Nuclear Targeting of Transforming Growth Factor-β-activated Smad Complexes*§

Upon stimulation by the transforming growth factor β (TGF-β), Smad2 and Smad3 are phosphorylated at their C termini and assemble into stable heteromeric complexes with Smad4. These complexes are the functional entities that translocate into the nucleus and regulate the expression of TGF-β target genes. Here we report that the TGF-β-activated phospho-Smad3/Smad4 complex utilizes an importin-independent mechanism for nuclear import and engages different nucleoporins for nuclear import compared with the monomeric Smad4. Within the heteromeric complex, phospho-Smad3 appears to dominate over Smad4 in the nuclear import process and guides the complex to its nuclear destination. We also demonstrate that the binding of phospho-Smad3 to Smad4 prevents Smad4 from interacting with the nuclear export receptor chromosome region maintenance 1. In this way, TGF-β signaling suppresses nuclear export of Smad4 by chromosome region maintenance 1 and thereby targets Smad4 into the nucleus. Indeed tumorigenic mutations in Smad4 that affect its interaction with Smad2 or Smad3 impair nuclear accumulation of Smad4 in response to TGF-β.

The transforming growth factor β (TGF-β)1 family of cytokines are critical regulators of embryonic development and homeostasis, controlling proliferation, differentiation, and apoptosis of many cell types (1). Signals from the TGF-β cytokines are transduced by a similar mechanism with the Smad family of transcription factors being the central components (2–4). Signals from TGF-β bound type I and type II receptor kinases (5). This phosphorylation event induces Smad2 and Smad3 to form stable complexes with Smad4, which subsequently accumulate in the nucleus (5–7). Previous studies have revealed a homotrimeric structure for C-terminally phosphorylated Smad2 or Smad3 (phospho-Smad2 or -Smad3) (8, 9) and a heterotrimeric configuration for phospho-Smad2 or Smad3 with Smad4 at a 2:1 (Smad2:3:Smad4) ratio (9, 10). Phospho-Smad2 was also suggested to form a heterodimer with Smad4 (8). It is the heteromeric complexes of Smad2 or Smad3 with Smad4 together with other transcription factors in the nucleus that activate or repress gene expression in response to TGF-β (3, 11, 12).

In the absence of TGF-β stimulation, Smad2, Smad3, and Smad4 are monomeric (13) and shuttle between the nucleus and cytoplasm independently (14, 15). The balance of nuclear import and export forces together with cytoplasmic retention mechanisms result in mostly cytoplasmic distribution of these Smad proteins (16, 17). Upon TGF-β signaling and the ensuing phosphorylation of Smad2/3, the Smads form multimeric complexes and become exclusively concentrated in the nucleus (14, 15). There has been much interest in understanding the machinery responsible for such signal-regulated nuclear enrichment of Smads. The importin β family of transport receptors mediate nuclear translocation of many macromolecules (18–20). The primary function of importin β is to bridge the association between their cargos and nucleoporins, the constituents of the nuclear pore complex (NPC) (18, 19, 21). However, previous studies have suggested an importin-independent nuclear import mechanism for unphosphorylated Smad2, Smad3, and Smad4 (22). Such a mechanism relies on the ability of Smad2, Smad3, and Smad4 to directly interact with phenylalanine-glycine (FG) repeat-containing nucleoporins including CAN/Nup214 and Nup153 (14, 22). In Smad2 and Smad3, the association with the nucleoporins is mediated by the C-terminal MH2 domains, whereas in Smad4, the nucleoporin-interaction domain is not yet defined (14, 22). Smad3 and Smad4 have also been reported to contain additional nuclear import activities in their N-terminal MH1 domains, which are mediated by importin β1 and importin α, respectively (23–26). However, most of these conclusions were based on studies of individual forms of Smad2, Smad3, and Smad4, which pertain only to Smad movement at the basal state. The question remains whether the TGF-β-activated forms, namely the homotrimeric and heterotrimeric complexes of Smad2 or Smad3 with Smad4, are imported into the nucleus via similar or distinct pathways.

Smad4 contains a typical nuclear export signal (NES) at the junction of its N-terminal MH1 domain and the linker region and is continuously exported out of the nucleus by chromosome region maintenance 1 (CRM-1) (27, 28). When CRM-1 is inactivated by the compound leptomycin B, Smad4 resides exclusively in the nucleus without TGF-β stimulation (27, 28). This suggests that, although the nuclear entry of Smad4 is a spontaneous event not requiring TGF-β signaling, CRM-1-mediated nuclear export must be suppressed by TGF-β in order to retain Smad4 in the nucleus. The mechanism for such inactivation of CRM-1-mediated Smad4 export remained elusive.

