Distinct Domain Utilization by Smad3 and Smad4 for Nucleoprotein Interaction and Nuclear Import*

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Smad proteins undergo rapid nuclear translocation upon stimulation by transforming growth factor-β (TGFβ) and in so doing transduce the signal into the nucleus. In this report we unraveled nuclear import mechanisms of Smad3 and Smad4 that are dependent on their interaction with FG-repeat-containing nucleoporins such as CAN/Nup214, without the involvement of importin molecules that are responsible for most of the known nuclear import events. A surface hydrophobic corridor within the MH2 domain of Smad3 is critical for association with CAN/Nup214 and nuclear import, whereas Smad4 interaction with CAN/Nup214, and nuclear import requires structural elements present only in the full-length Smad4. As exemplified by the different susceptibility to inhibition of import by cytoplasmic retention factor SARA (Smad anchor for receptor activation), such utilization of distinct domains for nuclear import of Smad3 and Smad4 suggests that nuclear transport of Smad3 and Smad4 is subject to control by different retention factors.

Signal from the transforming growth factor-β (TGFβ) cytokines originates at the cell surface upon engagement of TGFβ ligands with their corresponding Type I/Type II receptor complexes and is transduced into the nucleus by the family of Smad proteins (1). Smad proteins are phosphorylated by the corresponding receptor kinases activated upon ligand binding: Smad2 and Smad3 by TGFβ and activin receptors; Smads 1, 5, and 8 by bone morphogenetic protein (BMP) receptors. One key event accompanying ligand binding is the rapid movement of Smads, including the unphosphorylated Smad4, into the nucleus (4–7). As exemplified by the different susceptibility to inhibition of import by cytoplasmic retention factor SARA (Smad anchor for receptor activation), such utilization of distinct domains for nuclear import of Smad3 and Smad4 suggests that nuclear transport of Smad3 and Smad4 is subject to control by different retention factors.

Translocation of macromolecules across the nuclear envelope occurs via the nuclear pore complex (NPC), which consists of over 20 nucleoporins and many of them contain multiple phenylalanine-glycine (FG) dipeptide repeats (4–6). In many cases, movement of cargo molecules through the NPC is mediated by the importin β family of transport receptors, which bind cargo molecules and the FG dipeptide repeats simultaneously through separate domains (7). In the case of classical nuclear localization signal (NLS)-containing proteins, importin α bridges the interaction between the NLS motif and importin β (4–7). Another common element in importin β-mediated nuclear import is the small GTPase Ran in its GTP-bound form (RanGTP), whose binding to importin β disrupts all characterized association between importin β and their cargoes (4–7). Ran is mostly in its GTP-bound form in the nucleus and a GDP-bound form in the cytoplasm. Such Ran-GTP gradient across the nuclear envelope prompts release of cargo only in the nucleoplasm, ensuring that the cargoes will accumulate in the nucleus (4–7).

Since elucidation of the mechanism of importin β-mediated nuclear import, there have been efforts to investigate if similar principles apply to Smads. In Smad3, the N-terminal or MH1 domain contains a lysine-rich α-helix, which was suggested to interact with importin β and act as a NLS (8, 9). This interaction between importin β and the Smad3 MH1 domain appeared to be dependent on phosphorylation of the Smad3 C terminus in response to TGFβ (8, 9). Nuclear entry of Smad4 occurs in response to TGFβ signals, yet unlike Smad2 and Smad3 it is not phosphorylated (10). Smad4 contains a typical NES motif and is subject to strong nuclear export by the export receptor CRM-1 (11–13). A lysine-rich motif present in the Smad4 MH1 domain was recently proposed to function as a bipartite NES motif for interaction with importin α and facilitating nuclear import of Smad4 (14).

In contrast to the proposed involvement of importins in nuclear import of Smad3 and Smad4, studies on Smad2 have shown that its behavior in nuclear import assays is distinct from that of a classical NLS-containing substrate as it depends on direct interaction with FG-repeat containing nucleoporins without importin α or importin β as the bridging factor (15, 16). TGFβ promotes Smad2 nuclear import by attenuating its association with cytoplasmic retention factors while enhancing its affinity for nuclear retention factors, without affecting Smad2 affinity for nucleoporins (16).

