Repression of Bone Morphogenetic Protein and Activin-inducible Transcription by Evi-1*

Received for publication, December 20, 2004, and in revised form, April 1, 2005
Published, JBC Papers in Press, April 22, 2005, DOI 10.1074/jbc.M414305200

Tamara Alliston§§, Tien C. Ko§§§, Yanna Cao§, Yao-Yun Liang§, Xin-Hua Feng***, Chenbei Chang‡‡, and Rik Derynck§§§

From the ‡Departments of Cell and Tissue Biology and Anatomy, University of California at San Francisco, San Francisco, California 94143-0512, the Department of Surgery, Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555-0542, the Michael E. DeBakey Department of Surgery, Department of Molecular and Cellular Biology, Biology of Inflammation Center, and Baylor College of Medicine Cancer Center, Baylor College of Medicine, Houston, Texas 77030, and the §§Department of Cell Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Smads, key effectors of transforming growth factor (TGF)-β, activin, and bone morphogenetic protein (BMP) signaling, regulate gene expression and interact with coactivators and corepressors that modulate Smad activity. The coactivator Evi-1 exerts its oncogenic effects by repressing TGF-β/Smad3-mediated transcription, thereby blocking TGF-β-induced growth arrest. Because Evi-1 interacts with the highly conserved MH2 domain of Smad3, we investigated the physical and functional interaction of Evi-1 with Smad1 and Smad2, downstream targets of BMP and activin signaling, respectively. Evi-1 interacted with and repressed the receptor-activated transcription through Smad1 and Smad2, similarly to Smad3. In addition, Evi-1 repressed BMP/Smad1- and activin/Smad2-mediated induction of endogenous Xenopus gene expression, suggesting a role of repression of BMP and activin signals by Evi-1 in vertebrate embryogenesis. Evi-1 also repressed the induction of endogenous Smad7 expression by TGF-β family ligands. In the course of these studies, we observed Evi-1 repression of both transactivation and Smad binding to DNA. Thus, Evi-1 repressed BMP-induced histone acetylation, coincident with its repression of Smad7 gene expression. In this way, Evi-1 acts as a general Smad corepressor to inhibit TGF-β, activin-, and BMP-induced transcription.

† TGF-β family ligands, such as TGF-β, BMPs, and activin, regulate a number of cellular functions, including cell proliferation and differentiation, apoptosis, and matrix production. The actions of the TGF-β family ligands are particularly important during embryogenesis and tumorigenesis (1–4). BMP and activin signaling are required for gastrulation, mesoderm formation, tissue differentiation, and formation of extraembryonic tissues (1). TGF-β acts as a tumor suppressor by inducing the expression of the p15INK4B or p21CIP1 Cdk inhibitor genes to arrest proliferation of many cell types (2–4). Interference with TGF-β-induced p15INK4B and/or p21CIP1 expression, by oncogenic overexpression of c-Myc or c-Ski, leads to uncontrolled cell growth and tumorigenesis (5–7).

TGF-β family ligands signal through Smads, which regulate transcription by cooperating with DNA sequence-specific transcription factors (8–10). Among the Smads, Smad1, -5, and -8 relay signals from BMP-activated receptors, whereas Smad2 and Smad3 are the primary effectors of activin and TGF-β signaling, respectively. Upon ligand binding, the receptor complex phosphorylates and activates these Smads, resulting in dissociation of Smads from the receptor, heteromerization with Smad4, and nuclear translocation of the Smad complex. With the exception of Smad2, Smads bind DNA through their N-terminal MH1 domains. Stable interaction with select promoter sequences, however, occurs by association of the Smad complex with sequence-specific transcription factors. For example, in response to TGF-β, Smad2 and Smad3 interact with Sp1 at the p15INK4B or p21CIP1 promoters (11, 12). The receptor-activated Smads also bind directly, through their C-terminal MH2 domains, to the coactivators CBP/p300. This coactivator recruitment, together with the physical and functional interactions with sequence-specific transcription factors, confers ligand-induced transcriptional activation (8–10). Smads can recruit additional coactivators, such as SMIF, MSG1, or Swift, that enhance the transcription response or corepressors, including c-Ski, TGFIP, SIP1, SNIP1, or Tob, which decrease or inhibit ligand-induced transactivation (13).

Another Smad3 corepressor is the nuclear proto-oncogene, evi-1, which inhibits cellular responsiveness to TGF-β-induced growth arrest (14). The Evi-1 protein is a 145-kDa, 10-zinc finger-containing polypeptide that can function as a DNA sequence-dependent transcription repressor (15). Tar-
Evi-1 Repression of TGF-β Family-inducible Transcription

targeted inactivation of the evi-1 gene results in embryonic lethality because of defective heart, brain, and paraxial mesenchyme development and widespread hypocellularity (16). This phenotype is consistent with the expression of Evi-1 during embryogenesis and the role of Evi-1 as a potent activator of cellular proliferation (17). In adult tissues, Evi-1 is expressed at very low levels with the exception of the promyelocytic stage of myeloid differentiation, where it activates myeloid cell proliferation while inhibiting differentiation (18). The expression pattern and proliferative function of Evi-1 explains the occurrence of myeloid leukemias in humans with chromosomal rearrangement at the evi-1 locus. Most of these rearrangements cause Evi-1 overexpression and uncontrolled myeloid cell proliferation (15). A recent study using microarray analysis of gene expression in AML patients revealed that overexpression of Evi-1 correlated with poor treatment outcomes, illustrating the importance of tightly regulated Evi-1 expression and function (19).

