Distinct Oligomeric States of SMAD Proteins in the Transforming Growth Factor-β Pathway*

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Protein interactions are critical for the function of SMADs as mediators of transforming growth factor-β (TGF-β) signals. TGF-β receptor phosphorylation of SMAD2 or SMAD3 causes their association with SMAD4 and accumulation in the nucleus where the SMAD complex binds cofactors that determine the choice of target genes. We provide evidence that in the basal state, SMADs 2, 3, and 4 form separate, strikingly different complexes. SMAD2 is found mostly as monomer, whereas the closely related SMAD3 exists in multiple oligomeric states. This difference is due to a unique structural element in the MH1 domain of SMAD2 that inhibits protein-protein interactions in the basal state. In contrast to SMAD2 and SMAD3, SMAD4 in the basal state is found mostly as a homo-oligomer, most likely a trimer. Upon cell stimulation with TGF-β, SMAD proteins become engaged in a multitude of complexes ranging in size from SMAD2–SMAD4 heterodimers to assemblies of >650 kDa. The latter display the highest DNA binding affinity for the TGF-β-response elements of JUNB and collagen 7. These observations, all validated with endogenous SMAD proteins, modify previous models regarding the assembly and activity of SMAD complexes in the TGF-β pathway.

Transforming growth factor-β (TGF-β) family of secretory polypeptides regulate various important cellular processes such as differentiation, adhesion, tissue repair, and apoptosis of many different cell types. Included in this family are TGF-β, the bone morphogenetic proteins, the activins and the inhibins, the nodals, and some other related factors. Signaling by TGF-β is initiated upon its binding to two cell-surface receptors termed type I (TβRI) and type II (TβRII). Both receptors are serine/threonine kinases, and binding by TGF-β results in phosphorylation of TβRI by TβRII. The only substrates known to date for phosphorylated TβRI are the SMAD proteins. Upon phosphorylation of conserved serine residues at their extreme C terminus, the TGF-β-activated SMADs, SMAD2 and SMAD3 (or SMAD1 in the case of bone morphogenetic proteins), translocate into the nucleus. En route to the nucleus they associate with SMAD4 which is a shared partner (1). Both groups of SMAD proteins share a common structure with a conserved N-terminal (or MH1) and C-terminal (or MH2) domain and a more variable linker region that connects the two (1).

Upon entry into the nucleus, the receptor-activated SMAD complex can interact with a number of partner proteins that are cell type-specific and jointly with the associated SMADs provide DNA binding activity specific to particular downstream target genes. The activated SMAD complexes then recruit the transcriptional coactivators CBP/p300 (2–5) or the corepressors TGIF, Ski or Sno (6–8). The set of gene responses set in motion by TGF-β varies in different cell types and in response to different stimuli and is mediated by transcriptional coactivators CBP/p300 (2–5). SMADs may exist as oligomers in the basal state challenge the observation that overexpressed SMADs 2, 3, and 4 appeared as monomers (12). These results were obtained utilizing transfection of tagged SMAD constructs and therefore may or may not accurately reflect the state of endogenous SMADs. To address this controversy, we have undertaken an analysis of endogenous SMAD2 and -4 proteins so as to better understand their oligomeric composition in the absence of ligand and any changes that occur upon its addition.

MATERIALS AND METHODS

Cell Culture and Transfection—HaCaT and COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, penicillin/streptomycin, and fungizone. For transient transfections COS-1 cells were seeded in 10-cm dishes and transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. In cases where low levels of proteins were expressed, cells were transfected using DEAE-dextran.

Constructs—FLAG-tagged SMAD2 Δ exon 3 was a kind gift from M. Kato. FLAG SMAD3/2 and SMAD2/3 were constructed by polymerase chain reaction amplification of MH1 domain of SMAD3 and linker + MH2 of SMAD2 or vice versa and cloning into the pc52 expression vector. FLAG SMAD2 Δ 12aa containing a 12-amino acid deletion of...
residues 88–99 of SMAD2 was constructed by polymerase chain reaction amplification using appropriate primers and cloned into pCS2.