In this study, we investigate the nuclear import mechanisms of Smad3 and Smad4, using an in vitro nuclear import assay. Our observations using this assay suggest that nuclear import of Smad3 and Smad4 is mediated by mechanisms independent of importin β or importin α. The nuclear import capability of Smad3 is intrinsic, and does not require its phosphorylation by TGFβ receptors. Similar to Smad2, it is the C-terminal MH2 domain of Smad3 that contains the major nuclear import activity, owing to the presence of a series of hydrophobic surface

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The abbreviations used are: TGFβ, transforming growth factor-β; NLS, nuclear localization signal; GST, glutathione S-transferase; GFP, green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; HA, hemagglutinin; MBP, maltose-binding protein.

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42569

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Distinctive Nuclear Import Mechanisms of Smad3 and Smad4

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Luciferase Reporter Assays—Hela, HaCaT, and COS-1 cells were maintained in Dulbecco’s modified Eagle’s medium, and L17 cells were cultured in MEM. In all cases, the media was supplemented with 10% fetal bovine serum, penicillin, streptomycin, and fungizone. All transient transfections were carried out using Lipofectamine following the manufacturer’s manual (Invitrogen). Luciferase reporter assays were carried out as previously described, with constitutively active Renilla reporter as the internal control (16).

Plasmids and Mutagenesis—Full-length Smad3 with FLAG tag at its N terminus was cloned into the pQE80 vector to generate His-Flag-Smad3 (Qiagen). His-Smad3 was constructed by inserting full-length Smad3 into the pQE80 vector (Qiagen). Fragments corresponding to amino acids 1–155 and 146–425 of Smad3 were inserted into the EcoRI-XhoI sites of pGEX 4T1 (Amer sham Biosciences) to create GST-S3MH1 and GST-S3(L-MH2), respectively. GST-S4MH1 and GST-S4(L-MH2) contain amino acids 1–145 and 145–552 of Smad4, respectively, and were cloned into the pGEX 4T1 vector. GST-GFP-S3MH1 was described previously (9). The L-MH2 fragment of Smad3 was inserted into the L-MH2 domain of Smad4 (Clontech) by PCR cloning to generate GFP-S3(L-MH2). Plasmids encoding importin α, importin β, Ran, RanG�QL, were gifts from Dr. Dirk Gorlich (ZMBH, Heidelberg, Germany), and plasmids for NLS-GST-GFP and His-NTF2 were obtained from Dr. Gunter Blobel (Rockefeller University). Site-directed mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene). Protein Labeling—Purified recombinant proteins were labeled with fluorescein-5-maleimide or Texas Red C 2 maleimide (Molecular Probes), using protocols recommended by the manufacturer. The labeled proteins were exchanged into the desired buffer using Econo-Pac 10 DG columns (Bio-Rad).

Nuclear Import Assays—Permeabilization of Hela cells using digitonin, and the in vitro nuclear import assays were carried out as previously described (17). In cases where cytosol was used, rabbit reticulocyte lysate or Hela lysate, cytosol at 5–10 mg/ml were used as the source of importin α and importin β (17, 18). In assays where Hela-SBD was used, the procedure was as described previously (15). In import reactions reconstituted with recombinant factors, purified importin α and importin β at indicated concentrations were used, together with RanGDP (5 μM) and NTF2 (2.5 μM). An ATP-regenerating system containing 0.02 unit/ml creatine kinase (Sigma), 1 mM ATP (Sigma), and 5 mM phosphocreatine were present in all import reactions. Immunostaining after the nuclear import assays was conducted as described previously (15).

Protein-Protein Interaction Assays—GST pull-down experiments were performed as described previously (16). To test Smad2-Smad4 interactions, L17 cells were co-transfected with FLAG-Smad2 and HA-Smad4. As indicated, the cells were also transfected with the constitutively active TpRI(T204D) or the kinase-defective TpRI(K232R) (19). Cells were harvested 48 h after transfection, and whole cell lysate was prepared by sonication in 20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 20 mM NaF, 20 mM β-glycerol phosphate, 0.15% Nonidet P-40, 2 mM dithiothreitol, and protease inhibitors (Complete, Roche Applied Science). Anti-FLAG immunoprecipitation was done using agarose beads conjugated with the M2 anti-FLAG monoclonal antibody (Sigma).