Evi-1-mediated transformation and transcriptional repression require interaction with the corepressor CtBP (20, 21), which itself interacts with histone deacetylases (HDACs) (22). In addition to interacting with transcriptional corepressors, Evi-1 can also interact with the co-activators CBP and p300/CBP-associated factor, which have intrinsic histone acetylase activity (23). Coexpression of CBP reverses the transcription repression by Evi-1 at an artificial promoter that allows for Evi-1 binding (23); however, the physiological importance of this interaction at a natural promoter that is regulated by Evi-1 has yet to be determined.

Evi-1 has been shown to suppress TGF-β-induced signaling through direct interaction with Smad3. Thus, Evi-1 inhibits TGF-β-mediated Smad3 induction of a p15ink4B Cdk inhibitor reporter gene (14). This inhibition is thought to prevent the normal growth-inhibitory response to TGF-β and to enhance proliferation. Full repression of Smad3 activity requires interaction of Evi-1 with CtBP (20). Evi-1 was proposed to act as a Smad3-specific corepressor that decreases Smad3 DNA binding (14). The requirement of the Smad3 MH2 domain for Evi-1 repression of TGF-β-inducible transcription led us to investigate the physical interaction of Evi-1 with Smads 1–4, which have MH2 domains that are highly homologous to Smad3. Since the MH2 domain of Smad3 also interacts with CBP/p300 (13), it was conceivable that Evi-1 binding to the MH2 domain might interfere with coactivator recruitment, thus providing a mechanism for repression of Smad3-mediated transcription. We therefore examined the ability of Evi-1 to bind and repress the function of TGF-β-, BMP-, and activin-receptor activated Smads.

We observed that Evi-1 interacted with each Smad tested. Evi-1 repressed BMP/Smad1, activin/Smad2, and TGF-β-Smad3-induced transcription of reporter genes as well as endogenous Smad7 gene expression. Evi-1 also repressed BMP/Smad1 and activin/Smad2-mediated induction of endogenous Xenopus genes required for cell fate specification, suggesting a role for Evi-1 in development. Evi-1 did not interfere with CBP binding to Smad3; nor did it displace Smad3 from DNA. Rather, TGF-β-stimulated recruitment of Evi-1 and CtBP to the endogenous Smad7 promoter, resulting in decreased TGF-β-induced histone acetylation and transcription.

MATERIALS AND METHODS

Expression Plasmids—Coding sequences for C-terminally Myc-tagged or VP16-fused Evi-1 proteins or defined regions were generated by oligonucleotide- or PCR-based techniques from pME-Evi-1 and pME-Evi-1ΔZF1-7 (14), kindly provided by H. Hirai and inserted into the EcoRI-SalI sites of the mammalian expression plasmid pRK5 (24) or derivatives or into the retrovector vector LPCX (25). A neomycin-resist-
GST Fusion Proteins and in Vitro Protein-binding Assays—Plasmids pGEX-Smad1–4 have been described (29, 40). Equal amounts of GST or GST-Smad fusion protein bound to glutathione-Sepharose beads were incubated, as previously described, with 35S-labeled, in vitro translated nucleic acids (TNT translation kit; Promega) with similar specific radioactivity (29). Associated 35S-labeled proteins were detected by SDS-PAGE and autoradiography.

DNA Precipitation Assays—Biotinylated 5′ oligonucleotides containing two repeats of the consensus Smad binding element (GTCATAGAC) from the Smad7 promoter were synthesized and annealed with unbiotinylated complementary oligonucleotides to generate probes for DNA precipitation assays: SBE, 5′-AATGGTGCTAGCTGCTAGTGGTGG-3′. Immobilization of biotinylated oligonucleotide probes and absorption of nuclear extracts from transfected COS cells were carried out using streptavidin-coated Magna-sphere paramagnetic particles (Promega) following the manufacturer’s instructions and as described (11). After extensive washing, TdT-biotinylations were subjected to SDS-PAGE, followed by Western blotting using antibodies that recognized FLAG (M2)-, Myc (9E10)-, or HA (HA-11)-tagged proteins. In parallel, the cell lysates were immunoblotted to demonstrate the expression of transfected proteins.

RNA Isolation and Quantitative Real-time PCR—RNA was isolated using RNeasy reagents (Qiagen). cDNA was generated from equal amounts of total RNA precipitated with 2× proteinase K and phenol/chloroform extraction and ethanol precipitation. PCR was performed using 5 m MessageMACHINE kit (Ambion). The following DNA templates were used for reverse transcription (RT)-PCR.

