SMADs are transforming growth factor β (TGF-β) receptor substrates and mediators of TGF-β transcriptional responses. Here we provide evidence that the co-activators p300 and CBP interact with Smads 1 through 4. The biological relevance of this interaction is shown in vivo by overexpression of the adenovirus E1A protein and mutant forms of E1A that lack p300 or CBP-binding sites. Wild-type E1A, but not the mutants, inhibits SMAD-dependent transcriptional responses to TGF-β. E1A also inhibits the intrinsic transactivating function of the Smad4 MH2 domain. In addition, overexpression of p300 enhances SMAD-dependent transactivation. Our results suggest a role for p300/CBP in SMAD-mediated transcriptional activation and provide an explanation for the observed ability of E1A to interfere with TGF-β action.

Many biological processes are controlled at the level of transcriptional regulation. Minimally, two functions are required for transcriptional control: the ability to bind a specific DNA sequence and the ability to transactivate by recruiting and modulating the basal transcriptional machinery. SMAD proteins have been identified as the components mediating both these functions in transcriptional regulation by the transforming growth factor-β (TGF-β) family of cytokines (1, 2). SMADs located in the cytoplasm are directly phosphorylated by membrane serine/threonine kinase receptors that bind TGF-β or the related factors activin and BMPs. The phosphorylated SMADs then move into the nucleus as complexes that bind specific DNA sequences in target promoters, activating transcription. Although progress has been made in elucidating the DNA binding mechanism of SMAD complexes (3–8), the molecular basis for transactivation by SMAD remains unknown.

Three subgroups of SMAD proteins are known. The first, or receptor-regulated SMADs, include members that are phosphorylated by TGF-β and activin receptors (Smad2 and Smad3) (9–13) or BMP receptors (Smad1, Smad5, Smad8, and in Drosophila, Mad) (10, 14–18). Upon phosphorylation by the receptors at C-terminal serines, these proteins associate with members of the second group, or co-SMADs, which include Smad4 in vertebrates and Medea in Drosophila (12, 19–22). Smad4 acts as a shared partner of different receptor-regulated SMADs and is essential for various TGF-β, activin, and BMP responses (12, 19). The third group includes the antagonistic SMADs, Smad6, Smad7, and Drosophila Dad, which act as decoys by binding to activated receptors (23–26) or to receptor-activated SMADs (27) and yielding inactive complexes.

SMAD proteins contain highly conserved N- and C-terminal domains, known as the MH1 and MH2 domains, respectively (1, 2). The linker region between these domains contains mitogen-activated protein kinase phosphorylation sites that inhibit nuclear translocation (28). In the basal state, the MH1 and MH2 domains can interact, inhibiting each other’s functions (29). The MH1 domains of some SMADs have DNA binding activity (5, 7, 8), whereas the MH2 domains have transactivating activity (16). In addition, the MH2 domain is involved in homotrimeric interactions that are disrupted by inactivating mutations in tumor-derived forms of Smad2 and Smad4 (30). The MH2 domain also mediates specific interactions with activated TGF-β receptors (31, 32), with partner-SMADs (29) and, in the nucleus, with DNA-binding proteins such as FAST1 (4, 6). Discreet structural elements in the MH2 domain specify each one of these interactions (31, 32).

Although the MH1 domains of SMADs have DNA binding ability, studies on the SMAD regulated gene Mix.2 indicate that the SMAD complex requires FAST1 as a partner for efficient binding to a specific promoter sequence known as the “activin response element” (ARE) (3, 4, 6). FAST1, a member of the “winged helix” family of DNA-binding proteins (also known as the forhead or HNF-3 family), associates with incoming Smad2-Smad4 or Smad3-Smad4 complexes in the nucleus (4, 6). FAST1 may provide specific binding interactions, whereas the SMADs provide additional DNA contacts and the transactivating function (6).