Here we studied the homotrimeric phospho-Smad3 and the heterotrimeric phospho-Smad3/Smad4 complexes, which are the active forms of Smads in response to TGF-β. We demon-

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§The on-line version of this article (available at http://www.jbc.org) contains Supplemental Fig. 1.

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The abbreviations used are: TGF-β, transforming growth factor β; CRM-1, chromosome region maintenance 1; NPC, nuclear pore complex; NES, nuclear export signal; GST, glutathione S-transferase; FPLC, fast protein liquid chromatography; aa, amino acid; WGA, wheat germ agglutinin; NLS, nuclear localization signal.

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strate that these complexes are imported into the nucleus through an importin-independent mechanism. Although the monomeric Smad4 and Smad4 in complex with phospho-Smad3 are imported with comparable efficiency, the two forms employ different nucleoporins in their translocation across the nuclear pore. It also appears that phospho-Smad3 in the complex plays a more dominant role in guiding the complex through the nuclear pore and excluding Smad4 from the nucleolus. Our data also suggest that the nuclear import activity in phospho-Smad3 alone may be sufficient for the Smad4/phospho-Smad3 complex to enter the nucleus. Furthermore, we found that the heterotrimer formation with phospho-Smad3, but not its binding to the target DNA, physically prevents Smad4 from binding to CRM-1 and thus enables Smad4 to accumulate in the nucleus in response to TGF-β. Indeed, Smad4 mutants defective in interaction with phospho-Smad2 or Smad3 are impaired in nuclear accumulation and therefore are unable to function as tumor suppressor.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Immunocytochemistry—COS-1 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 units/ml), and streptomycin (100 units/ml, Invitrogen). Cells were transiently transfected with the indicated plasmids using Lipofectamine (Invitrogen) or FuGENE 6 (Roche Applied Science) following manufacturers' instructions. Immunocytochemistry was carried out as previously described (17). Cells were examined under a Nikon TE2000-S inverted fluorescent microscope, and the images were captured by a Spot RT-KE digital camera (Diagnostic Instruments).

Recombinant Proteins and Fluorescence Labeling—The expression vectors for His-CRM-1, His-importin β (45–462), and His-nuclear transport factor 2 were gifts from Drs. Maarten Fornerod (the Netherlands Cancer Institute, Amsterdam, the Netherlands), Dirk Görlich (Göttingen, Germany), and Gunter Blobel (Rockefeller University, New York City) respectively. The encoded proteins were expressed in Escherichia coli and purified using nitrilotriacetic acid-agarose beads following manufacturer's instructions (Qiagen).

Full-length Smad4 was expressed and purified as a GST fusion protein. The GST moiety was then cleaved by thrombin digestion, and the buffer was adjusted to 20 mM Tris, pH 8.5, 10 mM NaCl, and 0.1 mM EDTA before being loaded onto a DEAE-Sepharose column (Amersham Biosciences). Full-length Smad4 was eluted with 20 mM Tris, pH 8.3, and 150 mM NaCl. Purified Smad4 was labeled with fluorescein-5-maleimide following the vendor's protocol (Molecular Probe). After terminating the labeling reaction with 50 mM 2-mercaptoethanol, Smad4 was further purified by a Superdex 200 HR column on FPLC in 20 mM Hepes, pH 7.4, 100 mM NaCl, and 0.1 mM EDTA.

Generation of Phosphorylated Smad3 and Homotrimer—Smad3 (aa 144–419) without the C-terminal CSSVS sequence was cloned into the pTXB1 bacterial expression vector (New England BioLabs) upstream of the T7 promoter. The protein was expressed in E. coli and purified using nitrilotriacetic acid-agarose beads following manufacturer's instructions (Qiagen).

For detection of the Smad4-CRM-1 interaction, 5 μg of His-CRM-1, 10 μg of GST-Smad4N (aa 273–552), purified as previously described, was added in a 2-fold molar excess to phospho-Smad3(144–424) (29). Each heterotrimeric Smad complex was first purified by ion-exchange chromatography on a DEAE column, in which the uncomplexed Smad4 eluted in the flow-through fractions, whereas the heteromeric Smad complex eluted in a 100–300 mM NaCl gradient. The resulting Smad complex was further purified by a Superdex 200 size-exclusion column (Amersham Biosciences).

To generate the phospho-Smad3/full-length Smad4 heterocomplex, the full-length Smad4 purified and labeled as described above was incubated with 3 μl molar excess of phospho-Smad3(144–424) for 30 min at 4 °C and subsequently purified by a Superdex 200 size-exclusion column (Amersham Biosciences). Fractions containing complexed form of full-length Smad4 were pooled and concentrated before various experiments.

