 MH1 (lanes 1), Smad 3 MH1 NLS mutant K43N/K44Q (lane 5), GFP alone (lane 2), Smad 3 MH2 (lane 3), or Smad 4 MH1 (lane 4), all as GFP-fusions, were added to 2 µg of GST-importin β immobilized on glutathione beads. Lanes 6 and 7 represent another assay in which wild-type Smad 3 MH1 (lane 7) was compared with a second NLS mutant, Smad 3 MH1 K44E (lane 6). Bound proteins were eluted and detected after SDS-PAGE with anti-GFP antibody (lanes 8). Bound proteins were eluted and detected after SDS-PAGE with anti-GFP antibody (upper panel); total Smad protein input, representing 20% of that added to the binding reactions, is shown in the lower panel. B, a Smad 4 MH1 domain containing the E49K mutation exhibits enhanced binding to importin β. BOSC cell lysates containing wild-type Smad 4 MH1 or mutant Smad 4 MH1 E49K were incubated with GST-importin β attached to glutathione beads. Bound proteins were eluted and detected. Input represents 10% of that added to the beads. C, binding of Smad 3 MH1 domain to imp-β is inhibited by the IBB domain of imp-α. Increasing amounts of purified imp-α IBB domain were added to binding reactions containing 2 µg of GST-imp-β and also Smad 3 MH1 domain from lysates of 3 × 10^6 transfected BOSC cells. The first lane has no IBB. Lane 2 has 0.1 µg IBB, and each succeeding lane has twice the amount of IBB as the lane on its left. The molar ratio between IBB and GST-imp-β in lane 4 is roughly 3:1. Bound Smad 3 MH1 was eluted from the glutathione beads and quantified by an anti-GFP immunoblot. D, purified recombinant Smad 3 MH1 domain binds to purified importin β. Purified GST, GST-Smad 3 MH1, and GST-Smad 3 proteins were incubated with His-tagged importin β coupled to a Ni^2+-NTA column for 2–3 h. After extensive washing, bound proteins were eluted with 200 mM imidazole, resolved by SDS-PAGE, and stained with Coomassie blue. "Input" shows one third of the amount of Smad 3 proteins added to the binding reaction.

MH1 domain converted it into a predominantly nuclear form (Table I), we investigated whether this mutation also leads to a corresponding increase in imp-β binding. Not surprisingly, the Smad 4 MH1 E49K mutant displayed about 8–10-fold more specific binding than the wild-type protein (Fig. 2B), indicating that imp-β is responsible for the enhanced nuclear enrichment of the mutant Smad 4 MH1 protein.

The IBB (importin β binding) domain of imp-α is essential for dimerization of imp-α with imp-β. Isolated IBB, when introduced into cells, efficiently inhibits nuclear translocation through imp-β (14, 15). Thus we investigated whether the IBB domain would interfere with the interactions between Smad 3 and imp-β. Indeed, Fig. 2C shows that increasing amounts of purified IBB progressively inhibit the binding of Smad 3 MH1 to GST-imp-β. Similarly, binding of full-length Smad 3 to imp-β was also abolished by IBB (data not shown). This suggests that the binding of Smad 3 and importin α to importin β may be mutually exclusive.

Since we used BOSC cell lysates in all of the above binding assays, we could not rule out the possibility that Smad 3 binds to importin β through an adaptor molecule. To address this issue, we used purified recombinant Smad 3 proteins in the binding assay (Fig. 2D). Consistent with the above results, pure recombinant Smad 3 MH1 displayed strong binding to immobilized imp-β; the bound fraction corresponded to about 25% of the input (first panel). Purified intact Smad 3 showed a much reduced but still specific interaction with imp-β. This demonstrates that Smad 3 binds directly to imp-β.

Smad 3 Binding to Importin β Is Enhanced by Phosphorylation by the Type I TGF-β Receptor and Disrupted by Mutations in the NLS—FIG. 3 shows that the binding of full-length Smad 3 to imp-β is enhanced when it has been preincubated with the constitutively active type I TGF-β receptor (T204D). This receptor is used as a surrogate for normal TGF-β-triggered activation of Smad 3. Full-length Smad 3 synthesized in BOSC cells exhibits low but specific binding to imp-β (lane 1, panel 1), consistent with the results obtained in Fig. 1. Much greater binding was seen when Smad 3 was phosphorylated by the co-expressed T204D type I TGF-β receptor (lane 2), and no binding to imp-β was observed (lane 3) if the phosphorylated Smad 3 harbored a mutation (K43N/K44Q) in its NLS, which makes it incompetent for nuclear translocation (Table IB). This indicates that Smad 3 phosphorylation, induced by TGF-β treatment, results in an increased binding of its NLS to importin-β, which leads to nuclear import. The difference in imp-β binding to Smad 3 alone and to Smad 3 co-expressed with the activated type I receptor TβR1 T204D, quantified by scanning densitometry, was about 3-fold. Though not impressive, this difference can be functionally significant, since it represents a steady-state situation. Additionally, considering that less than 20–30% of Smad 3 exists as phosphorylated form in T204D-activated cells,2 the real difference between imp-β bindings of phosphorylated and unphosphorylated Smad 3 may be much greater than the observed 3-fold.