Evi-1 Represses Smad3-dependent Transcription Independently of DNA Binding—Evi-1 has been shown to repress TGF-β-induced growth inhibition and transcription reporter activity from the p15INK4B and 3TP promoters (14). At these promoters, TGF-β-activated Smad3/4 cooperates with sequence-specific transcription factors (i.e. Sp1 or c-Jun/c-Fos, respectively) (11, 42, 43). Therefore, we tested whether Evi-1 was able to repress TGF-β- and Smad3/4-mediated transcription independently of its interacting, sequence-specific transcription factors. Tandem Smad binding elements in the (SBE)4 promoter allow cooperative binding of the Smads and TGF-β-inducible transcription without the help of other sequence-specific transcription factors (34). Similarly to the previously tested promoters, Evi-1 repressed TGF-β- and Smad3/4-mediated transcription from the (SBE)4 promoter (Fig. 1A). This result suggests that Evi-1 directly targets the intrinsic transactivation function of Smad3 in the absence of coactivation of an interacting, sequence-specific transcription factor.

The zinc finger 1 (ZF1) domain of Evi-1 is required for Smad3 interaction, and the repressor domain of Evi-1 confers transcriptional repression (14). However, the domains of Smad3 required for physical interaction with Evi-1 were not fully defined. We therefore examined the domains of Smad3 required for interaction with Evi-1 using mammalian two-hybrid assays, in which the transcriptional activation correlates with the physical interaction in transfected cells (44). These assays required deletion of the repressor domain of Evi-1, which would otherwise repress the transcription activity of the VP16 transactivation domain (Fig. 1D). As shown in Fig. 1B, Evi-1 did not interact with the MH1 domain and linker segment of Smad3 in Gal-Smad3NL but showed a strong interaction with the MH2 domain of Smad3 in Gal-Smad3C, which was further enhanced upon TGF-β stimulation. As expected, deletion of the ZF1 domain abolished the interaction of Evi-1 with Smad3.

The repression of TGF-β/Smad3-induced transcription, even without cooperation with other sequence-specific transcription factors, and the interaction of Evi-1 with the MH2 domain of Smad3 suggested that Evi-1 might repress TGF-β/Smad3-induced transcription through repression of the transactivation function of the Smad3 MH2 domain. We tested this hypothesis under constant DNA binding conditions using the MH2 domain of Smad3 fused to a Gal4 DNA binding domain, using a Gal4 binding promoter as a reporter. As shown in Fig. 1C, Evi-1 repressed the transactivation function of Smad3, independent of DNA binding. Maximal repression required both the ZF1 and repressor domains, since deletion of either domain reduced Evi-1 repression. We conclude that Evi-1 targets the transactivation function of the Smad3 MH2 domain, independently of any possible effect of Evi-1 on DNA binding of Smad3.

**Results**

**Evi-1 Interacts with Smad1, -2, -3, and -4**—The interaction of Evi-1 with the MH2 domain of Smad3 (Fig. 1B and C), and the high conservation of this Smad domain led us to test whether Evi-1 interacts with other Smads in addition to Smad3. As shown using mammalian two-hybrid assays, Evi-1 without its repressor domain interacted with the receptor-activated Smad1, -2, and -3 and had a lower level interaction with Smad4. These interactions were increased in the presence of TGF-β and abolished when the ZF1 domain was deleted from Evi-1 (Fig. 2A). In principle, the ability of the Smads to heterodimerize with each other and the endogenous presence of Smad2, -3, and -4 in HepG2 cells could contribute to these in vitro interactions. However, Evi-1 still interacted with Smad1 or Smad2 in Smad3-deficient cells and with Smad1, -2, and -3 in
the Smad4-deficient MDA-MB-468 cells (Fig. 2B). Thus, Evi-1 interacted with all Smads tested, and this interaction depended in all cases on the ZF1 domain in Evi-1.

In GST adsorption assays, the ZF1 domain of Evi-1 interacted with GST-fused Smads 1–4 and not GST alone (Fig. 2C), and all four Smads were able to interact with the GST-fused ZF1 domain of Evi-1 but not with GST alone (data not shown). These data are consistent with the two-hybrid interaction data and confirm the role of the ZF1 domain in the in vivo interactions of Evi-1 with the Smads (Fig. 2A).

The interaction of Evi-1 with each of the four Smads was also observed by coimmunoprecipitation from transfected cell lysates (Fig. 2D). In these assays, Evi-1 coprecipitated with Smad3 or Smad4, albeit to a different extent. Under the same conditions, Evi-1 coprecipitated only weakly with Smad1 or -2, but reversible chemical cross-linking using dithiobis(succinimidyl propionate) sufficiently stabilized the Smad1-Evi-1 or Smad2-Evi-1 complexes to allow specific coprecipitation of Evi-1 with Smad1 or -2 (Fig. 2D).

To test the interaction of endogenous Evi-1 and Smad proteins, we performed coimmunoprecipitation assays in HEC-1b cells, the only one of ~60 cell lines screened to express high levels of Evi-1 (60). Endogenous Evi-1 and Smad3 interacted only following TGF-β stimulation (Fig. 2E). Together, data from mammalian two-hybrid, GST adsorption, and coimmunoprecipitation assays reveal that Evi-1 can interact with Smad1, -2, -3, and -4. The differing affinities presumably reflect the differential involvement of Evi-1 domains (data not shown), in
addition to ZF1, in Smad binding, as well as differences in the assays used.