Coactivators are a class of proteins essential for the transactivating function of a variety of transcription factors. Some of the best characterized coactivators are p300 and CBP (33–35). CBP and p300 are structurally and functionally conserved proteins. They possess intrinsic acetyltransferase activity capable of modifying chromatin organization (36, 37) and may also serve to bridge transcription factors and components of the basal transcriptional apparatus (33–35). They have been shown to enhance transcriptional activation by a variety of transcription factors such as p53, CREB, AP-1, STATs, MyoD, NF-κB, and steroid/nuclear receptors (33–35). To investigate how SMADs activate transcription, we decided to test whether SMADs interact with p300/CBP and require these proteins for transcriptional activation.
SMAD Interaction with p300/CBP

FIG. 1. Physical interaction of SMADs with p300 and CBP. A and B, p300 and CBP interact directly with the linker/MH2 domains of Smad1 and Smad2. Extracts from HaCaT cells treated with 100 pM TGF-β were incubated with the indicated recombinant GST fusion proteins. The unbound fraction (a) (10% of input) as well as the bound fractions (b) eluted from the beads were analyzed by Western immunoblotting using anti-p300 (A) or anti-CBP (B). Nonimmune rabbit serum (NRS) and anti-Myc antibody were used as negative controls. C and D, coimmunoprecipitation of p300 with SMADs. Whole cell extracts from HaCaT cells (C) or 293T cells (D) treated with TGF-β or BMP in the case where Smad1 was transfected) were immunoprecipitated with anti-Flag antibodies, and the presence of p300 in the precipitates was detected by immunoblotting using anti-p300 antibody.

Flag antibody were performed followed by Western immunoblotting with anti-p300 antibody. In these experiments, interactions were observed between p300 and SMADs 1, 2, and 3 (Fig. 1C). p300 was detectable in the Flag immunoprecipitates when cells were cotransfected with the SMADs but not in control cells transfected with p300 alone (Fig. 1C). We also saw an interaction of p300 with Smad4, albeit to a lesser extent than with Smad3 (Fig. 1D). The Smad3-p300 interaction was enhanced by TGF-β (data not shown). This is in contrast to the lack of interaction between p300 and the GST-Smad4 fusion protein.

E1A Specifically Inhibits SMAD-mediated Transactivation—The adenoviral 12 S oncoprotein E1A enhances progression through the cell cycle by binding the protein products of at least two different gene families: retinoblastoma (pRb) and p300/CBP (43, 44). These two families of proteins bind to distinct domains on E1A (38, 39, 45). Interestingly, E1A has also been shown to abrogate many TGF-β-mediated gene responses and TGF-β-induced growth inhibition (46–52). Whereas the ability of E1A to counteract growth inhibitory functions of TGF-β in certain cell lines is due in part to its ability to bind pRb (46, 52), its inhibitory effect on other TGF-β responses requires regions of the E1A protein that bind p300 (48, 49, 52). We therefore investigated the effect of E1A on transcriptional responses to TGF-β that are known to be mediated by the SMADs. For this we used the A3CAT construct which contains SMAD-responsive ARE sites from Xenopus Mix.2 driving expression of a CAT reporter (41). Both in Mv1Lu mink lung epithelial cells (Fig. 2A) and in HaCaT human keratinocytes (Fig. 2B), transfection of increasing amounts of wild-type E1A markedly inhibited transcriptional activation of the ARE. However, similar levels of two different deletion mutants of E1A lacking either residues 64–68 (Fig. 2A) or residues 2–29 (Fig. 2B) had little or no effect
on activation by TGF-β. These two deletions selectively eliminate the two p300-interacting regions of E1A without preventing interactions with Rb and related proteins (38, 39, 45). These results suggest a requirement of p300 function for transactivation by a SMAD-containing complex.

To confirm a requirement for p300 in SMAD-directed transactivation more directly, we tested the effect of wild-type and mutant E1A proteins on activation of a GAL4 reporter by a Gal4-Smad2 fusion protein. Gal4-Smad2 activates this reporter when cells are incubated with TGF-β (6). Cotransfection of wild-type E1A, but not an E1A mutant defective in p300 binding, inhibited transactivation by Gal4-Smad2 in HaCaT cells (Fig. 3A). Similar results were obtained in SW480.7 colon carcinoma cells that are defective in Smad4 and thus depend on exogenous Smad4 for Gal4-Smad2 activity (6, 12) (Fig. 3B). We also tested the effect of E1A on transactivation by Gal4-Smad4(MH2). This fusion contains the MH2 domain and part of the linker region of Smad4 and is transcriptionally active independent of TGF-β stimulation (6). Wild-type E1A inhibited transactivation by Gal4-Smad4(MH2), whereas the mutant E1A did not (Fig. 3A). Thus, under various well characterized conditions, the transactivating function of SMADs was inhibited by wild-type E1A, and this inhibition specifically required the p300 binding function of E1A.