The isolated Smad 3 MH1 domain binds much stronger to imp-β than does full-length Smad 3, as is evident by comparing lane 1 with lane 4 of Fig. 3. Indeed, normalized by input, the imp-β column retains over 20-fold more Smad 3 MH1 domain

TABLE I

| Protein | Localization |
|---------|-------------|
| Smad 3 MH1 | N |
| Smad 3 MH1 K43N/K44Q | N/E |
| Smad 3 MH1 K44E | E |
| Smad 3 MH1 ΔK43K44 | C |
| Smad 4 MH1 | E |
| Smad 4 MH1 E49K | N |

A. Location of isolated wild-type and mutant Smad 3 and Smad 4 MH1 domains

B. Location of wild-type and mutant Smad 3 proteins in control and TGF-β-stimulated cells

PROTEIN LOCALIZATION

Table:<ref>

| Protein | Localization |
|---------|-------------|
| Smad 3 | C |
| Smad 3 K43N/K44Q | C |
| Smad 3 K44E | C |
| Smad 3 ΔK43K44 | C |

Input represents 10% of that added to the beads. Means the localization falls between a nuclear profile and an even dispersion. In B, stably expressing MvLU1 cells were treated with or without 200 pM TGF-β for 1 h at 37 °C and then the GFP signal was recorded for each cell line. Data were abstracted from Ref. 6.

2 Z. Xiao, X. Liu, and H. F. Lodish, unpublished observations.
than does full-size Smad 3, consistent with the result obtained from the binding assay performed using purified recombinant Smad 3 and its MH1 domain (Fig. 2D). This supports our hypothesis that the NLS signal in intact unactivated Smad 3 is sequestered and is much less “exposed” than is the NLS on an isolated MH1 domain.

A Model for Ligand-induced Nuclear Translocation of Smad 3—Based on these findings, we propose a model for ligand-induced nuclear import of Smad 3 (Fig. 4). Under basal conditions Smad 3 resides in the cytosol, since its NLS motif in the MH1 domain is masked by an intramolecular interaction between MH1 and MH2. After TGF-β stimulation, the type I receptor phosphorylates Smad 3 on its C terminus and thereby induces a conformational change that opens up the complex and exposes the NLS motif. Importin β then binds directly to the NLS and carries Smad 3 (with its associated Smad 4) into the nucleus. Once the complex is inside the nucleus, Ran-GTP presumably binds to importin β, causing the release of Smad 3 (7, 8).

**DISCUSSION**

Nuclear import of most proteins requires both imp-α and -β, with imp-α as the adaptor between imp-β and the cargo protein. Only a few proteins undergo nuclear import via direct binding to imp-β without involvement of imp-α. These include the Rex protein of human T-cell leukemia virus type 1 (10), HIV-1 Rev (17, 18) and Tat proteins (18), ribosomal protein L23a (19), the T-cell protein tyrosine phosphatase (20), and cyclin B1 (16). Among these, the nuclear import of cyclin B1 also requires an activating phosphorylation that is dependent on the Cdc2 kinase. It is not certain whether or not these proteins also require Ran-GTPase for nuclear translocation (8).

Our data indicate that regulated nuclear import of Smad 3 may also involve binding to imp-β without the participation of imp-α. Furthermore, the binding of Smad 3 and imp-α to imp-β may be mutually exclusive. These conclusions rest mainly on in vitro binding studies, which showed that the Smad 3 MH1 domain binds strongly to imp-β, which is diminished or eliminated by mutations in the NLS. Full-size Smad 3 exhibits much weaker binding to imp-β, which is substantially enhanced after phosphorylation by the type 1 TGF-β receptor.

Despite the obvious similarity of its NLS motif to that of the classical T-antigen NLS, full-length Smad 3 does not accumulate in the nucleus nor does it bind to imp-α. This indicates that it is not possible to deduce a protein’s nuclear localization or importin-binding properties simply by inspection of its particular NLS sequence. Additionally, the Smad 3 NLS by itself is not sufficient to confer imp-β binding to a fused heterologous protein (data not shown), indicating that it will provide the proper interface for docking imp-β only in the context of an appropriate surrounding structure. Consistently, Smad 3 interacts with imp-β weakly under basal conditions and only upon Smad 3 phosphorylation does imp-β gain high affinity to Smad 3.

Thus we have defined a putative nuclear import pathway for Smad 3. Under basal conditions Smad 3 resides in the cytosol with its NLS motif in the MH1 domain masked by an intramolecular interaction between MH1 and MH2. After TGF-β stimulation, the type I receptor phosphorylates Smad 3 on its C terminus and thereby induces a conformational change that opens up the complex. Imp-β then binds directly to the exposed NLS and carries Smad 3 (with its associated Smad 4) into the nucleus.

**Acknowledgments**—We thank members of the Lodish group, especially Xiaxin Hua, for stimulating discussions.

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