Antibodies—Anti-SMAD2 antibodies were raised in rabbits by immunization with the recombinant linker region of human SMAD2 (residues 183–273) (13). They were affinity-purified with immobilized SMAD2 prior to use and cross-react with SMAD3. Anti-SMAD3–specific antibodies (14) were raised against synthetic peptides that correspond to residues 192–211 of human SMAD3 (Zymed Laboratories Inc.). Anti-SMAD4–specific antibodies (15) were raised in rabbits by immunization with a recombinant human SMAD4 peptide spanning residues 40–333 and were affinity-purified prior to use. The anti-FLAG and anti-HA (12CA5) monoclonal antibodies were from Sigma and Roche Molecular Biochemicals, respectively.

Gel Chromatography—COS-1 cells (48 h post-transfection) or untransfected HaCaT cells (untreated or treated with 200 pmol TGF-β) were washed twice with phosphate-buffered saline, scraped, and resuspended in lysis buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 1 mM dithiothreitol, and protease inhibitors. Whole cell extracts were prepared by rocking at 4 °C for 20 min and centrifugation at 10,000 g for 10 min followed by centrifugation at 50,000 g for 10 min to pellet cell debris. Cell supernatant was collected, and an aliquot (0.2 ml) was applied to a Superdex 200 gel filtration column (Amersham Pharmacia Biotech) pre-equilibrated with buffer containing 50 mM Tris (pH 8.0), 200 mM NaCl, and 5 mM dithiothreitol. The column was run at 0.2 ml/min, and 0.25-ml fractions were collected. An aliquot of each fraction was run on an SDS-PAGE gel and electrotransferred to nitrocellulose, probed with the relevant antibody, and visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) according to manufacturer’s instructions.

Immunoprecipitation and Immunoblotting—Fractions eluting from the column were pooled as indicated in the text and preclarred for 30 min with protein A-Sepharose beads and then incubated with the relevant antibody and protein A-Sepharose beads for an additional 3 h at 4 °C. The beads were washed four times with lysis buffer, and the immune complexes were eluted by boiling in SDS sample loading buffer and separated by SDS-PAGE. Proteins were then electrotransferred to nitrocellulose filters, probed with the relevant antibody, and visualized as described above.

Oligonucleotide Pull-down Assay—Fractions were pooled as indicated and preclarred. They were then incubated overnight at 4 °C with biotinylated oligonucleotides containing relevant SMAD-binding element following which the DNA-SMAD complexes were captured on streptavidin beads, and bound protein was visualized after separation on SDS-PAGE and electroblotting as mentioned above. The sequences of the oligonucleotides used for the assay are as follows: JunB, 5'-taatattccttctcagacagtctgtctgcctgtcttaagtgtctcacgtctagcgaattcggatcc-3'; Col 7, 5'-agggccaccaactagatgtctggtctgaagtccaggggccagccctgaggggtg-3'; and mt Col 7, 5'-aggcccacactgcttggctgaatcacaggagtgccggcgggacccatggcctcacgtctagcgaattcggatcc-3'.

RESULTS

Basal State of Endogenous SMAD2 and SMAD4—The availability of antibodies against the SMAD proteins allowed us to examine the oligomeric state of endogenous SMADs. Whole cell extracts from HaCaT cells, a human keratinocyte cell line extensively used in studies of TGF-β action, were subjected to size fractionation on a Superdex 200 column. Fractions were subjected to Western immunoblotting with affinity-purified anti-SMAD2 or anti-SMAD4 antibodies. SMAD2 migrated predominantly in fractions corresponding roughly to its molecular weight (55 kDa (16) (Fig. 1A, top panel; fractions 60–65). A small but reproducible proportion of SMAD2 also eluted in fractions 51–54 corresponding to a molecular mass of ~180 kDa. The low abundance of this species did not allow determination of its composition.