Cell Line Construction—To generate pSUPER-Smad3, the pSUPER vector (Dr. R. Agami, the Netherlands Cancer Institute) was digested with BglII and HindIII and the annealed oligos (5’-gatccgcGTCGACACCAAGTGCATC tcaagggagtGACCTTGTTTGTCACttggg3’ and 5’-agctttcctaaaag GCTGACACCAAGTGCATCttttctggg3’) were ligated into the vector (20). The 19-n Smad3 target sequence was amplified using amino acids 146–425 of Smad3, and the HaCaT cells were co-transfected with pSUPER-Smad3 and p Pur (Clontech) vectors at the ratio of 5:1 using Lipofectamine as described by the manufacturer (Invitrogen). At 24-h post-transfection, the medium was changed with addition of 1 μg/ml puromycin to start selection. Extract prepared from selected clones was examined by immunoblotting using anti-Smad2/3, anti-Smad3 (Zymed Laboratories Inc.), and anti-tubulin (Santa Cruz) antibodies.

RESULTS

Nuclear Import of Smad3 and Smad4 Is Independent of Added Cytosol—We evaluated nuclear import of recombinant full-length Smad3 and Smad4 using the in vitro nuclear import assay (17). Cytosol-independent nuclear accumulation of purified His-FLAG-Smad3 and His-Smad4 was observed, and inclusion of cytosol did not further enhance the efficiency of nuclear import (Fig. 1, A and B). In fact, there was a slight decrease in Smad4 nuclear import when cytosol was present (Fig. 1B). This could be due to the presence in cytosol of Smad4 retention factors or CRM-1, which exports Smad4 out of the nucleus (12, 13). Results in Fig. 1, A and B are in complete contrast to the cytosol-dependent nuclear import of the classical NLS-containing protein NLS-GST-GFP (Fig. 1C). Like all known active nuclear transport events, import of Smad3 and Smad4 in this assay was completely blocked by wheat germ agglutinin (WGA) regardless of the presence or absence of cytosol, suggesting that the observed nuclear import was an active process involving contact with nucleoporins (Fig. 1, A and B and data not shown) (17). This observation also supports the conclusion that for both Smad3 and Smad4, nuclear import in the presence or absence of cytosol is mechanistically similar.

The Smad anchor for receptor activation (SARA) protein is a cytoplasmic Smad retention factor that specifically binds to Smad2 and Smad3 by contacting the hydrophobic corridor on the MH2 domain (21, 22). Nuclear import of Smad3 was greatly diminished in the presence of the Smad-binding domain of SARA (SARA-SBD), amino acids 665–750 of SARA) (Fig. 1A). In contrast, SARA-SBD did not interfere with nuclear import of Smad4 (Fig. 1B), which is consistent with the fact that Smad4 does not interact with SARA (21). In the control experiment, NLS-driven nuclear import of NLS-GST-GFP was not affected by SARA-SBD (Fig. 1C), further supporting the specificity of SARA in blocking Smad3 nuclear import.

The L-MH2 Domain Harbors the Major Nuclear Import Activity of Smad3—GST fusion of the L-MH2 domain of Smad3 (amino acids 146–425), but not the MH1 domain (amino acids 1–155), displayed cytosol-independent nuclear import activity (Fig. 2A). Since the MH2 domain mediates SARA-SBD interaction, this observation suggested that SARA-SBD may inhibit Smad3 nuclear import by occluding the MH2 domain (Fig. 1A). However, neither the MH1 (amino acids 1–145) nor the L-MH2 (amino acids 145–552) domain of Smad4 exhibited significant nuclear import activity (Fig. 2B). The lack of nuclear import of Smad4 MH1 domain is not due to the presence of an NES in this construct, since inhibition of the NES by leptomycin B (LMB) did not enable nuclear accumulation of Smad4 MH1 (data not shown). We tested additional Smad4 L-MH2 constructs (amino acids 251–552 and 285–552), and the results were all negative (data not shown). Thus it appears that although the MH2 domain of Smad4 is similar to that of Smad2 and Smad3 (~40% sequence identity), different structural elements are responsible for Smad4 nuclear import.

