The Smad4 Activation Domain (SAD) Is a Proline-rich, p300-dependent Transcriptional Activation Domain*

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Transforming growth factor-β (TGF-β) family members signal through a unique set of intracellular proteins called Smads. Smad4, previously identified as the tumor suppressor DPC4, is functionally distinct among the Smad family, and is required for the assembly and transcriptional activation of diverse, Smad-DNA complexes. We previously identified a 48-amino acid proline-rich regulatory element within the middle linker domain of this molecule, the Smad4 activation domain (SAD), which is essential for mediating these signaling activities. We now characterize the functional activity of the SAD. Mutants lacking the SAD are still able to form complexes with other Smad family members and associated transcription factors, but cannot activate transcription in these complexes. Furthermore, the SAD itself is able to activate transcription in heterologous reporter assays, identifying it as a proline-rich transcriptional activation domain, and indicating that the SAD is both necessary and sufficient to activate Smad-dependent transcriptional responses. We show that transcriptional activation by the SAD is p300-dependent, and demonstrate that this activity is associated with a physical interaction of the SAD with the amino terminus of p300. These data identify a novel function of the middle linker region of Smad4, and define the role of the SAD as an important locus determining the transcriptional activation of the Smad complex.

TGF-β1 is the prototypic member of a large family of structurally related cytokines including the TGF-βs, activins, and bone morphogenetic proteins which regulate cell fate and extracellular matrix deposition through the transcriptional regulation of diverse gene targets. These ligands initiate cellular signals by associating with two classes of interacting transmembrane receptor serine-threonine kinases. Ligand binding to the type II receptor results in recruitment and transphosphorylation of type I receptors, which then signal downstream responses (1). Clues as to the mechanisms regulating downstream signaling responses have been provided by the discovery of Smad proteins as direct substrates of the TGF-β family of receptor kinases, and mediators of signals from the receptors to the nucleus.

Receptor-activated Smads (R-Smads) interact transiently with specific, ligand-activated type I receptors and are phosphorylated on highly conserved carboxyl-terminal (COOH-terminal)-SS(V/M)S motifs. Smad2 and Smad3 are specific mediators of TGF-β and activin signaling pathways, while Smad1, Smad5, and Smad8 are involved in bone morphogenetic protein responses (1). Following receptor activation, these Smad proteins translocate to the nucleus where they function as transcriptional regulators (2). Smads have a domain structure consisting of highly conserved amino (NH2)- and (COOH)-terminal regions, referred to as Mad homology 1 (MH1) and MH2 domains, respectively, and an intervening middle linker, which is of variable length and sequence. The MH2 domain contains the principal receptor serine-threonine kinase phosphoacceptor sites (1, 3), and determinants of specific Smad-receptor and Smad-Smad interactions (4), and is essential for transcriptional activation (2). Effector functions of the MH2 domain are inhibited by the MH1 domain (5), while the MH1 and linker regions of Drosophila MAD and mammalian Smad3 are responsible for their DNA binding (2).

Smad4 is functionally unique among the Smads, with an amino acid sequence more closely related to the Drosophila gene product Medea than to Mad (6). In contrast to the R-Smads, Smad4 is not regulated by phosphorylation, but acts as a common mediator of TGF-β, activin, and bone morphogenetic protein signaling responses (7–9). Following phosphorylation, R-Smads form hetero-oligomeric complexes with Smad4 which are then translocated to the nucleus (1, 3). Like the R-Smads, the MH2 domain of Smad4 is responsible for interaction with other Smad proteins, while autoinhibition of MH2 activity and DNA binding are mediated through its MH1 domain (5).

Smad4 is an essential component of transcriptional complexes mediating the activation of Smad-dependent target genes. The transcriptional activity of Smad4 has been ascribed to its capacity to associate with other Smad-transcription factor complexes on cis-acting elements of responsive promoters (10–13), and by participating in R-Smad interactions with the paralogous bridging co-activators CBP and p300 (14–18). Recent data also suggest that Smad4 plays an active role in recruiting other components of the transcriptional complex involved in target gene activation, including the transcriptional co-activator MSG1 (19). Although several studies have suggested that the C-terminal MH2 domain is essential for