In Vitro Nuclear Import and Export Assays—HeLa cells were permeabilized with 40 μg/ml digitonin (Calbiochem) and processed for in vitro nuclear import assays as described previously (17). All of the import substrates were used at 0.1 μg/μl and all of the import and export reactions include an ATP-regenerating system (1 mM ATP, 5 mM phosphocreatine, and 0.02 units/μl creatine phosphokinase). Where indicated, RanQ69L-GTP (5 μM), importin β(45–462) (10 μM), and wheat germ agglutinin (WGA) (0.5 μg/μl) were included in the import reactions. After 20 min of import reaction at room temperature, cells were fixed and stained with anti-Smad2/3 antibody to monitor nuclear translocation of Smad3. For the detection of Smad4 in these assays, the recombinant Smad4 was directly labeled with fluorescein-5-maleimide as described above.

In the in vitro nuclear export assay, proteins of interest were first allowed to enter the nucleus following the in vitro nuclear import assay protocol (17). After the import reaction, the cells were washed with transport buffer (20 mM Hepes, pH 7.6, 110 mM KAc, 2 mM MgOAc2, 2 mM EGTA) and further incubated with the transport mixture containing 1 μg of His-CRM-1, 1 μg of RanGDP, 0.2 μg of nuclear transport factor 2, 5 μl of rabbit reticulocyte lysate (Promega), 1 mM ATP, 0.2 mM GTP, 5 mM phosphocreatine, and 0.02 unit/μl creatine phosphokinase in a final volume of 20 μl. The export reaction was allowed to proceed for 10 min at room temperature, and then the cells were washed with the transport buffer and fixed with paraformaldehyde as described before (17).

Protein-Protein Interaction Assays—In GST pull-down assays, hemagglutinin-CAN1600 (aa 1600–2091) was overexpressed in COS-1 cells by transient transfection and cell lysates were prepared by sonication in the immunoprecipitation buffer (20 mM Tris, pH 7.4, 100 mM NaCl, 20 mM NaF, 20 mM β-glycerophosphate, 10% glycerol, 0.1% Nonidet P-40, and 2 mM dithiothreitol supplemented with protease inhibitors). The lysate was mixed with 10 μg of GST-Smad4 with or without 20 μg of phospho-Smad3(144–424) and incubated for 1 h at 4 °C before adding glutathione-conjugated agarose beads (Sigma). After additional incubation for 2 h at 4 °C with rotation, the agarose beads were washed three times in the immunoprecipitation buffer and the bound proteins were analyzed by Western blotting.

For immunoprecipitation experiments, COS-1 cells were transfected with FLAG-Smad4 wild type or mutant expression plasmids as indicated. Cells were treated with 100 μM TGF-β (Peprotech) 18 h post-transfection and lysed by sonication in the immunoprecipitation buffer. Immunoprecipitation was done using anti-Smad2/3 antibody at 4 °C overnight, and the immunocomplex was precipitated by protein A beads (Zymed Laboratories Inc.). After washing 3× with the immunoprecipitation buffer, proteins bound to the beads were resolved by SDS-PAGE and analyzed by Western blotting.

For detection of the Smad4-CRM-1 interaction, 5 μg of His-CRM-1, 10 μg of GST-Smad4N (aa 1–239), or 10 μg of GST-Smad4 were incubated in 20 mM Hepes, pH 7.9, 200 mM NaCl, 8% glycerol, and 2.5 mM 2-mercaptoethanol for 20 min before adding glutathione-conjugated agarose beads. As indicated, 20 μg of RanG69L-GTP and 1 mM GTP were included in the assay. After 2 h, the beads were washed in the same buffer for three times and the bound proteins were analyzed by Western blotting using anti-His conjugated with horseradish peroxidase (Roche Applied Science). As stated in the figure legends, 30 μg of phospho-Smad3(144–424) or 2 nmol (20.1 molar ratio to GST-Smad4) of Smad-binding element DNA element (5'-TAAGTCTAGAGCAGTGTTACGTTTCAGTC-3') were preincubated with GST-Smad4 prior to the addition of His-CRM-1.

RESULTS

Nuclear Import of the Phospho-Smad3 Complexes and the Interaction with CANINp214—To elucidate the nuclear import mechanisms for TGF-β-activated Smad complexes, we purified these complexes to test them in the in vitro nuclear import assay using digitonin-permeabilized HeLa cells (30). As the import substrate in this assay, the Smad3 fragment (aa 144–419, including the linker and the MH2 domain) was chemically ligated to a synthetic peptide (Cys-Ser-pSer-Val-
Phospho-Smad3 Harbors Sufficient Nuclear Import Activity to Drive Associated Smad4 into the Nucleus—The aa 273–552 region of Smad4 (also referred to as the AF fragment) interacts with phospho-Smad3, but it lacks intrinsic nuclear import activity as evidenced by the observation in this (Fig. 1D) and our previous studies (22) that the GST-Smad4AF fusion (GST-S4AF) did not enter the nucleus. However a substantial amount of GST-S4AF could be imported into the nucleus upon association with phospho-Smad3 (Fig. 1D). The extra GST moiety on the Smad4 AF fragment may have contributed to the slightly lower nuclear import efficiency compared with the heterotrimer without GST used in Fig. 1B. Nevertheless, the data suggest that the nuclear import activity provided by phospho-Smad3 alone is sufficient to carry the bound Smad4 fragment across the NPC.