The observation that the nuclear import activity of Smad3 resides in its L-MH2 domain differs from a previous report (9). We sought to clarify this issue by examining the exact constructs used in the study by Kurisaki et al. (9). In contrast to that report, only a very small proportion of nuclei imported the MH1-containing construct GST-GFP-S3MH1 in the import assay, even with cytosol present (Fig. 3A). We also observed only weak import activity in the MH2-containing construct GST-S3 (231–425) (Fig. 3A), which is similar to previously described observation using this construct (9). However, a much stronger
import was seen with the GST-S3(L-MH2) (amino acids 145–425) construct (Fig. 3A), which included the complete MH2 domain (amino acids 219–425) as defined by crystal structure, not sequence homology (23). This conclusion is further substantiated by the observation in HaCaT and Hela cells that the L-MH2 domain of Smad3 can drive the heterologous protein GFP into the nucleus in the absence of TGFβ stimulation (Fig. 3B). Moreover, when tested simultaneously on the same cells, GST-S3(L-MH2) was imported very efficiently, whereas GST-GFP-S3MH1 was mostly excluded from the same nuclei (Fig. 3C). Consistent with these results, excess amount of the L-MH2 domain but not the MH1 domain inhibited the import of full-length Smad3 (Fig. 3D), supporting the conclusion that the major nuclear import activity in Smad3 is located in its C-terminal L-MH2 domain. Furthermore, just like full-length Smad3, nuclear import of GST-S3(L-MH2) is also blocked by SARA-SBD (data not shown), suggesting that L-MH2 is the domain through which full-length Smad3 gets imported into the nucleus.

Nuclear Import of Smad2, -3, and -4 Is Independent of the Importin β Family—Our observations in Fig. 1 implied that cytosol, which is used as the source of importin β in these assays (17), was not essential for nuclear import of Smad3 and Smad4. This behavior is similar to that of Smad2 in previous reports (15). However, residual importin β factors may still be present after digitonin treatment and contribute to nuclear import. Therefore, we decided to investigate this possibility.

RanQ69L, a RanGTPase mutant defective in GTPase activity and therefore remains stably bound to GTP, effectively blocks importin β-dependent nuclear import by dissociating importin β family members from their cargoes (24, 25). This provides a rigorous test of whether nuclear import of Smad2, -3, and -4 is independent of importin β. Indeed, the presence of excess RanQ69L-GTP did not interfere with nuclear import of the l-MH2 domain of both Smad2 and Smad3, either in the presence or absence of cytosol (Fig. 4), nuclear import of Smad4 was also not affected by RanQ69L (Fig. 4). The efficacy of the RanQ69L protein was confirmed, as it substantially inhibited nuclear import of importin α, which is an importin β-dependent process (Fig. 4) (25). These results further validate our conclusion that nuclear import of Smad2, -3, and -4 does not require importin β family of transport receptors.

Artificially High Concentration of Importins Are Required for the Nuclear Import of MH1 Domains of Smads—Previous reports have suggested that the MH1 domain of Smad3 interacts with importin β1 (8, 9). It was also reported that the Smad4 MH1 domain is able to interact with importin α (14). To ascertain whether such interactions would result in nuclear import of Smad3 and Smad4 respectively, we compared their behavior in the nuclear import assay with importin α as a typical cargo of importin β, and NLS-GST-GFP as a typical cargo of importin α.