In contrast to SMAD2, SMAD4 eluted from the column in fractions 54–59 (Fig. 1A, bottom panel) that corresponds to a range of molecular weights (~120–150 kDa), higher than expected from a SMAD4 monomer (60 kDa) (17). A very small proportion of SMAD4 eluted as a minor peak in fractions 48–50. The chromatographic behavior of exogenous FLAG-tagged SMAD2 and SMAD4 from extracts of transfected COS-1 cells was similar to that of the endogenous proteins from HaCaT cells (Fig. 1B), indicating that the large size of SMAD4 did not result from an interaction with a saturable endogenous component.

SMAD4 Exists as a Homo-oligomer in Unstimulated Cells—To ascertain whether the major SMAD4 species represented an oligomer or as previously proposed (12), a monomer running anomalously, we tested the effect of a high salt concentration on the stability of this form (Fig. 2). Cell lysates prepared in lysis buffer containing 2 M NaCl were separated on a column that was preswashed and eluted in buffer containing 2 M NaCl. SMAD2 eluted in the same fractions as it did in low salt (compare Figs. 2A and 1A). SMAD3 was readily detected under these conditions also eluting as a monomer (Fig. 2A; the significance of this observation is described below). SMAD4, on the other hand, eluted in distinctly separated fractions (fractions

FIG. 1. Gel filtration profile of endogenous and exogenous SMAD2 and 4. A, HaCaT whole cell extracts were prepared and separated on a Superdex 200 gel filtration column. Fractions were collected and run on an SDS-PAGE gel, transferred to nitrocellulose, and probed with antibodies to either SMAD2 (upper panel) or SMAD4 (lower panel) as indicated. Fraction numbers are indicated at top of the gel. Molecular mass markers were run under the same conditions and are shown on top of arrows corresponding to the fraction that represents the peak of their elution profile. B, COS cells were transfected with either FLAG-tagged SMAD2 or FLAG-tagged SMAD4. 48 h post-transfection whole cell extracts were prepared and separated on a Superdex 200 gel filtration column as above. Fractions were run on an SDS-PAGE gel and probed with the anti-FLAG antibody.
with the peak now being in fractions 58–60. This behavior suggested that SMAD4 forms oligomers in the basal state whose stability is diminished by a high salt concentration. It is noteworthy that concentrations of NaCl up to 1 M did not affect the elution pattern of wild-type SMAD4 (data not shown) suggesting that the interactions giving rise to the basal SMAD4 complex are highly stable.

To determine whether the basal SMAD4 oligomer was generated by the association of multiple SMAD4 monomers, we cotransfected COS-1 cells with two vectors, one of them encoding SMAD4 with an N-terminal FLAG epitope tag and the other encoding SMAD4 with a C-terminal HA epitope tag. Cell lysates were subjected to immunoprecipitation with either the anti-FLAG or the anti-HA antibody followed by immunoblotting (WB) with either one of the two antibodies as indicated. Following detection by ECL, bands were quantified, and the results are presented as a graph. Percentage of immunoprecipitating species was calculated based on bands obtained upon immunoblotting 2.5% of the total lysates.

56–62) with the peak now being in fractions 58–60. This behavior suggested that SMAD4 forms oligomers in the basal state whose stability is diminished by a high salt concentration. It is noteworthy that concentrations of NaCl up to 1 M did not affect the elution pattern of wild-type SMAD4 (data not shown) suggesting that the interactions giving rise to the basal SMAD4 complex are highly stable.

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formed with whole cell extracts under conditions of 2 M NaCl showed no evidence of SMAD4 association (Fig. 2C), indicating that salt was effective in dissociating SMAD4 homo-oligomers into monomers. The difference between SMAD4-containing fractions in the absence and presence of 2M salt is not large, and in fact, SMAD4 monomers do not coelute with SMAD2. It is possible that the SMAD4 monomer generated in the presence of 2M NaCl is partially unfolded and has a higher Stokes radius than its native globular state at low salt concentration.