_Evi-1 Represses Smad1- and Smad2-mediated Transcription_—Because of the physical interactions of Evi-1 with Smads 1–4, we assessed whether Evi-1 could repress Smad1- and Smad2-mediated transactivation. We tested this hypothesis under constant DNA binding conditions using the MH2 domains of Smad1, -2, and -3 fused to the Gal4 DNA binding domain, using a Gal4 binding promoter as a reporter. As shown in Fig. 3A, Evi-1 strongly repressed the basal and receptor-induced transactivation of Gal4-Smad1, -2, and -3. Consistent with the previous report on Smad3 (14), the repression of Smad1 and Smad2 transcription was decreased when the ZF1 segment was deleted from Evi-1 (Fig. 3A). The requirement of the ZF1 domain for efficient repression is consistent with the interaction of MH2 domains of Smads with the ZF1 domain of Evi-1 (Fig. 1B and data not shown).

We then tested the ability of Evi-1 to repress Smad1-, Smad2-, and Smad3-mediated transcription using BMP, activin, and TGF-β-responsive promoter-reporter genes. We tested Smad1-mediated transcription using a BMP-responsive promoter segment derived from the Xenopus vent-2 gene. Consistent with previous reports (35), activation of ALK-6/BMP-RIB signaling enhanced transcription, and coexpression of Smad1 or Smad1/4 strongly activated transcription. In each case, Evi-1 strongly repressed the transcriptional activation of the Xvent-2 promoter (Fig. 3B). Similarly, we used a reporter plasmid containing a segment of the Xenopus goosecoid promoter that binds the transcription factor FAST-1 to measure Smad2-activated transcription. Activin signaling causes Smad2 to bind FAST-1, thereby activating transcription from the goosecoid promoter (45). In the presence of FAST-1, activin signaling conferred a low level transcription, which was strongly enhanced by coexpression of Smad2 or Smad2/4. Evi-1 strongly repressed the activin receptor- and Smad2-induced transcription (Fig. 3C). Consistent with the ability of Evi-1 to repress TGF-β/Smad3/Smad4-responsive induction of the PAI-1 promoter (14), Evi-1 also repressed TGF-β/Smad3/Smad4-responsive induction of the PAI-1 promoter (Fig. 3D). Therefore, Evi-1 repressed BMP/Smad1-, activin/Smad2-, and TGF-β/Smad3-mediated transactivation of their respective target gene promoters.

_Evi-1 Represses Activin- and BMP-responsive Endogenous Gene Expression in Xenopus Embryos_—Although aberrantly expressed in tumorigenesis (15), Evi-1 expression is predominantly restricted to embryonic stages and is down-regulated in adult tissues (17). During embryonic development, activin/nodal and BMP signals control patterning and cell fate determination in multiple processes (1), raising the possibility that their activities may be regulated by Evi-1 during development. We therefore tested whether Evi-1 inhibits BMP- and activin-inducible endogenous gene expression during Xenopus embryogenesis using the Xenopus ectodermal explant system. The changes in gene expression and cell differentiation in response to Smad1-mediated BMP signals and Smad2-mediated activin/nodal/Vg1 signals have been well studied in this system. In the
absence of growth factors, the ectodermal explants (i.e. animal caps) develop into atypical epidermis. However, incubation with TGF-β/H9252 family ligands or increased expression of the corresponding Smads induces mesoderm- and endoderm-specific gene expression. Specifically, activin/nodal/Vg1 and Smad2 induce dorsal mesoderm and endoderm, whereas BMP and Smad1 induce ventral mesodermal and endodermal markers (for a review, see Ref. 46).

We first analyzed the effects of Evi-1 on activin- and BMP-dependent mesendodermal marker induction. RNAs encoding activin or BMP2 were injected into the animal poles of two-cell stage embryos either alone, or together with different doses of Evi-1 RNA. Animal caps were dissected from the injected embryos at blastula stages and incubated to gastrula stages before processing them for RT-PCR assays for marker gene expression. As shown in Fig. 4A, activin induced the ventrolateral marker Xwnt8, the dorsal mesodermal marker chordin, and the endodermal marker Mix1 (lanes 3). Coexpression of Evi-1 with activin, however, suppressed the expression of all of these genes (Fig. 4A, compare lanes 4 and 5 with lane 3). Similarly, BMP2 induced the endodermal marker Mix1, as well as the ventral markers Xho3 and Msx1, and Evi-1 repressed the induction of these genes (Fig. 4A, compare lanes 7 and 8 with lane 6). The data thus indicate that Evi-1 blocks both activin and BMP signals in *Xenopus* animal caps.