The amount of endogenous importin β in the Hela cytosol we used was sufficient to support nuclear entry of importin α, but not enough to import the MH1 domain of Smad3 (Fig. 5A). In order to achieve higher relative concentration of importin β in the import reaction, instead of cytosol, we used purified recombinant importin β, RanGDP, and NTF2 to reconstitute nuclear import.
import as described previously (9, 26). Only under these conditions, with concentration of importin β in the micromolar range and a high ratio of importin β to Smad3MH1 (up to 2 importin β molecules per GST-GFP-S3MH1), did we observe nuclear import of the Smad3 MH1 domain (Fig. 5A, lower). In contrast, at the lowest importin β concentration, nuclear import of importin α already reached the plateau (Fig. 5A, upper). Similarly, endogenous importin α in cytosol allowed efficient

**FIG. 2.** Mapping the nuclear import activity in Smad3 and Smad4. GST fusions of MH1 and L-MH2 domains of Smad2 and Smad3 (A), and Smad4 (B), were tested in the nuclear import assay, with or without cytosol as indicated. Anti-GST immunostaining was used to detect GST fusion proteins after the import assays. Demarcations of the MH1, L (linker) and MH2 domains of the Smads are shown. The MH2 domain (MH2) was traditionally defined according to sequence homology, but based on crystal structure, the complete MH2 domains should include the region marked in pink (amino acids 262-273 in Smad2, amino acids 220-231 in Smad3, and amino acids 286-322 in Smad4).

**FIG. 3.** The L-MH2 is the major nuclear import domain of Smad3. A, GST fusion of the indicated portion of Smad3 was tested in the nuclear import assay, with or without addition of cytosol. Anti-GST immunostaining was used to detect the import substrates (green), and the nuclei were marked by DAPI (blue). B, expression plasmid encoding EGFP fusion of the Smad3(L-MH2) domain (GFP-S3(L-MH2)) was transfected into HaCaT and Hela cells, and subcellular localization of the fusion protein is indicated by the GFP signal (green). No TGFβ was added to the cells, and nuclei were marked by DAPI staining (blue). C, purified GST-S3(L-MH2) was directly labeled with Texas red (red), and used in the nuclear import assay simultaneously with GST-GFP-S3MH1 (green). Fluorescence of Texas red and GFP from the same cells was captured by confocal microscopy. D, His-FLAG-Smad3 was tested in the nuclear import assay, with 10 μmolar excess of either GST-S3MH1 or GST-S3(L-MH2) as competitors. Import of His-FLAG-Smad3 was monitored by anti-FLAG immunostaining (green). Nuclei were marked by DAPI staining (blue).
nuclear import of NLS-GST-GFP, but was not sufficient for import of the MH1 domain of Smad4 (Fig. 5B). In the reconstituted import assay with recombinant import factors, at the lowest concentration of importin α, nuclear import of NLS-GST-GFP already reached the maximum (Fig. 5B, upper), while we detected little import of Smad4 MH1 domain (Fig. 5B, lower). Only when importin α was increased to micromolar range in the reconstituted assay did the MH1 domain of Smad4 start to be imported into the nucleus (Fig. 5B, lower). The higher the importin α concentration we used, the higher the percentage of nuclei that imported the Smad4 MH1 domain (Fig. 5B, lower).

These observations suggest that while the Smad3 MH1 domain can interact with recombinant importin β and the Smad4 MH1 domain with recombinant importin α, the nature of such interactions is such that an artificially high concentration of the importins is required for nuclear import of these proteins in the nuclear import assays.

Smad3 and Smad4 Interact with FG-containing Nucleoporins—A series of hydrophobic surface elements on the MH2 domain of Smad2 mediate the interaction with SARA-SBD, as revealed by the crystal structure of the SARA-SBD bound to this MH2 domain (22). These surface elements of Smad2, referred to as the hydrophobic corridor, were shown to interact with the FG-repeat region of both CAN/Nup214 and Nup153, and such interaction is critical for nuclear import of Smad2 (16). The MH2 domain of Smad2 and Smad3 share 92% amino acid sequence identity and a very similar structure as revealed by x-ray crystal analyses (22, 23). The hydrophobic corridor is highly conserved in Smad3, but not Smad4, which is more divergent from Smad2 and Smad3 (Fig. 6A) (23, 27).