It was next important to ascertain whether the association that we could observe between two or more SMAD4 molecules was indeed representative of the predominantly oligomeric state of the protein or merely reflected a very minor population of spontaneously associating SMAD4 monomers. To this end COS-1 cells doubly transfected with FLAG-SMAD4 and HA-SMAD4 were lysed, and lysates were subjected to immunoprecipitation via one tag followed by immunoblotting for the other. Quantitation of the bands obtained showed that regardless of whether the anti-FLAG or the anti-HA antibody was used for the immunoprecipitation, the intensity of coimmunoprecipitating species was ~30–40% of the species seen by direct immunoprecipitation (Fig. 2D). Thus, conditions under which 8.1% of transfected FLAG-SMAD4 was immunoprecipitated by the anti-FLAG antibody, 3.5% of FLAG-SMAD4 was coimmunoprecipitated using the anti-HA antibody (Fig. 2D, left panel). Similarly, conditions under which the HA-antibody precipitated 6.7% of HA-tagged SMAD4, the anti-FLAG antibody brought down 2.1% of HA-tagged SMAD4 (Fig. 2D, right panel). Thus, over a third of total SMAD4 with one tag is coimmunoprecipitated with SMAD4 with the other tag. Note that these values underestimate the proportion of SMAD4 molecules that are homo-oligomers as they do not take into account those complexes formed by SMAD4 molecules containing the same epitope tag. Our results therefore suggest that a large proportion of SMAD4 is in a homo-oligomeric complex with itself.

The size of the basal SMAD4 complexes was compatible with either a dimer or a trimer. Attempts to visualize SMAD4 homotrimers in the present studies using cells cotransfected with three SMAD4 vectors containing different epitope tags met with negative results (data not shown). However, since the efficiency of the immunoprecipitation reaction with each of the pairs of SMAD4 combinations was not all uniformly high, our inability to see trimers could be the result of low sensitivity of the assay or interference between epitopes at the same termini of more than one SMAD4 molecule in the same complex. Given this and the previous physical and genetic evidence that SMAD4 can form trimers, the endogenous SMAD4 homo-oligomers could be homotrimers.

*Endogenous SMAD3 Differs from SMAD2 in Its Chromatographic Behavior*-SMAD3 has been previously reported to be a monomer in the basal state when overexpressed in COS-1 cells (12). To ascertain whether this applies to endogenous SMAD3 as well, HaCaT cell extracts fractionated over Superdex 200 were subjected to Western immunoblotting using an antibody that specifically recognizes SMAD3 (but not SMAD2). SMAD3 was detected in many different fractions extending over most of the elution profile (Fig. 3A). A distinct peak, however, was observed in fractions 34–37, corresponding to
molecular masses of ~650–700 kDa. Similar but not identical results were obtained with our polyclonal antibody against SMAD2, which cross-reacts with SMAD3. In this case, SMAD3 can be distinguished from SMAD2 by its faster electrophoretic mobility (Fig. 3B, top panel). In the same experiment, SMAD2 and SMAD4 eluted in their characteristic positions (Fig. 3B, top panel). Detection of SMAD3 in these eluates using the anti-SMAD2 antibody was variable from experiment to experiment. For example, SMAD3 was not detectable in the experiment shown in Fig. 1A at that fluorographic exposure. This may be due to the spreading of SMAD3 over several fractions and the lower titer of our anti-SMAD2 antisera toward SMAD3. Furthermore, as shown in Fig. 2A, endogenous SMAD3 eluted exclusively as monomers in the presence of 2 M NaCl. Thus it appears that endogenous SMAD3, unlike SMAD2, is predominantly found in oligomers in the basal state. Although a small fraction of SMAD3 did elute as a monomer, the vast majority of the SMAD3 protein is engaged in complexes of varying molecular weight and, presumably, varying composition.