Nuclear Import of the Phospho-Smad3 Complexes Is Importin-independent but Nucleoporin-dependent—We further probed the requirements for nuclear import of the phospho-Smad3 complexes. RanQ69L, a mutant form of the RanGTPase that is a potent inhibitor of all known importin β family-mediated nuclear imports (31, 32), did not affect the transport of phospho-Smad3 into the nucleus (Fig. 2A). Under the same assay conditions, the efficacy of RanQ69L-GTP was confirmed by its ability to block the classic nuclear localization signal (NLS)-mediated importin/cytosol-dependent nuclear import

pSer) with the indicated serine residues phosphorylated. This homogenously phosphorylated Smad3, identical to the TGF-β-activated form of Smad3, readily assembles into a homotrimer complex by itself and a heterotrimer complex at 2:1 ratio with Smad4 (aa 273–552) under physiological conditions (Fig. 1A) (10). Resolved by Superdex 200 size-exclusion column, the homotrimer and heterotrimer phospho-Smad3 complexes were eluted in fractions corresponding to molecular mass of over 100 kDa (Fig. 1A, fractions 15–16), whereas the monomers were eluted much later (fraction 21, data not shown). This allowed us to purify the complexes for use in the nuclear import experiments.

Both the homotrimeric and heterotrimeric forms of phospho-Smad3 were imported into the nucleus without cytosol in the import assay (Fig. 1B). The addition of cytosol neither enhanced nor reduced the level of phospho-Smad3 accumulating in the nucleus, suggesting that the importin molecules may not be necessary for nuclear import of the phospho-Smad3 complexes (Fig. 1B). In a GST pull-down experiment, the phospho-Smad3/Smad4 complex interacted with the FG-repeat domain of the nucleoporin CAN/Nup214 (Fig. 1C). The level of interaction was not considerably different from that exhibited by the monomeric Smad4 (Fig. 1C). This and our previous observations suggest that the complex formation between phospho-Smad2/3 and Smad4 does not affect their association with FG-containing nucleoporins such as CAN/Nup214 (14). Therefore, in both the nuclear import assay and interaction with nucleoporins, the general characteristics of the phospho-Smad3 complexes are similar to those of monomeric Smads (22).
monomeric and the heteromeric Smad3 make contact with a similar group of nucleoporins in migrating across the NPC.

These observations suggest that the phospho-Smad3 homotrimers and heterotrimers, assembled in response to TGF-β signal, enter the nucleus via an importin-independent mechanism that involves direct contact with nucleoporins. Some of these nucleoporins must also be engaged by importin β in its translocation across the nuclear pore. Such features in the nuclear import mechanism were also observed for unphosphorylated Smad2 and Smad3 (17, 22). These observations suggest that the basal state nucleocytoplasmatic shuttling of Smad3 and the nuclear translocation of TGF-β-activated phospho-Smad3 complexes are mediated by similar transport mechanisms.

**Distinction in Nuclear Import of Monomeric and Phospho-Smad3-associated Smad4**—We next compared nuclear import of monomeric Smad4 and its complex with phospho-Smad3. Using Superdex 200HR gel-filtration chromatography, the monomeric Smad4 and the phospho-Smad3/Smad4 complex can be readily separated and we pooled the fractions containing the heteromeric phospho-Smad3/Smad4 complex for the nuclear import assays (Fig. 3A). Because we used excess homotrimeric phospho-Smad3 to maximize Smad4 incorporation into the complex and the gel filtration column cannot resolve the newly formed heterotrimer and the Smad3 homotrimer, the fractions we pooled also contained phospho-Smad3 heterotrimer (Fig. 3A).

However, because Smad4 was labeled with fluorescein maleimide prior to incubation with phospho-Smad3, we could specifically follow the movement of Smad4 in the context of the heterotrimer.