The results from our import assays raised the possibility that similar to Smad2, direct contact with nucleoporins facilitates the importin-independent nuclear import of Smad3 and Smad4. Indeed, recombinant MBP-fused FG-repeat domain of CAN/Nup214 (amino acids 1805–2091) (MBP-CAN) interacted with the full-length Smad2, Smad3, and Smad4, with comparable affinity in a pull-down assay (Fig. 6B). We tested GST fusions of the MH1 and L-MH2 domains of Smad2, -3, and -4 for their ability to interact with the FG-repeats of CAN/Nup214 (HA-CAN1600, amino acids 1600–2091). The L-MH2 domain of Smad3, which is nearly identical to that of Smad2, was able to bind HA-CAN1600 (Fig. 6C). Only weak binding to HA-CAN1600 was detected with the L-MH2 domains of Smad4, and almost no binding with any of the MH1 domains (Fig. 6C). Therefore for all the tested constructs, their affinity for CAN/Nup214 correlates well with their activity in nuclear import assays.

Two point mutations in the hydrophobic corridor of Smad3, V356R and W406A, resulted in much decreased affinity for FG-repeat region of CAN/Nup214 (HA-CAN1600) and Nup153 (HA-Nup153C, aa1156–1476) (Fig. 7A). More importantly, these mutations also resulted in compromised nuclear import activity (Fig. 7B). As a comparison, the P403H mutation, which is located outside of the hydrophobic corridor (Fig. 6A), did not affect Smad3 interaction with the nucleoporins and did not weaken Smad3 nuclear import either (Fig. 7, A and B). These effects are similar to those of the corresponding mutations in Smad2 (16).

In order to determine the specificity of the effect of these mutations on the activation process of Smads, we tested them in vivo, in transfected HaCaT human keratinocytes. The mutations did not affect the ability of Smad3 to be phosphorylated by TGFβ receptor and the subsequent interaction with Smad4 (Fig. 7C). In HaCaT cells with endogenous Smad3 specifically depleted by RNA interference (20), transfection of wild-type Smad3 resulted in TGFβ-induced activation of the Smad-specific transcriptional reporter construct 4xSBE-luc; and as expected, transfection of the import-defective Smad3V356R and W406A mutants failed to activate the same reporter gene after TGFβ stimulation (Fig. 7D). These data support the conclusion that the hydrophobic corridor in Smad3 is responsible for interaction with FG-containing nucleoporins and is crucial for its intrinsic nuclear import activity.

DISCUSSION

In this study, we analyzed the molecular machinery that mediates nuclear import of Smad3 and Smad4. Using the nuclear import assay, which directly measures nuclear transport activity, we reached the conclusion that Smad3 and Smad4 are imported into the nucleus via a mechanism that is independent of importin α and importin β family of import receptors. We demonstrate that Smad3 and Smad4 are capable of interaction with the nucleoporin CAN/Nup214, and this interaction is required for nuclear import. Our observations suggest that Smad3 and Smad4 are imported into the nucleus via direct but different modes of contact with nucleoporins.

Smad3 Nuclear Import Mediated by the Hydrophobic Corridor—We observed the following characteristics of Smad3 nuclear import: first, the MH2 domain is the major driving force...
of Smad3 nuclear import; second, the hydrophobic corridor in the MH2 domain contributes to Smad3 interaction with CAN/Nup214 and Nup153, and is required for Smad3 nuclear import; and third, nuclear import of Smad3 can occur independently of importin/β and importin/α.

As the recombinant Smad3 protein used in our assays was produced in Escherichia coli, which lacks TGF-β-like kinase activity (28), our results suggest that nuclear import of Smad3 can take place without it being phosphorylated by TGF-β receptor. However, the spontaneous nuclear import of Smad3 is prevented by the SARA-SBD domain, which occludes the hydrophobic corridor in the MH2 domain (22, 23). This observation, together with the report that Smad3 loses interaction with SARA upon TGF-β stimulation in the cell (Tsukazaki et al., Ref. 21), suggest that TGFβ enhances Smad3 nuclear import partly through dissociating Smad3 from cytoplasmic retention factors such as SARA. Direct biochemical assays have shown that receptor-mediated phosphorylation decreases Smad affinity for SARA (15, 21, 23).

