A Novel E1A-like Inhibitor of Differentiation (EID) Family Member, EID-2, Suppresses Transforming Growth Factor (TGF)-β Signaling by Blocking TGF-β-induced Formation of Smad3-Smad4 Complexes*

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Some retinoblastoma gene product-interacting viral proteins, such as E1A and HPV-16 E7, antagonize TGF-β signaling in various systems. E1A interacts with Smad3 and interferes with the interaction of Smad3 with p300 (16). E7 interacts with Smads and blocks Smad3 binding to its target sequence on DNA (17). E1A-like inhibitor of differentiation (EID)-1 and EID-1-like inhibitor of differentiation-2 (EID-2) were recently identified as novel p-retinoblastoma-interacting cellular proteins and block muscle differentiation (18–20). Inhibition of differentiation by EID-1 correlates with its ability to inhibit p300 histone acetyltransferase (HAT) activity (18). Unlike EID-1, EID-2 does not block p300 activity and associates with both class I histone deacetylase (20). In this study, we examined whether EID-2 might target the Smad proteins that mediate TGF-β signaling. Here, we showed that EID-2 inhibits Smad3-Smad4-induced transactivation through binding to Smad3. E1A-like inhibitor of differentiation-2 (EID-2) was recently identified as an endogenous suppressor of TGF-β signaling.

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‡ The abbreviations used are: TGF-β, transforming growth factor-β; HDAC, histone deacetylase; EID, E1A-like inhibitor of differentiation; HAT, histone acetyltransferase; GST, glutathione-S-transferase; HA, hemagglutinin; SBE, Smad-binding element; PAI-1, plasminogen activator inhibitor 1; PIAV, protein inhibitor of activated signal transducer and activator of transcription.

MATERIALS AND METHODS

Cell Culture—COST cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin (PSG; Invitrogen). HepG2 and Mv1Lu cells were maintained in mini-
24 h after TGF-β/H11032
Values were normalized with the quantified by using Enhanced Luciferase Assay Kit (BD Biosciences).

glycerol, 5 mM EDTA, and protease inhibitor mixture (Complete, Roche)
cells with either 3TP-Lux (A) or SBE4-luc (B). Luciferase activity was measured 24 h after TGF-β1 stimulation. C, GAL4 fusion constructs were transfected into HepG2 cells along with the reporter plasmids, and then cells were incubated in the presence or absence of TGF-β1. Data shown are means of triplicate measurements from one representative transfection.

Fig. 1. EID-2 represses TGF-β-induced transcriptional activation.

EID-2 was co-transfected into HepG2 cells with either 3TP-Lux (A) or SBE4-luc (B). Luciferase activity was measured 24 h after TGF-β1 stimulation. C, GAL4 fusion constructs were transfected into HepG2 cells along with the reporter plasmids, and then cells were incubated in the presence or absence of TGF-β1. Data shown are means of triplicate measurements from one representative transfection.

Plasmid Constructs—pcDNA3-T7-EID-2 (20), FLAG-tagged Smad2 and Smad3, Myc-tagged Smad4, and FLAG-tagged Smad3 deletion constructs were described previously (17). Plasmids encoding glutathione-S-transferase (GST)-tagged EID-2 wild type and mutants were generated by PCR using oligonucleotides that introduced a 5'-BamHI site and a 3'-SpeI site; they were then subcloned into pEBG eukaryotic expression vector. EID-2 versions tagged with hemagglutinin (HA) were generated in the pEBB vector.

Transfection and Reporter Assays—HepG2 cells were transiently transfected with 3TP-Lux (21), SBE4-luc (22), and the internal control pCMV-β-gal in six-well plates using Lipofectin (Invitrogen) according to the manufacturer’s instructions. After 24 h transfection, cells were treated with 5 ng/ml TGF-β1 for 24 h in media. Luciferase activity was quantified by using Enhanced Luciferase Assay Kit (BD Biosciences). Values were normalized with the β-galactosidase activity. All assays were performed in triplicate and represented as the mean (± S.E.) of three independent transfections.