We then examined the effects of Evi-1 on marker gene induction by Smad1 or Smad2. RNAs coding for Smad1 or Smad2 were injected alone or with Evi-1 RNA into the animal poles of two-cell stage embryos, and animal caps from injected embryos were obtained and analyzed as above. Smad2 expression induced expression of Xwnt8, chordin, and Mix1, similarly to activin (Fig. 4B, lane 3). Coexpression of Evi-1 repressed the induction of these mesendodermal genes by Smad2 (Fig. 4B, compare lanes 4 and 5 with lane 3). Similarly, the markers induced by Smad1 (i.e. Mix1, Xhox3 and Msx1) were inhibited by Evi-1 to different degrees (Fig. 4B, compare lanes 7 and 8 with lane 6). We also note that Evi-1 alone blocked the endogenous expression of Msx1 in animal caps (Fig. 4, A and B, lanes 2). Since Msx1 is a direct downstream target of BMP signals (47, 48) and its expression relies on endogenously expressed BMP ligands in the animal caps, our result demonstrates that Evi-1 blocks endogenous as well as ectopically activated BMP signals. Therefore, in addition to its role in promoting cell proliferation in embryogenesis, Evi-1 may also regulate cell fate determination by its repression of activin and BMP signaling.

### Evi-1 Represses Endogenous Smad7 Transactivation

To evaluate the effect of Evi-1 on endogenous mammalian gene expression, we generated retrovirally infected, control, and Evi-1-expressing C2C12 cells. In contrast to LPCX control cells, the LPCX-Evi-1 C2C12 cells expressed Myc-tagged Evi-1 (Fig. 5A). Since Evi-1 repressed the transactivation of the synthetic
(SBE), promoter with Smad binding elements similar to those in the Smad7 promoter (Fig. 1A), we first assayed the transcription activation from the 0.5-kbp Smad7 promoter segment in the Smad7-Luc reporter in the retrovirally infected C2C12 cells. Consistent with previous reports (49, 50), TGF-β, BMP, and activin receptor signaling activated the Smad7 reporter in control C2C12 cells. The activation by each of the three ligands was absent or much reduced in cells expressing Evi-1 (Fig. 5B). We then evaluated the regulation of endogenous Smad7 expression by Evi-1. Smad7 mRNA expression was induced by BMP-2 in control C2C12 cells, but this induction was blocked by retroviral Evi-1 expression in C2C12 cells (Fig. 5C). Similarly, TGF-β induced Smad7 mRNA expression 4.3-fold after 3 h, and this induction of Smad7 mRNA by TGF-β was nearly absent in Evi-1-expressing cells (1.39-fold induction over untreated) (Fig. 5D). Together, the data from reporter and endogenous gene expression experiments in Xenopus and mammalian systems demonstrate that Evi-1 represses BMP, activin,
and TGF-β receptor signaling through its physical and functional interactions with Smad1, -2, and -3.

**Evi-1 Binding to DNA Is Enhanced by Smad3 and CBP**—The ability of Evi-1 to repress Smad transactivation when DNA binding was kept constant (Figs. 1C and 3A) suggested that Evi-1 may employ alternative strategies to repress transcription, in addition to the previously proposed displacement of Smad binding to DNA (14). We therefore investigated possible mechanisms of Evi-1 repression of Smad transactivation. The binding of Evi-1 to the MH2 domain and the consequent repression of the transactivation function of Smad3 (Fig. 1C) raised the possibility that Evi-1 might repress transcription by blocking the interaction of CBP with the C-terminal sequence of Smad3. Moreover, Evi-1 has been shown to interact with CBP (23), and this was confirmed in coimmunoprecipitation assays (data not shown). Therefore tested whether increasing levels of Evi-1 would interfere with the Smad3 association with CBP using coimmunoprecipitation assays (Fig. 6A). Surprisingly, Evi-1 enhanced the interaction of Smad3 with CBP, suggesting stabilization of the Smad3-CBP complex. Conversely, increasing levels of CBP also enhanced the interaction of Evi-1 with Smad3 (Fig. 6B). These data suggest that Evi-1 does not repress Smad activity by displacing the coactivator CBP but rather enhances the stability of the Smad3/CBP interaction.

The ability of Evi-1 to repress transcription when Smad binding to DNA was kept constant (Figs. 1C and 3A) and the Evi-1 interaction with the MH2 domain, but not the DNA-binding MH1 domain, of Smad3 (Fig. 1B) led us to test the effect of Evi-1 on binding of Smad3 to the Smad binding element from the Smad7 promoter. At this promoter, Smads bind to the SBE sequence independently of other transcription factors (51). DNA precipitation assays showed the formation of a Smad3-DNA complex upon TGF-β receptor activation. Coexpression of Evi-1, in the absence or presence of coexpressed CBP, did not decrease the binding of Smad3 to DNA (Fig. 6C). Identical results were observed using electrophoretic mobility shift assays (data not shown). CBP was also recruited to the

![Image](https://example.com/image.png)

**Fig. 6. Smad3 and CBP stabilize recruitment of Evi-1 to DNA.** A and B, Cell lysates were prepared from COS cells transfected with expression plasmids for tagged CBP, Evi-1, or Smad3 as indicated. The presence of proteins in FLAG or Myc immunoprecipitation (IP) complexes or in cell lysates (IB) was detected by Western analysis with the indicated antibodies. A, increasing Evi-1 levels stabilize the interaction of Smad3 with CBP. B, increasing CBP levels stabilize the Smad3/Evi-1 interaction. C, DNA precipitation assays evaluated the binding of each cotransfected protein in cell lysates to biotinylated SBE oligonucleotide (left panel). Western blotting of whole cell lysates shows the expression level of each tagged protein (right panel). D, enzymatically inactive CBP F1541A is as effective as wild-type CBP in facilitating recruitment of Evi-1 to DNA-bound Smad3.
Smad-binding DNA sequence and also did not displace Smad3 from DNA. Interestingly, Evi-1 binding to the Smad-binding DNA sequence was only observed in the presence of coexpressed CBP and Smad3. Thus, the repression of Smad3-mediated transcription by Evi-1 does not result from a displacement of Smad3 from the DNA or from competitive interference with CBP association with Smad3. Instead, recruitment of Evi-1 to the Smad-binding DNA sequence is enhanced by Smad3 and CBP.