Smad4, either in complex with phospho-Smad3 (120 kDa) or by itself (60 kDa), was imported into the nucleus independent of cytosol (Fig. 3B). The intensity of signal in the nucleus is similar between the monomeric and the complexed Smad4, suggesting that the association with phospho-Smad3 did not further accelerate nuclear import. RanQ69L-GTP did not affect nuclear import of either the monomeric or the complexed Smad4, arguing for importin-independent mechanisms (Fig. 3B). However, importin β-(45–462) did not significantly inhibit nuclear import of the monomeric Smad4, suggesting that, in the process of nuclear translocation, Smad4 interacts with a set of nucleoporins distinct from those recognized by importin β. This is in contrast to the Smad4 in complex with phospho-Smad3 in which the nuclear import was substantially inhibited by importin β-(45–462) (Fig. 3B) WGA, a more general blocker of the NPC, prevented both the monomeric and complexed forms of Smad4 from entering the nucleus (Fig. 3B), confirming that nuclear import of the monomeric Smad4 is indeed an active process (34).

The above data suggest that both the monomeric and complexed forms of Smad4 are capable of nuclear import in an importin-independent manner, but the nucleoporin contacts crucial for the complex may not be essential for nuclear import of the monomeric Smad4. Combining the observations in Figs. 1C, 2, and 3, it appears that, for the phospho-Smad3/Smad4 complex, the import route utilized by phospho-Smad3 may be dominant over the pathway for Smad4.

**Phospho-Smad3 Guides Smad4 Away from the Nucleolus**—In nuclear import assays, we noticed that, once in the nucleus, phospho-Smad3 is mostly excluded from the nucleolus (Fig. 4A). Unphosphorylated Smad3 also exhibited a similar pattern of localization in this and our previous studies (Fig. 2B) (17, 22). On the other hand, when the Smad4 monomer was imported into the nucleus, it was distributed rather evenly throughout the nucleus (Fig. 4A). Interestingly when Smad4 was associated with phospho-Smad3, it resided mainly in areas outside of the nucleolus, assuming a pattern similar to that of phospho-Smad3 (Fig. 4A). In cells treated with TGF-β, Smad4

![Fig. 2. Importin-independent nuclear import of phospho-Smad3 complexes. A, the homotrimeric and heterotrimeric phospho-Smad3 complexes as in Fig. 1 were tested in nuclear import assays without cytosol but in the presence of RanQ69L-GTP or importin β (impβ(45–462)) as indicated. Detection of imported phospho-Smad3 and the nuclei was the same as in Fig. 1. Compared with the BSA control, Impβ(45–462) abrogated nuclear import of phospho-Smad3 complexes, whereas RanQ69L-GTP had no effect. DAPI, 4,6-diamidino-2-phenylindole. B, nuclear import analysis of an NLS-containing cargo NLS-GST-green fluorescent protein (NLS-GST-GFP). The importin-mediated nuclear import of NLS-GST-green fluorescent protein was strictly dependent on cytosol and was inhibited by RanQ69L-GTP, importin β-(45–462), and WGA. Immunostaining with an anti-GST antibody was used to detect NLS-GST-green fluorescent protein. C, nuclear import assay of unphosphorylated Smad3. GST-Smad3 (aa 144–424) was expressed and purified from E. coli and tested in the nuclear import assay as in A.

Fig. 2B). The N-terminally truncated importin β (aa 45–462) stably associates with and masks a subset of nucleoporins to block active nuclear import (33). Indeed, the nuclear import of phospho-Smad3 in either the homotrimeric or the heterotrimeric complex was largely abolished by importin β-(45–462), suggesting that contact with importin β-targeted nucleoporins is important for nuclear translocation of phospho-Smad3 homotrimers and heterotrimers (Fig. 2A). Similar observations were made with full-length importin β (Supplemental Fig. 1). Furthermore, the unphosphorylated version of the same fragment of Smad3 (GST-Smad3LMH2) was imported with similar efficiency compared with phospho-Smad3 in the complexes and was also prevented from entering the nucleus by importin β (aa 45–462) (Fig. 2C). This is consistent with the idea that the...
Thus TGF-\(\beta\) must somehow inactivate CRM-1 to drive Smad4 accumulation in the nucleus. In response to TGF-\(\beta\), in addition to forming complexes with Smad2 or Smad3, Smad4 is also bound to DNA (35). Thus we began by investigating whether phospho-Smad3 interaction or DNA binding would interfere with physical interaction between Smad4 and CRM-1.

In GST pull-down assays using purified recombinant proteins, Smad4 directly bound CRM-1 in a RanGTP-dependent manner (Fig. 5A), which is expected for a typical export substrate of CRM-1 (36). More importantly, when Smad4 was associated with phospho-Smad3, we no longer detected significant interaction between Smad4 and CRM-1, even in the presence of RanGTP (Fig. 5B). On the other hand, preincubation with the Smad-binding element oligonucleotide did not affect Smad4 interaction with CRM-1 (Fig. 5C). Similar observations were made using both the N terminus (aa 1–239) and full-length Smad4 (Fig. 5C).