Immunoblotting and Immunoprecipitation—HepG2 or COS7 cells were used for the detection of protein-protein interaction in vivo. HepG2 cells were transiently transfected with the indicated plasmids. After 24 h transfection, cells were switched to 0.2% serum overnight, and treated with 5 ng/ml TGF-β1 for 2 h. Cells were lysed in a buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and protease inhibitor mixture (Complete, Roche Applied Science). Extracts were separated by SDSPAGE followed by electrophoresis to polyvinylidene difluoride membranes and probed with polyclonal or monoclonal antisera, followed by horseradish peroxidase-conjugated anti-rabbit, anti-mouse IgG and visualized by chemiluminescence, according to the manufacturer’s instructions (Pierce). For immunoprecipitation the cell lysates were incubated with the appropriate antibody for 1 h, followed by incubation with Gamma-bind beads (Amersham Biosciences) for 1 h at 4 °C. Beads were washed four times with the buffer used for cell solubilization. Immune complexes were then eluted by boiling for 3 min in 2 x Laemmli buffer, and then extracts were analyzed by immunoblotting as described above. GST pull-down assay was performed by incubation with glutathione-Sepharose 4B beads (Amersham Biosciences) with each extract for 1 h. After washing the beads four times with the buffer used for cell solubilization, immunoblots were performed.

Generation of Stable Cell Lines Expressing EID-2—For stable expres-

sion of EID-2, HepG2 or Mv1Lu cells were transfected with pcDNA-T7-EID-2 expression plasmid. A day after transfection, cells were split and selected for neomycin resistance. Neomycin-resistant colonies were pooled after 2 weeks of selection, expanded, and analyzed.

For cell proliferation assay, EID-2-expressing cells were plated in 30-well plates at a density of 5 x 10^4 cells per well in 0.5 ml of assay medium (minimal essential medium/0.2% fetal bovine serum). After incubating for 22 h in the presence or absence of TGF-β1, cells were pulse-labeled with 0.5 μCi of [³H]thymidine for 2 h at 37 °C. Cells were fixed, trypsinized, solubilized, and transferred to scintillation vials to measure radioactivity as described previously (23).

siRNA Methods—We used the siRNA design tool (Dharmacon Inc., Lafayette, CO) to identify target siRNAs. The EID-2-specific sequence was 5'-UGCCGAUAUCAGCGAAdTdT-3' (EID-2 nucleotides 648–664; GenBank accession number AY251272). HeLa cells were seeded at 30% density the day before transfection. Transfections were performed by using TransIT-TKO reagent (Mirus, Madison, WI), according to the manufacturer’s instructions, with 200 pmol of siRNA and 10 μl of transfection reagent/10 cm dish for HeLa cells.

RESULTS

EID-2 Inhibits Smad3-dependent Transcription in TGF-β Reporter Assays—To analyze the effect of EID-2 on TGF-β/Smad-dependent transcription, HepG2 cells were cotransfected with an EID-2 expression construct and either the TGF-β-responsive 3TP-lux reporter construct or the SBE4-luc, which contains four tandem repeats of SBE (Smad-binding element) (22). Introduction of EID-2 suppressed the TGF-β-induced transcriptional activity of these reporter gene constructs in a dose-dependent manner (Fig. 1, A and B). Basal transcription from the SBE4-luc reporter was also suppressed by EID-2 (Fig. 1B). The repression of the SBE4-luc reporter activity by EID-2 suggests that it may directly inhibit the transcriptional activation of Smad complexes. To determine whether EID-2 can directly suppress Smad transcriptional activity, we used a heterologous reporter assay in which the GAL4 DNA-binding domain was fused to various Smad proteins. GAL4-Smad2, GAL4-Smad3, or GAL4-Smad4 expression constructs were co-
transfected with a luciferase reporter construct (G5E1b-lux), which contained five GAL4-binding sites upstream of the AdE1b TATA box. As shown in Fig. 1C, TGF-β/H9252 treatment did not induce transcription by the minimal GAL4-DNA binding domain, and EID-2 did not have any effect on its transcription. However, EID-2 strongly suppressed TGF-β/H9252-induced transcriptional activity of GAL4-Smad3 fusion protein and to a lesser extent GAL4-Smad2 or GAL4-Smad4 fusion proteins (Fig. 1C), demonstrating that EID-2 can directly suppress Smad-mediated transcriptional activation.