An interaction between the Smad3 MH1 domain and importin β1 was reported previously, and was proposed to mediate Smad3 nuclear import (8, 9). However, several lines of evidence in our study argue against such conclusion. First, when tested on the same cells, nuclear import of the L-MH2 domain of Smad3 is much stronger than that of the MH1 domain (Fig. 4, A and C). The previous assertion that the MH2 domain of Smad3 contains only weak nuclear import activity is based on the construct GST-S2 (231–452) (Fig. 4A) (9). However the co-crystal structures of SARA-SBD complexed with the MH2 domain of Smad2 or Smad3, indicated that the N-terminal boundary of the hydrophobic corridor extends to the β1-strand of the MH2 domain, which starts at residue 262 in Smad2 and 220 in Smad3 (22, 23). Therefore, the Smad3 MH2 (amino acids 231–425) construct, which was used in previous studies by others (9), is missing this critical region of the hydrophobic corridor, which would explain why only weak nuclear import activity was observed. Our present mutational analysis of Smad3 indeed shows that the portion of the hydrophobic corridor involving the β1-strand (the portion that also includes Val-356 and Trp-406, see Fig. 6A) is crucial for binding to nucleoporins and nuclear import. Second, only when recombinant importin/β was added to the import reaction to achieve artificially high molar ratio to GST-GFP-S3MH1 did we begin to observe some nuclear import by the MH1 domain of Smad3. Third, for importin β to function as an import factor, it needs to bind to nucleoporins and RanGTP in addition to the cargo protein, and it is unclear if binding the MH1 domain of Smad3

![High concentration of importin β and importin α is needed to import the MH1 domains of Smad3 and Smad4 into the nucleus, respectively.](image-url)
would affect those interactions (29, 30). Moreover, it was recently suggested that importin β factors can serve as chaperones for exposed basic domain aside from its roles in nuclear import (31). Therefore an interaction with importin β may not necessarily relate to nuclear import.

In summary, our biochemical and functional data are consistent with the conclusion that Smad3 nuclear import is primarily mediated by direct interactions with nucleoporins via the hydrophobic corridor, and is independent of importins. Smad4 Nuclear Import by Distinctive Nucleoporin-interacting Domain—Although not phosphorylated by the TGFβ receptor, Smad4 undergoes TGFβ-induced nuclear accumulation (10). Recent reports also revealed a dynamic nucleocytoplasmic shuttling of Smad4, with its nuclear export mediated by CRM-1 (12, 13). How the nuclear import of Smad4 is accomplished is unclear, but it was proposed that a Lys-rich region in the MH1 domain may serve as a classical NLS (12, 14).

In the nuclear import assays we observed, first, that Smad4 nuclear import is independent of cytosol. The nature of the nuclear import assay also indicates that Smad4 is intrinsically capable of entering the nucleus without the need for TGFβ stimulation. Second, nuclear import of Smad4 is not inhibited by RanQ69L, suggesting that the importin β family of transport receptors is not necessary for this process. Third, unlike Smad2 and Smad3, Smad4 nuclear import is not blocked by SARA-SBD, consistent with the idea that an unique domain in Smad4 is recognized by the import machinery. In addition, biochemical analyses indicated that like Smad2 and Smad3, Smad4 interacted with the FG-repeat region of CAN/Nup214. The evidence from biochemical and nuclear import analyses suggest that Smad4 is imported into the nucleus via direct contact with FG-containing nucleoporins, through a domain distinct from the hydrophobic corridor in Smad2 and Smad3.

Although Smad4 shares considerable sequence homology in its MH2 domain to that of Smad2 and Smad3, at the tertiary structure level they are substantially different (Fig. 6A). This is particularly so with regard to the hydrophobic corridor that is present only in Smad2/3 but not in Smad4, which is responsible for interaction with both CAN/Nup214 and SARA (Fig. 6A). This information from x-ray crystal structures is reflected in our functional data that Smad4 nuclear import is impervious to SARA and that the L-MH2 region of Smad4 is inefficient at binding FG-containing nucleoporins and carrying out nuclear import. It appears that Smad4 interaction with CAN/Nup214 and its nuclear import ability require structural integrity in the whole protein, since neither the MH1 nor the several L-MH2 domains of Smad4 we tested were able to mediate CAN/Nup214 association or nuclear import. Given the divergence in domains involved in NPC contact by Smad4 and Smad2/3, Smad4 nuclear import is expected to be controlled by different factors.