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§ The abbreviations used are: TGF-β, transforming growth factor-β; R-Smad, receptor-activated Smad; MH1, Mad homology domain 1; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; SAD, Smad activation domain; ARF, activin response factor; ARE, activin response element; CBP, cAMP-binding protein.
mediating Smad4 transcriptional activation (11, 20). Recent studies show that a Smad4 mutant lacking the whole MH2 domain retains the capacity to activate transcription (21). Furthermore, we previously showed that the Smad4 MH2 domain is interchangeable with the Smad1 MH2 domain, and that a 48-amino acid segment within the middle linker called the Smad4 activation domain, or SAD, is required for the activation of Smad4-dependent signaling responses (7). These data provide evidence that elements within the middle linker region of Smad4 are required for the activation of Smad4-dependent transcriptional responses, but it is unknown how these regulate the function of Smad4.

In this study, we show that the SAD is in fact a transcriptional activation domain which is both necessary and sufficient for the activation of transcription by Smad4. Mutants lacking the SAD are still able to form complexes with other Smad family members and associated transcription factors, but cannot activate transcription by these complexes. We show that the SAD has intrinsic, p300-dependent, transcriptional activity, and determine that this activity is associated with a physical interaction between the SAD and the NH2 terminus of p300. These findings identify a novel function for the middle linker region of Smad4, and define the role of the SAD as an important locus determining the transcriptional activation of the Smad complex.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Expression Constructs**—MDA-MB468, NMuMg, and COS-1 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics. Cells were transfected with the indicated constructs using Superfect (Qiagen) or LipofectAMINE (Life Technologies), according to the manufacturer's protocols. 3′FLAG-tagged Smad4 mutant and deletion constructs, and 5′ double Myc-tagged Smad2 were generated by polymerase chain reaction using a proofreading polymerase and subcloned into pCDNA3 (Invitrogen) or the pSG424 (22) expression vectors. All polymerase chain reaction-generated products were sequenced using the dideoxynucleotide method.

**Transcriptional Response Assays**—The 3TP-Lux reporter was used to measure TGF-β-induced gene expression, while pG5E1B-Luc, containing an upstream activating sequence linked to a luciferase reporter, was co-transfected with the indicated Gal4-Smad fusion protein constructs in heterologous DNA binding assays. For the ARE-reporter assays, ARE-Luc containing three tandem repeats of the ARE linked to a luciferase vector were transiently transfected with the indicated constructs, with or without the activated TβRI (T204D), and serum starved overnight 24 h later. Cells were then fixed and permeabilized, as described previously (24), and FLAG epitopes detected by incubating with the anti-FLAG M2 monoclonal antibody overnight at 4 °C. This was followed by incubation with a goat anti-mouse fluorescein isothiocyanate secondary antibody, and mounting in medium containing 4,6-diamino-2-phenylindole (Vectorshield, Vector Labs). The percentage of nuclei containing p300. These findings identify a novel function for the middle linker region of Smad4, and define the role of the SAD as an important locus determining the transcriptional activation of the Smad complex.

**RESULTS**

**Smad4 SAD Deletion Results in Loss of Function**—Smad4 restores TGF-β responsive p3TP-Lux reporter gene activation when co-transfected into Smad4 null MDA-MB468 cells (7). Using the same functional assay, we have previously shown that deletion of the COOH-terminal portion of the middle linker region of Smad4 (amino acids 275–322), the Smad4 activation domain (SAD), results in loss of function, and that the NH2 terminus of Smad4 enhances ligand-dependent reporter gene activation in Smad1/Smad4 chimeras (7). The mechanism whereby Smad4(275–322) interferes with Smad-mediated signaling is unknown. To explore this further, we used a heterologous transcriptional activation assay to determine the ability of this Smad4 mutant to restore transcriptional activity of a Gal4-Smad2 fusion protein, which has previously been shown to be dependent on Smad4 expression (11). Co-transfection of Smad4 with Gal4-Smad2 restored ligand-dependent transcriptional activation of the Gal4 reporter gene in Smad4-null MDA-MB 468 cells, while Smad4(275–322) only weakly restored transcriptional activity of the fusion protein, despite comparable levels of Gal4-Smad2 fusion protein expression (Fig. 1A).