Because our results with endogenous SMAD3 differed significantly from what has been reported with overexpressed SMAD3 (12), we decided to investigate further this discrepancy. To this end, we transfected COS-1 cells with a vector encoding FLAG-tagged SMAD3. When a large amount of vector was transfected, the overexpressed FLAG-SMAD3 in the cell lysates eluted from Superdex 200 mostly in the monomer position (Fig. 3C, top panel, fractions 60–65), in agreement with previous observations (12). However, when a lower amount of vector was used in the transfections to achieve a lower level of FLAG-SMAD3 expression, the protein eluted from Superdex 200 in fractions corresponding to the 200–400-kDa range, with no protein seen in the monomer position (Fig. 3C, bottom panel). This complex elution profile was different from that of endogenous SMAD3 (compare with Fig. 3, A and B). In both cases, however, the resulting chromatography profile included multiple SMAD3 complexes of high molecular weight and very little SMAD3 monomer. These results suggested that in the basal state endogenous SMAD3 is engaged in multiple associations with saturable components, and manipulation of the levels of SMAD3 by expression of exogenous protein markedly alters the size distribution and composition of these complexes.

**Role of the MH1 Domain Insert in the Oligomerization of SMAD2**—The principal structural difference between SMAD2 and SMAD3 is the presence of a 30-amino acid insert in the N-terminal domain (or MH1 domain) of SMAD2 (18). This insert is the result of alternative splicing, but the SMAD2 form containing the insert is the predominant form in most tissues (16). This insert is located immediately N-terminal to the conserved β-hairpin element that mediates direct contact with DNA in receptor-regulated SMADs (11). In SMAD2 the presence of this insert prevents direct contact with DNA (11, 20, 21). Thus in attempting to find a reason for the different oligomeric state of SMADs 2 and 3, we decided to focus primarily on this structural difference.

We tested FLAG-tagged versions of SMAD2 with the MH1 domain replaced by that of SMAD3 (SMAD3/2 construct), a complete deletion of the insert (SMAD2 Δ exon 3) (22) or a partial deletion (SMAD2 Δ 12aa). When expressed at low levels in COS-1 cells (Fig. 4, top three panels), the chromatographic properties of all these products were similar to those of FLAG-SMAD3 (compare with Fig. 3B, lower panel). Conversely, a FLAG-tagged version of SMAD3 with the MH1 domain replaced by that of SMAD2 (SMAD2/3 construct) eluted mostly as a monomer under the same conditions (Fig. 4B, last panel; compare with Fig. 1). These results strongly suggest that the monomeric appearance of SMAD2 in the basal state is an inherent property of the insert in its MH1 domain. In addition to preventing interaction of SMAD2 with DNA in the activated state, the presence of this insert limits the ability to interact with other proteins in the basal state.

To verify that the overexpressed SMAD2 and SMAD3 remained capable of interactions with known partners despite their presence as monomers, we examined their interaction with SARA, because this is the best characterized SMAD-binding protein under basal conditions (23). FLAG-tagged SARA from transfected COS-1 cells eluted from the Superdex 200 column at a position corresponding to 350–400 kDa (Fig. 5A), which is twice its predicted molecular weight (180 kDa). When FLAG-SARA and FLAG-SMAD3 were overexpressed together and subjected to gel filtration, they coeluted in fractions 33–36. Some FLAG-SARA trailed in the fractions in which it eluted when overexpressed alone, and some FLAG-SMAD3 eluted as an uncomplexed monomer (Fig. 5B). FLAG-SMAD2 behaved in a similar manner (Fig. 5C). The FLAG-SMADs overexpressed alone in parallel transfections in the same ex-
periment behaved largely as monomers (refer to Figs. 1B and 3B, top panels). Thus, coexpression with SARA causes SMAD2 and SMAD3 to form high molecular weight complexes. It is conceivable that these complexes contain multiple SMADs molecules in association with SARA. More importantly, the results of this experiment suggest that SMAD3 overexpressed alone (Fig. 3C, top panel) behaves as a monomer not because of an intrinsic inability to bind to other components but because SMAD3-interacting proteins are too limiting for the assembly of normal complexes under these conditions.