**EID-2 Interacts with Smads**—To examine the possibility that EID-2 interacts directly with Smad proteins in vivo, COS7 cells were transfected with FLAG-tagged Smad2, Smad3, or Myc-tagged Smad4 constructs. Cell extracts were subjected to GST pull-down assay using glutathione-Sepharose beads followed by immunoblotting with anti-FLAG or anti-Myc antibody. Expression of GST, GST-EID-2, and Smads was monitored as indicated. B, T7-EID-2 was transfected into HepG2 cells with the FLAG-tagged Smad2, Smad3, and Myc-tagged Smad4 constructs. Cells were treated with TGF-β1 for 2 h. Cell extracts were subjected to immunoprecipitation using anti-FLAG or anti-Myc antibody and Gamma-bind beads (Amersham Biosciences), followed by immunoblotting with anti-T7 antibody. The expression of EID-2 and Smads was monitored as indicated. C, interaction between endogenous EID-2 and Smads. Lysates of HepG2 cells untreated (−) or treated (+) with TGF-β for 1 h were subjected to EID-2 immunoprecipitation and immunoblotting with either a Smad2/3 antisera or a Smad4 antisera. D, schematic drawings of Smad3 truncation mutants are shown (upper panel). FLAG-tagged Smad3-deletion mutants were transfected into COS7 cells with GST-EID-2. Cell extracts were subjected to GST pull-down assay using glutathione-Sepharose beads followed by immunoblotting with anti-FLAG antibody.

interaction of EID-2 with Smads occurs in a ligand-dependent manner, we performed immunoprecipitation experiments in HepG2 cells with a T7-tagged EID-2 expression plasmid and FLAG/Myc-tagged Smad expression constructs in the presence or absence of TGF-β stimulation. The interaction between EID-2 and Smad2, Smad3, or Smad4 was not increased in the presence of TGF-β (Fig. 2B). To demonstrate the interaction between endogenous EID2 and Smad3, lysates of HepG2 cells untreated or treated with TGF-β1 for 1 h were subjected to EID-2 immunoprecipitation and immunoblotting with either a Smad2/3 antisera or a Smad4 antisera. Endogenous EID-2 associates with either Smad3 or Smad4 without ligand stimulation, and TGF-β treatment further increases the interaction (Fig. 2C).

To determine the domain of Smad3 responsible for interaction with EID-2 in vivo, we performed immunoprecipitation assays using various FLAG-tagged Smad3 expression constructs (Fig. 2D), along with a GST-tagged wild-type EID-2 construct. The C-terminal deletion mutants lacking the MH2 or
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L+MH2 domains of Smad3 were unable to bind to EID-2, whereas the N-terminal mutants in which MH1 or MH1+L were deleted interacted with EID-2 (Fig. 2D). The middle linker region is not likely to interact with EID-2 because MH1+L did not interact with EID-2. These results showed that the MH2 domain of Smad3 contained the EID-2 interaction domain.

Internal Portion of EID-2 Is Important for the Interaction with Smad3—We tried to determine which region of EID-2 is responsible for the suppression of TGF-β-induced transcriptional activity and the interaction with Smad3. We have generated various truncation mutants lacking either the N terminus or C terminus, as described previously (20). The full-length EID-2 suppressed TGF-β-induced transcriptional activity. Both N-terminal and C-terminal truncation mutants still retained the same suppressive property (Fig. 3A). The suppressive activity of these deletion mutants well correlated with their ability to interact with Smad3. As shown in Fig. 3B, the GST-tagged EID-2 mutant (1–100), which lacks the internal region and C terminus, did not interact with Smad3, whereas the N-terminal deletion mutant (101–236) was able to interact with Smad3. These results suggest that the internal portion of EID-2 is responsible for the interaction with Smad3 and may mediate its suppressive activity on TGF-β-induced transcriptional activity.

Down-regulation of Endogenous EID-2 Increases TGF-β Transcriptional Activity—We also performed the loss of function studies using EID-2-specific siRNA to evaluate the specific effect of EID-2 on TGF-β signaling. We tried to reduce endogenous EID-2 expression through RNA-mediated interference using EID-2-specific siRNA. Transfection of the EID-2 siRNA (~100–200 nM) resulted in a 70–90% decreases in protein levels (Fig. 4A). When transfected with EID-2-specific siRNA, transcriptional activity induced by TGF-β was increased (Fig. 4B).