To determine if the differences in ability to activate transcription were dependent on Smad4 directly, we performed the same experiments using Gal4-Smad4 fusion constructs. Transfection of Gal4 fusion proteins containing full-length Smad4, or a truncated Smad4 encompassing amino acids 266–552, showed a ligand dependent activation of the Gal4 reporter, with a larger absolute activation by the 266–552 construct, consistent with relief of autoinhibition by the MH1 domain (5,
Transcriptional Activity of Smad4

Smad4 SAD Deletion Does Not Affect the Known Cytoplasmic Functions of Smad4—Interactions between Smad4 and R-Smad proteins are particularly sensitive to deletion and mutation within the COOH-terminal domain of Smad4 (25–27). As the Smad4(Δ275–322) deletion is in close apposition to the MH2 domain (amino acids 323–552), we felt that a possible explanation for its lack of function was through disruption of the MH2 tertiary structure. We therefore tested the known cytoplasmic functions of the MH2 domain of Smad4, including hetero-oligomerization with R-Smads and nuclear translocation of Smad-containing complexes. Smad4(Δ275–322) formed heteromeric complexes with Smad2 (Lane 4, Fig. 2A), and, as described for the wild type molecule (11, 28), underwent ligand-dependent nuclear translocation in the presence of Smad2. We sought to determine if loss of the SAD would alter the DNA binding ability of the Smad-FAST1 complex. We performed gel shift experiments using lysates from Smad4-null MDA-MB468 cells transfected with various components of the ARF and a γ32P-labeled ARE probe. No TGF-β-inducible ARE binding complexes were detected in cells transfected with vector alone, FAST1, or Smad2 and FAST1. However, co-transfection with either wild type Smad4 or the Smad4(Δ275–322) SAD deletion mutant yielded TGF-β-inducible gel-shifted complexes (Lanes 9 and 11, Fig. 3A). To confirm that wild type Smad4, Smad4(Δ275–322), Smad2 and FAST1 all participated in these ARE-binding complexes, we used an

Is Distinct from Its Stabilizing Effects on Protein-DNA Complexes—To determine how deletion of the SAD disrupts the nuclear functions of Smad4, we reconstituted a defined transcriptional response from Xenopus in mammalian cells. Activin and TGF-β signaling induce the formation of an activin response factor (ARF) that contains Smad2, Smad4, and FAST1, and binds to the activin response element (ARE) on the Xenopus Mix.2 promoter (10, 11, 29). Smad4 is a critical component of this complex, enabling transcriptional activation of the ARE-containing reporter construct (10, 11). Previous studies have shown that wild type Smad4 forms ternary complexes with FAST1 in the presence of Smad2 (10, 11). We reproduced these findings with wild type Smad4, and also showed that the Smad4(Δ275–322) mutant co-immunoprecipitates with FAST1 in the presence of Smad2. We sought to determine if loss of the SAD would alter the DNA binding ability of the Smad-FAST1 complex. We performed gel shift experiments using lysates from Smad4-null MDA-MB468 cells transfected with various components of the ARF and a γ32P-labeled ARE probe. No TGF-β-inducible ARE binding complexes were detected in cells transfected with vector alone, FAST1, or Smad2 and FAST1. However, co-transfection with either wild type Smad4 or the Smad4(Δ275–322) SAD deletion mutant yielded TGF-β-inducible gel-shifted complexes (Lanes 9 and 11, Fig. 3A). To confirm that wild type Smad4, Smad4(Δ275–322), Smad2 and FAST1 all participated in these ARE-binding complexes, we used an

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bodies directed against the epitopes expressed on these constructs to supershift the complexes. TGF-β-responsive NMuMg cells were used as these gave a reproducibly high level of protein expression following transient transfection (Fig. 3E). In these cells, ligand-dependent gel-shifted complexes are seen in the absence of exogenous Smad4 (Fig. 3B), presumably as a result of binding to complexes containing endogenous Smad4. Incubation with Myc antibody reduced the mobility of the ARE-binding complex (Lanes 4, 8, and 12), confirming the presence of Myc-Smad2 and/or Myc-FAST1 in all of the complexes, while the FLAG antibody shifted a component of the complex either with transfected wild type Smad4, or the Smad4(Δ275–322) deletion mutant (Lanes 9 and 13). The FLAG antibody did not lead to any supershift in lysates from cells transfected with FAST1 and Smad2 alone (Lane 5), confirming the specificity of these findings. These data show that the Smad4(Δ275–322) deletion mutant participates in ligand-dependent DNA binding complexes.