It was reported that the MH1 domain of Smad4 could interact with importin α, leading to the hypothesis that Smad4 import into the nucleus is mediated by the classic NLS pathway involving importin α and importin β (14). However, our observations argue against such a notion. First of all, the nuclear import of Smad4 is insensitive to RanQ69LGTP, which rules out participation of importin β factors in this process. Second, GST-Smad4MH1 was not imported into the nucleus under the same condition that supported nuclear import of NLS-GST-GFP, suggesting that interaction between Smad4 MH1 domain and importin α does not functionally result in
nuclear import unless artificially high concentrations of importin α was used.

**Regulation of Nucleocytoplasmic Shuttling of Smad2, Smad3, and Smad4 by TGFβ**—We have obtained evidence suggesting spontaneous and TGFβ-independent nuclear import activity in Smad2, -3, and -4 (15). In addition, we and others (12, 13, 16, 32, 33) have characterized continuous nuclear export of Smad2, -3, and -4. In the case of Smad2 and likely Smad3, direct contact with nucleoporins underlies not only nuclear import but also nuclear export (16, 32). On the other hand, though Smad4 import appears to be mediated by direct contact with nucleoporins, the CRM-1 export receptor is responsible for its exit from the nucleus (12, 13).

Therefore, the import and export machineries for Smad2, -3, and -4 are all constitutively active. How then does TGFβ signal modulate the transport and retention forces in order to promote nuclear accumulation of these three Smads?

Phosphorylation in response to TGFβ needs not change the affinity of Smad3 for CAN/Nup214 in order to facilitate nuclear accumulation of Smad3 (16). Like Smad2, Smad3 loses affinity for SARA as a result of TGFβ-induced phosphorylation of its C terminus (15, 23). The crystal structure of phosphorylated Smad2 shows a conformational change in the disposition of the β′-strand that affects a critical SARA contact site and may explain the observed loss of affinity for SARA (34). This change most likely occurs also in Smad3, as the regions in question are highly conserved (23). In addition, Smad3 in TGFβ-treated cells has substantially increased association with Smad4 binding to target sites on the DNA (35). Hence, as a consequence to TGFβ signaling, the nuclear retention forces are expected to overcome cytoplasmic retention, causing Smad3 to concentrate in the nucleus.

Inactivation of the CRM-1 export receptor by LMB is sufficient to cause exclusive nuclear localization of Smad4, with no requirement for TGFβ signal (12, 13). This suggests that Smad4 import machinery is constitutively active and is able to restrict Smad4 in the nucleus, as long as Smad4 can be disengaged from CRM-1 once in the nucleus. We can speculate that TGFβ-promoted association of Smad4 with phosphorylated Smad2/3, other transcription cofactors and DNA all have the potential to interfere with Smad4 interaction with CRM-1, resulting in accumulation of Smad4 in the nucleus (10, 34).

So far, for Smad2, -3, and -4, all the experimental evidence is consistent with the hypothesis that TGFβ signal does not influence the nuclear import and export process itself, but rather modifies the affinity of these Smads with their binding partners which retain them in either nucleus or cytoplasm. It is the change in affinity for retention factors on both sides of the nuclear envelope that leads to enhanced nuclear localization of Smad2, -3, and -4 in response to TGFβ. This mode of nucleocytoplasmic redistribution of signal transduction molecules represents a significant departure from the more general
importin-dependent process that is central to other pathways, raising the possibility that Smad proteins evolved as nucleocytoplasmic transporters with an ancestral MH2 fold (36) that eventually became engaged in the sensing and transduction of signals from membrane receptors to the DNA.

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Distinct Domain Utilization by Smad3 and Smad4 for Nucleoporin Interaction and Nuclear Import
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