Expression of EID-2 Represses TGF-β-mediated Transcriptional Activation—To investigate further the role of EID-2 in TGF-β signaling, we generated HepG2 and Mv1Lu cells stably expressing EID-2. We examined the TGF-β-induced transcriptional activities in HepG2 and Mv1Lu cells expressing EID-2. As shown in Fig. 5, expression of EID-2 markedly repressed the TGF-β-induced reporter activities (3TP-Lux and SBE4-Luc) in both stable cell lines (Fig. 6 and Fig. 7).

EID-2 Has Little Effect on TGF-β-induced Smad2 or Smad3 Phosphorylation but Inhibits Endogenous Smad3-Smad4 Complex Formation—The transcriptional activity of both Smad2 and Smad3 depends upon their phosphorylation by activated TβRI. Therefore, we examined whether EID-2 regulates TGF-β-stimulated Smad2/3 phosphorylation. EID-2 expression had little effect on TGF-β-stimulated endogenous Smad2/3 phosphorylation in Mv1Lu cells expressing EID-2 compared with the control cells (Fig. 6A), suggesting that EID-2 effects is positioned downstream of Smad2 and Smad3 phosphorylation. Because EID-2 interacts with receptor-activated Smad2, Smad3, and co-Smad, Smad4, we examined whether EID-2 might regulate the formation of Smad3-Smad4 and/or Smad2-Smad4 complexes. Mv1Lu cells stably expressing EID-2 and vector control cells were incubated in the presence or absence of TGF-β for 1 h, and whole-cell extracts were prepared. To investigate Smad2-Smad4 or Smad3-Smad4 complex formation, total-cell extracts were immunoprecipitated with anti-Smad2 or anti-Smad3 antibody, and then Smad2-bound Smad4 or Smad3-bound Smad4 were examined by Western blot analysis using anti-Smad4 antibody. As shown in Fig. 6B, the level of Smad3-bound Smad4 was markedly decreased in Mv1Lu cells expressing EID-2 compared with vector control cells. However, the association of Smad2 with Smad4 was not affected by the EID-2. This result suggests that the primary mechanism by which EID-2 suppresses Smad-dependent transcription is likely dependent on its ability to block TGF-β-induced Smad3-Smad4 complex formation.

Expression of EID-2 Inhibits TGF-β/Smad-mediated Transcriptional Responses—To investigate the functional significance of EID-2-Smad interaction, we examined whether EID-2 inhibits endogenous TGF-β-responsive genes. Vector control or EID-2-expressing HepG2 cells were analyzed for the expression of several TGF-β/Smad-responsive genes by Western blot analysis. Cells were treated with TGF-β for 20 h, and Western blot analysis of p21 and c-Myc was performed. TGF-β treatment markedly increased in the level of p21 protein in control HepG2 cells, whereas the induction of the p21 protein level by TGF-β was significantly inhibited in EID-2-expressing HepG2 cells (Fig. 7A). TGF-β-mediated suppression of c-Myc level was also slightly inhibited in EID-2-expressing HepG2 cells. We next examined the effect of EID-2 on the expression of cyclin inhibitory proteins (p15 and p27) and G1/Cdk (Cdk2 and Cdk4) regulated by TGF-β in Mv1Lu cells expressing EID-2. In control Mv1Lu cells, TGF-β-induced expression of p15 but did not affect the level of p27 expression. As shown in Fig. 7B, EID-2 greatly inhibited the TGF-β-mediated induction of p15 and had...
no effect on the expression of p27 in EID-2-expressing Mv1Lu cells. TGF-
β is known to inhibit expression of Cdk2 and Cdk4 in control Mv1Lu cells. However, EID-2 prevented TGF-β-mediated suppression of Cdk2 and Cdk4 expression. We also ex-
amined the expression of plasminogen activator inhibitor 1 (PAI-1) by Northern blot analysis. Endogenous PAI-1 gene expression was induced after treatment with TGF-
β in control Mv1Lu cells, but the TGF-β-inducible level of PAI-1 mRNA was
moderately repressed in the EID-2-expressing Mv1Lu cells (Fig. 7C). In most cell types, TGF-β exhibits an antiprolifera-
tive response, which is most characteristic in epithelial cells as their cell cycle is arrested in the early G1 phase (24). To exam-
ine the effect of EID-2 on the growth-inhibitory pathway down-
stream of TGF-β, we performed thymidine uptake assays in EID-2-expressing Mv1Lu cells and control cells. As shown in
Fig. 7D, TGF-β potently inhibited the proliferation of control
Mv1Lu cells, whereas EID-2-expressing cells became slightly
resistant to TGF-β growth inhibitory activity.