Effect of p300 Overexpression on TGF-β-dependent Transactivation—Finally, using transient transfection assays, we directly evaluated the effect of p300 on the transcriptionally competent complex formed in response to TGF-β/activin. Mink lung epithelial cells were transfected with FAST1, the ARE reporter construct A3Lux, and p300 vectors. p300 overexpression further increased TGF-β-dependent transactivation of this reporter (Fig. 4). The stimulatory effect of p300 was observed only when FAST1 was present, suggesting that an excess of p300 can enhance the transcriptional activity of the ARF complex. However, the effect of p300 was small under all conditions tested, suggesting that the endogenous levels of p300 and functionally related proteins are not rate-limiting for TGF-β-induced transactivation.

DISCUSSION

SMADs are central components of transcriptional complexes that bind to specific sites in TGF-β target promoters and activate transcription. Whereas the DNA binding ability of SMAD complexes has begun to become clear (3–8), the mechanisms that control transactivation by this complex have remained unknown. Here we provide evidence that SMADs can interact with p300 and CBP, and these interactions are functionally important in transactivation. Using GST-SMAD fusion proteins, we show that both the BMP mediator, Smad1, and the TGF-β/activin mediator, Smad2, can interact with p300 and CBP from cell extracts. The SMAD MH2 domain and linker regions are sufficient for this interaction. Focusing on p300, we provide further evidence for an interaction between this protein and Smads 1, 2, and 3 by coprecipitation from cell extracts.
In these coimmunoprecipitation experiments, we also observed an interaction between p300 and Smad4. However, a GST-Smad4 fusion protein does not bind p300 or CBP from cell extracts under our assay conditions. Smad4 may have a weaker affinity for p300 than Smad1 or Smad2. Alternatively, the interaction between Smad4 and p300 may be mediated by association with endogenous Smads 1 or 2.

Evidence for a requirement of p300 in TGF-β transcriptional responses and SMAD-dependent transactivation is provided by results using adenovirus E1A protein that binds and inactivates p300/CBP. As a model system to investigate SMAD-mediated transcriptional responses, we used an ARE-dependent reporter gene. The ARE is a physiological response element, and its activation by a SMAD complex in response to TGF-β signaling is the best characterized to date (3, 4, 6).

Using this reporter construct, we show that wild-type E1A inhibits TGF-β-induced transactivation from the ARE in different cell lines. Most importantly, this effect appears to be dependent on the ability of E1A to bind p300, because two different E1A mutants containing small deletions that selectively eliminate p300 binding do not inhibit ARE transactivation. Furthermore, overexpression of p300 enhanced significantly the ability of TGF-β to activate a transcriptional response. The limited extent of this enhancement by p300 suggests that the endogenous levels of p300 or functionally related proteins are not limiting for these responses.

The present results provide a mechanistic explanation for the previously observed ability of E1A to inhibit TGF-β responses. E1A has been reported to inhibit both proliferative responses to TGF-β in fibroblasts (50) and antiproliferative responses to TGF-β in keratinocytes (46, 52). E1A has also been reported to inhibit specific gene responses to TGF-β, including the up-regulation of junB (47, 50), p15\textsuperscript{ink4b} (49), and p21\textsuperscript{cip1} (49). The ability of E1A to inhibit TGF-β growth inhibitory responses is attributable in part to its ability to bind hypophosphorylated pRB (46, 52). However, studies using mutant E1A constructs have shown that E1A can inhibit TGF-β responses independent of its pRB binding function (48, 52). Furthermore, the ability of E1A to inhibit both TGF-β-induced expression of p15\textsuperscript{ink4b} and p21\textsuperscript{cip1} and TGF-β-mediated growth inhibition have been mapped to the region of E1A that binds p300 (48, 49, 52). These observations raised the possibility that p300 might be involved in TGF-β transcriptional responses.

Our present results with the ARE reporter show that the p300 sequestering ability of E1A inhibits transcriptional responses that are directly mediated by SMADs. This conclusion is further supported by the observation that E1A inhibits transactivation of a Gal4 reporter by a Gal4-Smad2 fusion protein. Furthermore, the ability of E1A to specifically inhibit transactivation by Gal4-Smad4(MH2) suggests that p300 is required for the intrinsic transcriptional activity of a SMAD MH2 domain. In sum, our results suggest a role for p300/CBP in SMAD-mediated transcriptional activation and provide an explanation for the observed ability of E1A to interfere with TGF-β action.

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