The ability of the coactivator CBP to acetylate Evi-1 (23) and the surprising result that CBP enhanced recruitment of Evi-1 to the Smad3-binding DNA sequence led us to evaluate the role of the histone acetyltransferase activity in this recruitment (Fig. 6D). We therefore compared the effects of CBP and a CBP point mutant, CBP (F1541A), that lacks acetyltransferase activity in DNA binding assays (30). Both wild-type and mutant CBP were equally effective in recruiting Evi-1 to the Smad3-binding DNA sequence, indicating that the acetyltransferase activity is dispensable for this function of CBP.

**Evi-1 Recruits CBP to the Smad7 Promoter to Block TGF-β-induced Histone Acetylation**—We then used chromatin immunoprecipitation to evaluate the recruitment of Evi-1 to the endogenous Smad7 promoter in response to TGF-β. In these assays, we used the LPCX-Evi-1 C2C12 cells that expressed Myc-tagged Evi-1 and LPCX control cells that were also used in the gene expression assays in Fig. 5. Chromatin associated with Myc-tagged Evi-1 was isolated, and the Evi-1-bound Smad7 promoter sequences were quantified using real-time PCR analysis. As shown in Fig. 7A, Evi-1 bound to the Smad7 promoter at 60 min after the addition of TGF-β. No binding above the background, using a mouse IgG antibody, was observed in the absence of added TGF-β or in control C2C12 cells that do not express Myc-Evi-1. The binding of Evi-1 to the Smad7 promoter following TGF-β stimulation is consistent with the TGF-β-dependent interaction of Smad3 with Evi-1 (Fig. 2E) and the observation that Evi-1 only binds to SBE sequences in the presence of TGF-β-activated Smad3 (Fig. 6C).

Because Evi-1 interacts with CBP and its associated HDACs to repress transcription of reporter genes (20–22), we evaluated the effect of Evi-1 on histone acetylation of the endogenous Smad7 promoter by chromatin immunoprecipitation using an antibody specific for acetylated histone H4 (Fig. 7B). In control LPCX-C2C12 cells, TGF-β stimulation conferred a 3.2-fold increase in histone H4 acetylation at the Smad7 promoter after 2 h, consistent with the induction of Smad7 mRNA expression by TGF-β. However, the induction of histone H4 acetylation by TGF-β was reduced to 1.5-fold in Evi-1-expressing cells.

We also examined the binding of endogenous CtBP to the Smad7 promoter by chromatin immunoprecipitation. Like Evi-1 (Fig. 7A), CtBP is recruited to the Smad7 promoter within 60 min of TGF-β treatment, resulting in a more than 60-fold increase in CtBP binding (Fig. 7C). CtBP binding is subsequently lost following release of Evi-1 from the Smad7 promoter in LPCX-Evi-1 cells (data not shown). We also found that, in the LPCX cells that do not express Evi-1, the CtBP that binds to the Smad7 promoter in the absence of TGF-β is rapidly released upon TGF-β treatment (Fig. 7C, inset). These results suggest that the TGF-β-induced interaction of Evi-1 with the Smad7 promoter stabilizes and enhances CtBP recruitment.

It has previously been shown that mutation of the CtBP interaction domain of Evi-1 partially reverses the repression exerted by Evi-1 on TGF-β-induced transcription from the 3TP reporter. A similar, partial rescue was observed using trichostatin A, an inhibitor of HDACs (20). Whereas these observations implicate CtBP and associated HDACs in the repression of TGF-β-induced gene expression by Evi-1, they also revealed the role of additional repression domains in Evi-1 that are
Evi-1 Repression of TGF-β Family-inducible Transcription

Discussion

Based on the reported physical interaction of Evi-1 with the highly conserved MH2 domain of Smad3 (14), we investigated the interaction of Evi-1 with Smads 1–4. Evi-1 interacted with each of the Smads tested, albeit with differing efficiencies depending on the assays. Consistent with these physical interactions, Evi-1 repressed Smad1- and Smad2-mediated transactivation by BMP and activin receptors, respectively. Therefore, Evi-1 repression is not restricted to Smad3 but affects Smad-mediated transactivation in response to other TGF-β-related proteins, such as BMP and activin.