Therefore, by either inducing a conformational change or directly masking the NES motif of Smad4, TGF-\(\beta\)-activated Smad3 prevents the interaction between Smad4 and CRM-1. Although, in Smad4, the DNA-binding domain is close to the CRM-1-binding NES motif, our data suggest that Smad4 association with its DNA target and CRM-1 is not mutually exclusive.

Smad4 in Complex with Phospho-Smad3 Is Not Exported by CRM-1—We next sought to determine functionally whether CRM-1 is able to export the Smad4/phospho-Smad3 complex using an in vitro nuclear export assay. Under our assay condition, as well as previously reported, the digitonin treatment and subsequent washing largely depletes endogenous CRM-1, so CRM-1-mediated nuclear export can be reconstituted in vitro by adding back recombinant CRM-1 (37).

Monomeric and complexed forms of Smad4 were first allowed to enter the nucleus through the in vitro nuclear import assay. The cells were then washed to remove unimported substrates and then further incubated with nuclear export mixture containing recombinant CRM-1 or bovine serum albumin as a control (Fig. 6). Indeed, when recombinant CRM-1 was added but not the bovine serum albumin control, the originally imported monomeric Smad4 was mostly exported out of the nu-

**Fig. 4.** Phospho-Smad3 targets Smad4 away from the nucleolus. A, after nuclear import of the homotrimeric and heterotrimer complexes, the cells were stained with anti-Smad2/3 antibodies to reveal the subnuclear location of Smad3 (left). Fluorescein isothiocyanate (FITC)-labeled Smad4 monomer or its complex with phospho-Smad3 was allowed to enter the nucleus, and the fluorescent signal indicates the location of FITC-Smad4 (right). B, COS-1 cells were transfected with FLAG-Smad4 and Smad3. Cells were stained with an anti-FLAG antibody with or without prior TGF-\(\beta\) treatment.

**Fig. 3.** Nuclear import of monomeric and complexed Smad4. A, size-exclusion chromatography profiles of full-length Smad4 (upper gel) or its complex with phospho-Smad3 (lower gel). Fluorescein-labeled full-length Smad4 was resolved by a Superdex 200 column before (upper gel) and after (lower gel) co-incubation with excess phospho-Smad3. The eluted fractions were run on SDS-PAGE, and the Coomassie Blue-stained gels are shown. After incubation with phospho-Smad3, the majority of Smad4 was incorporated into a higher molecular weight (MW Std.) complex (fractions 14 and 15). Fractions containing the molecular standards are also indicated. B, nuclear import assay of the monomeric Smad4 and its complex with phospho-Smad3. Purified fractions corresponding to monomeric Smad4 and Smad4/phospho-Smad3 complex (fractions 14 and 15 in A) were tested in the nuclear import assay. The inclusion of cytosol (cytosol), RanQ69L-GTP, importin \(\beta\) (Imp\(\beta\)45–462), or WGA is as indicated. Smad4 was directly labeled with fluorescein, and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

in the nucleus was also excluded from the nucleolus (Fig. 4B).

Therefore, it seems that, in the context of the heterotrimer complex, phospho-Smad3 restricts the movement of Smad4 in the nucleus and targets Smad4 away from the nucleolus. This observation also suggests that phospho-Smad3 and Smad4 most probably move as a unit during both nuclear translocation and subsequent movement within the nucleus.

Smad4 Association with Phospho-Smad3 Prevents CRM-1 Binding—Smad4 is capable of entering the nucleus with or without TGF-\(\beta\) stimulation. At the basal state, CRM-1-mediated nuclear export prevails over the nuclear import of Smad4, resulting in exclusion of Smad4 from the nucleus. Data shown in Fig. 3 demonstrated that nuclear import of Smad4 at the basal and TGF-\(\beta\)-stimulated states is equally efficient, although the mode of nuclear translocation may be different.
Smad4 Mutants with Impaired Phospho-Smad2/3 Interaction Are Defective in TGF-β-induced Nuclear Accumulation—Smad4 is a tumor suppressor that is frequently mutated in a number of cancers, most notably pancreatic and colon cancers (38, 39). Many of these cancer-associated Smad4 mutants contain missense point mutations, and it is not clear which aspect of Smad4 function is impaired by these mutations. Conceivably, Smad4 nuclear accumulation in response to TGF-β is essential for its tumor suppressor function and is targeted for cancer-associated mutations. To test this hypothesis, we examined two cancer-associated mutants of Smad4, R361C and D351H, for their interaction with endogenous Smad2/3 in transfected COS-1 cells. Consistent with x-ray crystallography analyses (8), the R361C and D351H mutants exhibited little interaction with Smad2 or Smad3 in response to TGF-β in cells (Fig. 7A). In parallel, the R361C and D351H mutants were much less efficient in their accumulation in the nucleus in response to TGF-β compared with the wild-type Smad4 (Fig. 7B). Such reduced nuclear accumulation was not due to impaired nuclear import activity of these mutants, because when...
the CRM-1 function was inhibited by leptomycin B, both mutants became concentrated in the nucleus to the same extent as the wild type (Fig. 7C).