**DISCUSSION**

We have shown in this study that EID-2 inhibits TGF-β
transcriptional responses. EID-2 can interact strongly with
Smad3 but weakly with Smad2 and Smad4. EID-2 has no effect
on the level of TGF-β-induced Smad2 and Smad3 phosphor-
ylation. However, it inhibits the formation of TGF-β-dependent
Smad3-Smad4 complex, leading to a reduction in Smad3-de-
pendent transcription.

EID-1 and EID-2 comprise a novel family of inhibitors of
differentiation with distinct functions from those known nega-
tive regulators of differentiation. EID-1 inhibits myogenic dif-
ferration by blocking the HAT activity of p300 (18, 19).
EID-1 exhibits no homology to known proteins, including
bHLH factors and HDACs. Functionally, EID-1 exhibits similarity with adenovirus E1A or "twist" in terms of inhibition of HAT activity (18, 19). EID-2 was mainly expressed in heart, skeletal muscle, kidney, and liver. EID-2 inhibited MyoD-dependent transcription and blocked muscle differentiation in cultured cells like EID-1. However, EID-2 neither bound to p300 nor inhibited p300-dependent transcription, but associated with class I HDACs. EID-2 was localized exclusively in the nucleus and the existence of the nuclear localization signal in the N-terminal portion of EID-2 was postulated (20). Therefore, localization of EID-2 may explain why EID-2 did not block TGF-β-induced phosphorylation of Smad2 and Smad3, but blocked the complex formation of TGF-β-dependent Smad3-Smad4. EID-2 may interact with Smads even without TGF-β stimulation when transiently overexpressed, whereas endogenous EID-2 might only interact with Smad3 localized in the nucleus. Recently, a novel modulator of TGF-β signaling pathway, TRAP-1-like protein, has been shown to inhibit the formation of Smad3-4 complexes in the absence of effects on phosphorylation of Smad3, whereas it affects Smad2 phosphorylation (25). It was proposed that TRAP-1-like protein might regulate the balance of Smad2 and Smad3 signaling by localizing Smad4 intracellularly. Because EID-2 is only localized in the nucleus, EID-2 may act as a molecular switch to alter the balance of the Smad3-specific versus the Smad2-specific arm of TGF-β signaling in the nucleus.

EID-2 binds to the class I HDACs through its N terminus (20). Our mapping study of the Smad3 binding domain in EID-2 protein shows that the internal domain of the EID-2 is required in the interaction with Smad3 (as shown in Fig. 3B). The
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N-terminal deletion mutant (33–236), which does not bind to class I HDAC, still interacts with Smad3. Therefore, one of the mechanisms that underlie suppression of TGF-β/Smad3-dependent transcriptional responses by EID-2 could be the recruitment of an HDAC complex. Certain oncogene products and cellular proteins have been shown to functionally inactivate Smad3 through the recruitment of an HDAC complex. Chromosomal rearrangements resulting in the fusion products AML/Evi-1 (26) or AML/MDS/Evi-1 (27) are frequent in myeloid leukemia and myelodysplasia. Evi-1 represses Smad-induced transcriptional activation by recruiting C-terminal-binding protein as a corepressor. Because a specific HDAC inhibitor, trichostatin A, alleviates Evi-1-mediated repression of TGF-β signaling, it has been suggested that Evi-1 may recruit an HDAC complex through the CtB to the Smad complex. c-Ski is the cellular homologue of the v-ski oncogene product and has been shown to bind to Smad3 strongly and repress Smad transcription (PIASy, a member of the PIAS family) has been shown to interact with Smads and repress TGF-β signaling. Recently, protein inhibitor of activated signal transducer and activator of transcription (PIASy, a member of the PIAS family) has been suggested that Evi-1 may recruit an HDAC complex

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