The ability of Evi-1 to repress BMP and activin signaling raises the possibility that Evi-1 may function as a modulator of Smad signaling in development. BMPs, activin, and related TGF-β proteins are well known regulators of embryonic patterning and cell fate specification (46). Deficiencies in BMP and activin signaling, analyzed by targeted gene inactivation, result in embryonic lethal phenotypes with mouse embryos possessing severe defects in multiple organs (1). Likewise, mice lacking Evi-1 die in utero with widespread hypocellularity and multiple defects in various tissues (16, 17) that partially overlap with those in mice that lack signaling molecules in TGF-β family pathways. In addition, the developmental expression pattern of Evi-1 overlaps partially with that of TGF-β, activin, or BMP signaling components (16, 17). These data suggest that Evi-1 may regulate TGF-β-related signaling during early vertebrate embryogenesis, consistent with the direct repression of Smad1- and Smad2-stimulated induction of endogenous genes required for mesendodermal cell fate specification by BMP and activin in Xenopus embryo assays (Fig. 4). Thus, in addition to the role of Evi-1 in the repression of TGF-β/Smad-mediated growth inhibition in tumorigenesis (14), we propose a role for Evi-1 as a regulator of Smad-mediated signaling during development.

We also showed that Evi-1 represses endogenous Smad7 expression, the first demonstration that Evi-1 inhibits TGF-β family-induced expression of an endogenous gene. TGF-β, activin, and BMPs induce Smad7 expression through Smad-mediated transcription, and Smad7 inhibits effector Smad activation, thus allowing Smad7 to exert a negative feedback effect on TGF-β signaling. Accordingly, ectopic Smad7 expression in Xenopus explants inhibits mesoderm induction by activin and ventralizing signals by BMPs in a dose-dependent manner (52, 53). The regulation of Smad7 expression by Evi-1, as shown in this report, may therefore have important consequences in development. Since Evi-1 represses Smad signaling directly, and indirectly through inhibition of Smad7 expression, the graded expression of inhibitory and inductive signals (Evi-1, Smad7, and BMP and activin ligands) may contribute to cell fate specification and differentiation during embryonic development.

Our results also provide insight into the mechanism of Evi-1-mediated repression of effector Smads. Transcriptional repression can result from several strategies. These include displacement of the DNA binding of a transcriptional activator, interference with coactivator recruitment, and direct functional repression through recruitment of HDACs. Based on studies of the p15Ink4B or 3TP promoters, where Smad3 is recruited to DNA by its interactions with Sp1 or c-Jun/c-Fos, respectively (11, 42, 43), Evi-1 was originally proposed to repress TGF-β/Smad3-activated transcription by displacing Smad3 from DNA (14). However, we found that Evi-1 repressed Smad function even when DNA binding was kept constant using a Gal4 DNA binding domain (Figs. 1C and 3A). We also found that Evi-1 repressed Smad3-mediated transactivation of the Smad promoter, where DNA binding is mediated only by the Smad3 MH1 domain (34) instead of through the interaction of Smad3 with other sequence-specific transcription factors. At this promoter, Evi-1 did not displace Smad3 from the SBE DNA sequence but rather was recruited through its interaction with Smad3 to the SBE sequence (Fig. 6C). Therefore, Evi-1 uses alternative strategies to repress Smad transactivation.

Some reports provide evidence that competition between coactivators and corepressors for interaction with a sequence-specific transcription factor defines the level of transcription. For example, the coactivators GRIP, CBP, or p300 compete with the corepressor SMRT for binding to the same domain of the transcription factor HNF4α (54). Since Evi-1 interacted with the MH2 domain of Smad3, which directly binds to the coactivators CBP or p300 (29), we evaluated whether competition of Evi-1 with CBP/p300 for Smad3 binding might contribute to Evi-1-mediated repression. Contrary to our hypothesis, interactions of Evi-1 and CBP with Smad3 were not mutually exclusive; rather, CBP enhanced the Smad3 interaction with Evi-1, and Evi-1 enhanced Smad3 interaction with CBP. Our data suggest that physical interactions between Smad3, CBP, and Evi-1 allow formation of a stable complex that is presumably the basis for our observation that efficient recruitment of Evi-1 to a Smad-binding DNA sequence required the participation of Smad3 and CBP.

The participation of Evi-1 and CBP in the Smad-DNA complex suggests the involvement of acetylation and deacetylation activities. CBP has intrinsic acetylation activity (55), whereas Evi-1 recruits CtBP (20, 21), which in turn interacts with HDACs (22). Acetylation of transcription factors has been shown to regulate coactivator and corepressor recruitment. For example, acetylation of MyoD increases its affinity for coactivators (56), whereas acetylation of the orphan nuclear receptor RIP140 prevents corepressor recruitment (57). Regulated acetylation may also impact Evi-1 function, since Evi-1 has been proposed to be acetylated by CBP, P/CAF, and GCN5 (23, 58). The acetylase activity of CBP activity is, however, not essential for efficient recruitment of Evi-1 to the Smad3-binding DNA sequence, since both wild-type CBP and the acetylase-deficient CBP mutant are equally effective in recruiting Evi-1 to DNA. This is similar to a recent observation that p300 can recruit HDAC6 independently of its acetylase activity (59). Thus, CBP/p300 may act as a scaffold that stabilizes corepressor complex recruitment.