**Effect of TGF-β on SMAD Oligomerization and Interaction with Target DNA Sequences**—To examine the effect of TGF-β on the oligomeric state of endogenous SMADs, HaCaT cells were treated with TGF-β for 45 min prior to preparation of whole cell lysates and then analyzed by gel filtration (Fig. 3B, bottom panel). Although a portion of SMAD2 and SMAD4 still eluted in the same fractions as they did in the absence of ligand, much of the protein eluted in almost every fraction up to a molecular mass of over 650 kDa. This is to be expected since SMADs have been shown to associate with many nuclear proteins including DNA-binding cofactors and transcriptional coactivators and corepressors, in response to TGF-β (see Ref. 24 and references therein). This is in contrast to the formation of simple SMAD2-SMAD4 complexes previously reported using overexpressed proteins (12), conditions under which SMAD2 and SMAD4 would be in large molar excess over their endogenous natural partners.

To identify fractions containing SMAD2-SMAD4 complexes in the sample from TGF-β-treated cells, we pooled fractions and subjected them to immunoprecipitation with the anti-SMAD2 antibody followed by anti-SMAD4 immunoblotting (Fig. 6A). SMAD2 and SMAD4 were associated with each other in all high molecular weight fractions eluting from the column. Interestingly, SMAD2-SMAD4 complexes were readily detected in fractions beyond fraction 58, which is in the 100–120-kDa range, suggesting the presence of heterodimers.
Previous work has shown that at physiological levels of these proteins, binding of a TGF-β-induced SMAD2-SMAD4 complex to the cognate site in the promoter region of a target gene requires association with a specific DNA-binding cofactor (25–26). We therefore directly tested whether the association of SMAD2 and -4 into high molecular weight complexes did indeed reflect such a phenomenon. Fractions from TGF-β-treated cell extracts were prepared, separated and fractions pooled as above. The three panels show the results of DNA-binding assays performed with the pooled fractions and the indicated oligonucleotide.

**DISCUSSION**

SMAD2, -3, and -4 have previously been shown to be capable of homo-oligomerization (9–10). This property has been shown to be a function predominantly of the MH2 domain, with some contribution from the MH1 domain (9–10). However, these results were obtained utilizing coimmunoprecipitation assays with overexpressed tagged SMAD proteins. More recently, Kawabata et al. (12) also using transfected tagged SMAD proteins reported that SMAD proteins 1–7 are present as monomers in the absence of ligand stimulation. In an attempt to resolve this issue, we have investigated the oligomeric status of endogenous SMAD2, -3, and -4. In experiments where exogenous SMADs were used, care was taken to replicate *in vivo* conditions as closely as possible before extrapolating from the results. Our studies reveal that whereas SMAD2 elutes predominantly in fractions corresponding to its molecular mass of ~55 kDa, SMAD4 elutes in fractions that correspond to molecular weights larger than a monomer. SMAD3 on the other hand is strikingly different from both SMAD2 and -4 and appears to be distributed over a wide range of fractions.

Our experiments with endogenous and exogenous SMAD4 strongly support its existence as a homo-oligomer in the basal state. Our coimmunoprecipitation experiments with differently tagged SMAD4 molecules showed that fractions containing SMAD4 can contain minimally two molecules in a complex. Furthermore, these oligomers represent a fairly large proportion of the SMAD4 population. Given that the elution peak includes fractions in the size range of 110–160 kDa, it is difficult to predict whether they are dimers or trimers based on size alone. However, determination of the crystallographic structure of the MH2 domain of SMAD4 suggests that it is capable of forming homotrimers in solution (11). In addition, there is now genetic evidence to suggest that some of the tumor-derived missense mutations in SMAD4 map to the conserved monomeric interfaces of the trimer (11). Thus, the formation of trimers may be an important aspect of SMAD4-mediated signaling *in vivo*. Indeed, we have found that salt concentrations up to at least 1 M do not dissociate wild-type SMAD4 homo-complexes, indicating that SMAD4 homo-oligomer interactions are of high affinity. Our results therefore contradict the previous conclusion that SMAD4 exists predominantly as a monomer (12). The previous interpretation that SMAD4 is a monomer that elutes anomalously was solely based on the fact that a SMAD4 oligomerization mutant (D537E) migrated similarly upon gel filtration. However, it should be noted that this interfacial mutation causes dissociation of the protein only in the presence of 1 M NaCl (11), and the protein may well migrate similar to wild-type SMAD4 at a low salt concentration.