The ability of wild type Smad4 to participate in these transcriptional complexes correlates with its ability to enhance ligand-dependent transcriptional activation of the ARE-luciferase reporter both in Smad4 null cells (Fig. 3C), and in TGF-β-responsive NMuMg cells (Fig. 3D). The Smad4(Δ275–322) mutant only weakly enhanced ARE-luciferase activity in Smad4 null cells when compared with the Smad4-independent activation response (Fig. 3C). Furthermore, in NMuMg cells, both basal and ligand-dependent activation of the ARE luciferase reporter were reduced in the presence of Smad4(Δ275–322) (Fig. 3D), indicating that Smad4(Δ275–322) has dominant negative effects on TGF-β signaling. In these experiments, various defined components of this transcriptional complex were expressed at comparable levels (Fig. 3E). Taken together, these data indicate that maximal activation of Smad-dependent transcriptional responses by Smad4 requires the SAD, and that this activity is distinct from its ability to participate in these transcriptional complexes.

The SAD Is a Proline-rich Transcriptional Activation Domain That Binds p300—Although we have shown that Smad4 mutants lacking the SAD only weakly activate transcription of Smad-dependent transcriptional complexes, we were unable to identify any defects in the other known biochemical functions of Smad4. Analysis of the SAD amino acid sequence (Fig. 4A) shows that it is rich in proline residues, much like the transcriptional activation domains of other well characterized tran-
scriptional activators including AP-2 and CTF/NF-1 (30, 31). To test our hypothesis that the SAD acts as an intrinsic transcriptional activation domain, we performed heterologous activation assays using a Gal4-SAD fusion protein. These experiments demonstrate that the SAD is a strong, ligand-independent transcriptional activator, with levels of activation comparable to or higher than the most active Gal4-Smad4 fusion construct (Fig. 4B).

Recent studies suggest that the transcriptional activity of Smad proteins is dependent on their interaction with the paralogous bridging co-activators CBP and p300, linking the Smad-DNA binding complex to the basal transcriptional machinery (14–18, 32). In order to determine if SAD transcriptional activity was dependent on interaction with these co-activators, we co-transfected adenoviral E1A, an inhibitor of CBP/p300 activity (33), along with Gal4-SAD, in the heterologous activation assay. E1A overexpression markedly reduced the level of Gal4-SAD transcriptional activity, while a mutant form of E1A lacking the CBP/p300-binding site (Δ2–36) (34) had no effect on this response (Fig. 4C). The levels of Gal4-SAD protein expression were unaffected by E1A transfection. To confirm that this effect was dependent on p300 activity, we co-transfected increasing amounts of p300 along with E1A in the Gal4-SAD reporter assay. p300 partially relieved E1A inhibition of Gal4-SAD (Fig. 4D). This indicates that transcriptional activation by the SAD is functionally dependent on p300.

These studies suggest that there is a functional co-operativity between CBP/p300 and the SAD, but do not define the nature of this interaction. To determine if the p300-dependent activation of Gal4-SAD is mediated by a direct interaction with p300, we performed co-immunoprecipitation assays. Initially we looked for interactions between endogenous p300 and epitope-tagged Smad4 constructs. We used Smad4 266–552 as the backbone for these studies as this was expressed at high levels following transfection, has previously been shown to interact with p300 and Smad2 (5, 18), and is transcriptionally active (Fig. 1B). In these studies, the SAD deletion mutant was still able to bind to endogenous p300 in a ligand-dependent manner (Fig. 5A, Lanes 7 and 9). Furthermore, binding of both the wild type and the SAD deletion mutant was enhanced by overexpression of Smad2 (Lanes 5 and 9), indicating that the principal interaction of Smad4 with p300 may be indirect, mediated by stronger interactions between the R-Smad, Smad2, and p300, and the R-Smad with Smad4.

As our functional data provided evidence supporting the role of the SAD as a CBP/p300-dependent transcriptional activation domain, we went on to determine whether the SAD itself could independently interact with p300. For this, GST-p300 frag-
ments purified by affinity chromatography (Fig. 5B), were incubated with lysates from COS cells transiently transfected with the indicated constructs and the lysates analyzed by immunoprecipitation and Western blotting, as indicated. TβR-1* indicates transfection with the activating point mutant of the TGF-β type 1 receptor (T204D). B and C, the SAD interacts with the NH₂-terminal region of p300. Equal amounts of bacterially produced GST fusion fragments of p300 containing the CH1 (GST-N), CH2 (GST-M), or CH3 (GST-C) regions (B), were incubated with cell lysates from COS cells transiently transfected with Gal4-SAD, and precipitated by an anti-GST antibody (C). Co-precipitated Gal4-SAD was detected following SDS-PAGE by Western blotting using an anti-GAL4 antibody (top panel). Coomassie Blue staining of p300 fusion proteins demonstrates equal expression (bottom panel).