Therefore, the TGF-β-induced heterotrimer formation between Smad4 and Smad3 is a critical event that enables Smad4 to evade nuclear export by CRM-1. In this way, the TGF-β signal promotes the accumulation of Smad4 in the nucleus without accelerating its rate of nuclear import.

**DISCUSSION**

TGF-β triggers phosphorylation of Smad2 and Smad3 and consequently prompts their association with Smad4. These complexes are the physiologically active forms of Smads in TGF-β signaling. In this study, we found that the TGF-β-activated Smad complexes enter the nucleus via an importin-independent mechanism similar to that utilized by monomeric Smads, although they appear to employ different nucleoporins in the process. The assembly of the phospho-Smad3/Smad4 complex has a more pronounced impact on nuclear export in that interaction with phospho-Smad3 protects Smad4 from being engaged by CRM-1. Therefore, by disabling the nuclear export machinery, TGF-β-pho-Smad3 protects Smad4 from being engaged by CRM-1. This is independent of each other. However, immediately in response to TGF-β/H9252, Smad2 or Smad3 is tightly associated with its target genes in the nucleus, these mutations disrupt Smad4 function as a tumor suppressor.

**Nuclear Import of Monomeric Versus Multimeric Smad3 and Smad2**—In Smad4-null cells, TGF-β-induced nuclear accumulation of Smad2 or Smad3 appears to be normal (40). On the other hand, at the basal state, Smad4 can concentrate in the nucleus as a result of inactivation of CRM-1 by leptomycin B when Smad2 and Smad3 still reside mostly in the cytoplasm (27, 28). So clearly, nuclear import of these Smads is independent of each other. However, immediately in response to TGF-β, Smad2 or Smad3 is tightly associated with Smad4 and the question is whether the complex would utilize a different pathway for entering the nucleus because of the conformational changes.

Individually, Smad2 and Smad3 can interact with FG-repeat-containing nucleoporins through a hydrophobic corridor in the MH2 domain (14, 22). Although the same MH2 domains mediate the interaction between Smad2/3 and Smad4, we demonstrate that the heterotrimerization between phospho-Smad2/3 and Smad4 apparently does not affect contact with the nucleoporins. Consistent with this finding, like the monomeric Smads, the multimeric Smad complexes can be imported into the nucleus by direct contact with nucleoporins without the participation of importins.

In the context of either monomeric or trimeric complexes, the linker plus MH2 domain of Smad3 is sufficient for nuclear import (Figs. 1 and 2) (22). In terms of nuclear entrance* per se*, the formation of the heterotrimeric or homotrimeric complex does not afford phospho-Smad3 much advantage. Moreover, the behavior of unphosphorylated Smad3 and phospho-Smad3 was identical in all of the nuclear import assays that we have performed, suggesting that the two forms of Smad3 transverse the NPC through similar mechanisms.

**Nuclear Import of Monomeric Versus Complexed Smad4**—Less is known regarding the domain in Smad4 that makes the link to its target genes in the nucleus, these mutations disrupt Smad4 function as a tumor suppressor. Interestingly, when a subset of nucleoporins (including CAN/Nup214) was masked by importin β (45–462), only the heterotrimer but not the monomeric Smad4 was prevented from entering the nucleus. This suggests that the monomeric Smad4 has alternative routes to transverse the NPC without the participation of CAN/Nup214. Perhaps this reflects less restriction imposed on the monomeric Smad4 in the nuclear pore compared with the bulkier heterotrimer.

In contrast, importin β (45–462) blocked nuclear import of both unphosphorylated and phosphorylated Smad3. Thus our data are most consistent with the hypothesis that, in the phospho-Smad3/Smad4 complex, the interaction with nucleoporins is mediated mostly by Smad3 and the nucleoporin-contact regions of Smad4 may be occluded upon association with phospho-Smad3. Even with no contribution from Smad4, phospho-Smad3 may provide enough nucleoporin contact for the whole complex to migrate across the NPC, as suggested by the data in Fig. 1D.