Whereas the acetylase function of CBP is not required for Evi-1 recruitment to the Smad-binding DNA sequence, TGF-β-induced acetylation is observed at the Smad7 promoter (Fig. 7B), presumably a result of the recruitment of CBP by Smad3 (29). In cells expressing Evi-1, however, Smad3-mediated recruitment of Evi-1 to the promoter resulted in decreased histone acetylation (Fig. 7B), probably due to the co-recruitment of CtBP and associated HDACs with Evi-1 in response to TGF-β (Fig. 7C) (20, 21). Reduction of CtBP levels, through the use of small interfering RNA, decreased the repression of TGF-β-
induced Smad7 expression by Evi-1. This observation suggests that reduced histone acetylation, concomitant with Evi-1 and CtBP recruitment, is an important determinant in the repression of TGF-β-induced Smad7 expression by Evi-1. However, consistent with previous findings (20, 21), our results (Fig. 7D) additionally implicate CtBP-independent mechanisms in the repression of TGF-β/Smad3 signaling by Evi-1.

In conclusion, our results demonstrate that Evi-1 interacts with not only Smad3 but with all Smads tested, thus allowing Evi-1 to repress BMP-, activin-, and TGF-β-induced, Smad-mediated transcription. Upon TGF-β stimulation, Evi-1 and CtBP are recruited to the Smad7 promoter, where they inhibit TGF-β-induced histone acetylation and transcription activation. This is the first report of Evi-1 recruitment to and repression of Smad function by Evi-1. However, additional recruitment to and repression of Smad function by Evi-1 may have a role in development in addition to its previously proposed role in tumorigenesis.

Acknowledgments—We thank H. Hirai, R. Goodman, J. Massagué, M.G. Rosenberg, R. Tjian, K. Yamamoto, T. Kouzarides, M. Whitman, L. Attisano, J. Wrana, B. Vogelstein, C. Niehrs, I. Ihle, C. Bartholomew, Y. Shi, and K. Cho for providing essential plasmids.

REFERENCES

1. Chang, H., Brown, C. W., and Matsuz, M. M. (2002) Endocr. Rev. 23, 787–823
2. Siegel, P. M., and Massagué, J. (2003) Nat. Rev. Cancer 3, 807–812
3. Derynck, R., Akhurst, R. J., and Balmain, A. (2001) Nat. Genet. 29, 117–129
4. Wakefield, L. M. and Roberts, A. B. (2002) Curr. Opin. Genet. Dev. 12, 22–29
5. Feng, X. H., Liang, Y. Y., Liang, M., Zhai, W., and Lin, X. (2002) Mol. Cell 9, 133–143
6. Sesane, J., Le, H. V., and Massagué, J. (2002) Nature 419, 729–734
7. Sun, Y., Liu, X., Eaton, E. N., Lane, W. S., Lodish, H. F., and Weinberg, R. A. (2000) J. Biol. Chem. 275, 577–584
8. Derynck, R., and Zhang, Y. E. (2000) Nature 402, 577–584
9. ten Dijke, P., and Hill, C. S. (2004) Trends Biochem. Sci. 29, 265–273
10. Attisano, L., and Wrana, J. L. (2002) Science 296, 1646–1647
11. Feng, X. H., Lin, X., and Derynck, R. (2000) EMBO J. 19, 5178–5189
12. Pardali, K., Kurisaki, A., Moren, A., ten Dijke, P., Kardassis, D., and Mousta-kas, A. (2000) J. Biol. Chem. 275, 29244–29256
13. Mou斯塔kas, A., Souchelnytskyi, S., and Heldin, C. H. (2001) J. Cell Sci. 114, 4359–4369
14. Kurokawa, M., Mitani, K., Irie, K., Matsuyama, T., Takahashi, T., Chiba, S., Yazaki, Y., Matsumoto, K., and Hirai, H. (1998) Nature 394, 92–96
15. Hirai, H. (1999) Int. J. Biochem. Cell Biol. 31, 1367–1371
16. Hoyt, P. R., Bartholomew, C., Davis, A. J., Yutzey, K., Gamer, L. W., Potter, S. S., Ihle, J. N., and Mucenski, M. L. (1997) J. Biol. Chem. 272, 2815–2822
17. Perkin, A. S., Mercer, J. A., Jenkins, N. A., and Copeland, N. G. (1991) Development 111, 479–487
18. Morishita, K., Parganas, E., Matsugi, T., and Ihle, J. N. (1992) Mol. Biol. Cell. 3, 128–189
19. Vakil, P. J., Verhaak, R. G., Beijen, M. A., Erpelinc, C. A., Barjesteh van Vaalwijk von Doorn-Khorsavani, S., Boer, J. M., Beverloo, B. H. M., Mees- house, M. J., van der Spek, P. J., Lowenberg, B., and Delwel, R. (2004) N. Engl. J. Med. 350, 1617–1628
20. Izutsu, K., Kurokawa, M., Imai, Y., Maki, K., Mitani, K., and Hirai, H. (2001) Blood 97, 2815–2822
21. Palmer, S., Brulet, J. P., Kilbey, A., Fulton, R., Walker, M., Crossley, M., and Bartholomew, C. (2001) J. Biol. Chem. 276, 25884–25894