In order to define the functional significance of this Smad4 SAD-p300 interaction, we sought to determine whether overexpression of the SAD-binding region of p300 could interfere with transcriptional activation by the SAD. Initially, we used the Gal4-Smad4 266–552 construct in these studies as this has particularly strong ligand-dependent transcriptional activation when compared with the wild type Smad4 fusion protein (Fig. 1B). Overexpression of an NH₂-terminal p300 construct (1–1736), which contains the SAD-binding site but lacks the glutamine-rich transactivation domain of p300, strongly repressed both basal and ligand-dependent activation of Gal4 Smad4 266–552. In contrast, the COOH-terminal p300 construct (1737–2414), which lacks the SAD-binding site but has the capacity to bind to R-Smads, only partially inhibited this transcriptional response (Fig. 6A). This is in keeping with the observation that p300 (1737–2414) only partially blocks TGF-β-dependent activation of the p3TP-Lux reporter, and probably reflects inhibition of R-Smad binding to endogenous p300 (32).

As Smad4 266–552 contains the SAD(275–322), we sought to determine whether p300 (1–1736) might be interfering with the transcriptional activation of Gal4-SAD by inhibiting p300-dependent transcriptional activation of the SAD. Transfection of increasing amounts of p300 (1–1736) inhibits the transcriptional activity of Gal4-SAD, while the COOH-

Fig. 5. Physical interactions between Smad4 and p300. A, Smad2-dependent binding of Smad 4 to endogenous p300. COS-1 cells were transfected with the indicated constructs and the lysates analyzed by immunoprecipitation and Western blotting, as indicated. TβR-1* indicates transfection with the activating point mutant of the TGF-β type 1 receptor (T204D). B and C, the SAD interacts with the NH₂-terminal region of p300. Equal amounts of bacterially produced GST fusion fragments of p300 containing the CH1 (GST-N), CH2 (GST-M), or CH3 (GST-C) regions (B), were incubated with cell lysates from COS cells transiently transfected with Gal4-SAD, and precipitated by an anti-GST antibody (C). Co-precipitated Gal4-SAD was detected following SDS-PAGE by Western blotting using an anti-GAL4 antibody (top panel). Coomassie Blue staining of p300 fusion proteins demonstrates equal expression (bottom panel).
The NH₂-terminal MH1 domain of Smad4 is required to stabilize Smad4 capable of activating transcription of a variety of target genes both in homologous and heterologous reporter gene assays. Smad deletion mutants do not affect R-Smad-Smad4 hetero-oligomerization, nor the ability of Smad4 to participate in ligand-dependent DNA binding of Smad-containing transcriptional complexes. This contrasts with mutations and deletions within the COOH-terminal MH2 domain of Smad4, which interfere with transcriptional responses by disrupting hetero-oligomerization of Smad4 with R-Smads (26, 35, 36). Thus, while Smad4 requires its COOH terminus to interact with R-Smads and form ligand-dependent transcriptional complexes, this activity is distinct from the transactivating activity of Smad4 which requires the SAD.

The only other inactivating deletion in the mid linker region which has been studied (2323–301) is a splice variant of Smad4 found in MDA-MB231, a breast cancer cell line (37). Unlike the SAD deletion, which can inhibit TGF-β-dependent responsive reporter gene activity, this mutant lacks dominant negative activity, suggesting that the deletion of additional sequences upstream of the SAD may interfere with other functions of Smad4, for example, its association with other R-Smads in the cytoplasm. Partial mapping of Smad4 transactivator domains using heterologous DNA binding assays confirms that Gal4-Smad4 (266–552) strongly activates transcription of the luciferase reporter in a ligand-dependent manner, while deletion of the SAD in this construct completely abolishes this activity. Our experiments in Smad4 null cells also show that the transactivating activity of Gal4-Smad2 is absolutely dependent on the presence of exogenous Smad4, and that this response is markedly reduced following deletion of the SAD. These data confirm that Smad4 is involved in mediating Smad-dependent transcriptional responses, and support our conclusion that this activity is dependent on the SAD.