Endogenous and overexpressed SMAD2 elute largely as monomers, although a small portion is oligomeric. Thus SMAD2 does not appear to form extensive homo-oligomeric complexes in the basal state, at least not many that are stable. If SMAD2 is indeed tethered by cytoplasmic retention factors such as SARA (23), or bound to cytoskeletal components (32), one would expect that it would migrate in fractions of molecular masses larger than 55 kDa. It is possible, however, that most of these interactions are transitory and/or weak such that SMAD2 is in a constant equilibrium between bound and unbound states, and the process of cell extract preparation and gel filtration may be too stringent to keep these complexes intact. SMAD3, in contrast to SMAD2, is readily observed in multiple oligomeric complexes. Endogenous and low expression levels of exogenous SMAD3 have similar but not identical elution profiles. Overexpressed SMAD3 behaves identical to endogenous or exogenous SMAD2. Thus, altering the level of SMAD3 in the cell has strong effects on the complexes recovered after gel filtration. It therefore appears that SMAD3 has a higher affinity than SMAD2 for saturable cellular components.

It is intriguing that two proteins as homologous as SMAD2...
and SMAD3 differ so dramatically in their oligomerization properties. The main difference between the two is the presence, in the MH1 domain of SMAD2, of a short sequence of amino acids that lie immediately proximal to the residues that contribute to DNA binding of the SMADs (11). In the activated state, this insert prevents direct contact of SMAD2 with DNA. Our work now demonstrates that in the basal state this 30-amino acid sequence inhibits interactions with cellular proteins and thus is primarily responsible for the difference in behavior between SMAD2 and SMAD3. This may be achieved by direct occlusion or by inducing a conformational change that limits access of potential SMAD-interacting proteins to their binding sites on SMAD2. Interestingly, this insert has been conserved through vertebrate evolution suggesting that it serves an important function(s). Nevertheless, it is important to keep in mind that the differences between SMAD2 and -3 proteins within the cell may not necessarily be as dramatic as what is observed in cell-free extracts. Both proteins may interact with the same cellular components, except that the affinity of SMAD2 for these partners may be below the threshold that withholds dissociation under the conditions of gel filtration. Regardless, our results raise the possibility that some of the functional differences observed between SMAD2 and SMAD3 could be due to the differential ability of these two proteins to interact with other protein partners in a manner controlled by the MH1 insert.

Addition of TGF-β induces extensive redistribution of SMAD oligomers. It is interesting that complexes corresponding to a molecular weight roughly that of a SMAD2-SMAD4 heterodimer (~100–120 kDa) can be readily obtained (see Fig. 6A). Our results are consistent with the possibility that a SMAD4 homotrimer (or homodimer) in the basal state is converted to a SMAD2-SMAD4 heterodimer in response to TGF-β. It may well be that these are the first complexes that form upon TGF-β addition and serve as the basis upon which functional SMAD transcriptional complexes are built. Not unexpectedly, some of the SMAD4 containing complexes in fractions 54–57 showed binding to COL7. These fractions contain predominantly SMAD2-SMAD4 hetero-oligomers and SMAD4 homo-oligomers and therefore would be expected to bind DNA. The lack of observable binding to the JUNB oligonucleotide may reflect differential binding affinity for different sites. Our observation that large SMAD complexes of over 500 kDa are the ones with the highest affinity for binding to the TGF-β response elements in JUNB and COL7 was a surprise. It has been shown that binding to a TGF-β response element only requires SMADs and a DNA-binding cofactor. All SMAD DNA-binding cofactors identified to date are proteins of less than 120 kDa. Our results therefore suggest that the association of SMAD2-SMAD4 with such factors is concomitant with their association with additional components, likely to include the coactivator p300 or CREB-binding protein (300 kDa). Activated SMAD complexes may therefore bind to cognate DNA sites as fully formed transcriptional coactivator assemblies.

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