Furthermore, we observed that, as a component of the phospho-Smad3/Smad4 complex, Smad4 was excluded from the nucleolus, similar to that of phospho-Smad3 complexes and different from the monomeric Smad4. This finding further supports the notion that, during nuclear translocation and subsequent movement within the nucleus, phospho-Smad3 plays a directing role in the heterotrimer complex. Our observation is consistent with a recent report that, in living cells, TGF-β substantially reduces the mobility of Smad4 in the nucleus, indicating that the heterotrimer formation imposes restriction on intranuclear movement of Smad4 (16).

**Inactivation of CRM-1-mediated Nuclear Export of Smad4**—The subcellular localization of Smad4 is controlled by the counteracting nuclear import and export forces. Because TGF-β stimulation does not noticeably accelerate nuclear import of Smad4, it most likely acts upon the CRM-1-mediated export to promote Smad4 accumulation in the nucleus.

The NES motif of Smad4, which is recognized and bound by CRM-1, is located in the N terminus of its linker region (aa 138–149) close to the hairpin loop structure (aa 79–89) that is responsible for DNA binding (41). Furthermore, functional analyses suggested the involvement of a broader region (aa 43–135) in Smad4 binding to DNA (42). Thus one hypothesis would be that DNA binding could block CRM-1 association with Smad4. However, our data indicate that DNA does not perturb CRM-1 association with Smad4.

Instead, we demonstrate that the phospho-Smad3/Smad4 heterotrimerization prevents interaction between Smad4 and CRM-1. Because the heterotrimerization is mediated by the MH2 domains of Smad2/3 and Smad4, two mechanisms may account for the block of CRM-1/Smad4 interaction. First, the heterotrimerization may cause physical occlusion of the CRM-1-binding site on Smad4. Although there is no evidence of direct contact among the linker domains or the MH1 domains of the Smad subunits within the heterotrimer, interactions among the C-terminal MH2 domains may bring the linker and MH1 domains of all three subunits into close proximity and thereby interfere with CRM-1 binding to Smad4. Alternatively, the heterotrimerization may induce a long-range conformational change in the linker and MH1 regions of Smad4, which renders Smad4 incapable of recognizing CRM-1. At present, the available crystal structures do not include the NES motif of Smad4 and further experiments are needed to resolve this issue.

CRM-1 is responsible for excluding many transcription factors from the nucleus until the appropriate signals arrive. How signaling events inactivate CRM-1 activity specifically toward certain transcription factors is largely unknown. In the case of...
the transcription factor NF-AT, the NES motif overlaps with the calcineurin-binding site and, upon calcium signaling, the binding of calcineurin by NF-AT directly competes off CRM-1 (43). In the case of TGF-β signaling, our present study suggests that a more elaborate intermolecular interaction is involved to disengage Smad4 from CRM-1. This is also the first example for Smads in which a protein interaction event in the MH2 domain consequently affects the binding of another Smad partner at the distant MH1-linker region.

Returning Smads to the Cytoplasm—Although some phospho-Smad2 and -Smad3 in the nucleus may be targeted for ubiquitin-mediated degradation (44), when the TGF-β signal subsides, the bulk of Smad2, Smad3, and Smad4 returns to the cytoplasm. Such redistribution of Smads correlates with dephosphorylation of Smad2 and Smad3 (15). However, it is unclear whether the dephosphorylation event precedes nuclear export.

Our study suggests that the dissociation of Smad4 from phospho-Smad3 (or Smad2) is a prerequisite for nuclear export of Smad4 by CRM-1. Because the affinity of phospho-Smad3 or -Smad2 for Smad4 is high, our observation would argue that dephosphorylation of Smad3 or Smad2 occurs first in the nucleus before Smad4 can be released from Smad2 or Smad3 and exported by CRM-1.

One challenge to this hypothesis is that, although CRM-1 is unable to export the phospho-Smad3/Smad4 complex, one cannot exclude the possibility that yet unidentified export factors may be able to deport the complex out of the nucleus and so the dephosphorylation event may not occur only in the nucleus. Can Smads exit the nucleus as a complex? In a nucleocyttoplasmic shuttling assay, the bidirectional transnuclear envelope movement of Smad2 was much reduced once the cells received TGF-β treatment (14), suggesting that most of the phosphorylated Smad2 was immobilized in the nucleus. Therefore, the heterotrimeric complex may stay only in the nucleus until its dissociation, triggered by the dephosphorylation of Smad2 and Smad3.

Because the unphosphorylated Smad2/3 and the heterotrimer with Smad4 can interact with nucleoporins with similar affinity from inside the nucleus, it raises the question of what keeps the heterotrimeric complex in the nucleus? If the complex formation does not prevent contact with the NPC, other elements necessary for nuclear export of Smad2/3 must be disabled as a consequence of the heterotrimization. Answers to these questions must await further knowledge on the nuclear export mechanism of Smad2 and Smad3.

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