We now demonstrate that the SAD is an intrinsic transcriptional activation domain, rich in proline residues, and that it is not only necessary, but also sufficient to activate maximal Smad-dependent transcriptional responses. Similar proline-rich domains have been described in a number of other transcriptional activators such as AP-2 and CTF/NF1 (30), suggesting a common mechanism of action. A feature of these activation domains is that they interact with diverse components within the general transcriptional machinery, recruiting multicomponent complexes of proteins into juxtaposition with the transcription factor-DNA complex. In this context, the crystal structure of a transcriptionally active Smad4 fragment (273–552) has recently been solved (38), providing key insights into the structural basis for the transcriptional responses mediated by the SAD. This structure contrasts with the previously published structure of an inactive Smad4 fragment (319–543) (25) as the additional residues stabilize a previously disordered structure within the MH2 domain of Smad4, forming a glutamine-rich extension from the trimeric core. Interestingly, this glutamine-rich extension is reinforced by the SAD, which is stabilized by flanking sequences that interact with the structural core of Smad4. The proline-rich, hydrophobic surface of the SAD is located on the same surface as the solvent accessible glutamine-rich extension of the MH2 domain at the periphery of the trimeric disc, suggesting that this energetically unfavor-
able hydrophobic surface could be stabilized by interaction with a transcriptional co-factor. This model provides a structural basis for the unique functional role we have ascribed to the SAD.

Residual ligand-dependent reporter gene activation of the SAD deletion constructs suggests that other domains of Smad4 may also contribute to the maximal activation of Smad transcriptional complexes. Artificial nuclear localization of Smad4 using an estrogen receptor fusion protein indicates that loss of the COOH-terminal 37 amino acids prevents Smad4-dependent transcriptional activation (27). This result resembles our findings with the SAD deletion mutant, and suggests that there may be an additional transcriptional activation domain in the COOH terminus of Smad4. This would explain our observation that there is residual, ligand-dependent activation by Smad4 SAD deletion construct, as stimulation by the COOH terminus may still be competent despite a marked reduction in the overall transcriptional activity caused by the SAD deletion mutant. This is also consistent with the structural model of the transcriptionally active Smad4 fragment, which proposes that functional activity of the SAD occurs in the context of its physical relationship with an additional transcriptional activation domain in the COOH terminus of Smad4 (38).

In order to define the mechanism of transcriptional activation by the SAD, we sought to determine whether there were physical and/or functional interactions between the SAD and the paralogous transcriptional adaptor proteins CBP and p300, which are essential for the recruitment of transcriptional complexes to the basal general transcriptional machinery (39). A wide range of transcriptional activators interact with CBP and p300, and recent studies have shown that the transcriptional activity of Smad4 proteins also depends on the binding of R-Smads to CBP and p300 (14–18, 32). The identification of the COOH terminus of CBP/p300 as the R-Smad interaction site (14–18, 32) contrasts with our demonstration of a physical interaction between the SAD and the NH2-terminal region of p300. However, we have also shown that both wild type and the SAD deletion mutant of Smad4 co-immunoprecipitate with endogenous p300 in a ligand-dependent fashion, and that this interaction is enhanced by addition of exogenous Smad2. This fits with previously published data indicating that the principal interaction between Smad4 and p300 requires the participation of an R-Smad in the transcriptional complex (14, 16), and indicates that while the principal Smad4-CBP/p300 interaction occurs through an indirect physical association mediated by the R-Smad, direct interaction of the SAD with CBP/p300 occurs as a secondary event in the assembly of an active transcriptional complex.

The functional significance of this interaction between the SAD and p300 is underscored by our demonstration that transcriptional activity of the SAD is inhibited by p300-dependent fashion. Furthermore, the transcriptional activity both of Smad4, and of the SAD alone, are strongly repressed by overexpression of a p300 construct which lacks the R-Smad-binding site but has the capacity to bind to the SAD. Taken together, our findings define a novel function of the middle linker region of Smad4 in the regulation of transcriptional responses, and suggest a model in which interaction between the Smad4 SAD and CBP/p300 is essential for efficient transcriptional activation by the Smad complex. In this model, R-Smads initially recruit CBP/p300 into the transcriptional complex, bringing the Smad complex into apposition with the basal transcriptional machinery. At the same time, the hetero-