Both Max and TFE3 Cooperate with Smad Proteins to Bind the Plasminogen Activator Inhibitor-1 Promoter, but They Have Opposite Effects on Transcriptional Activity*¶†§

Transforming growth factor (TGF-\(\beta\)) regulates gene expression in large part through combinatorial interactions between members of the Smad family and other transcription factors. The basic helix-loop-helix leucine zipper (bHLHZIP) protein TFE3 and Smad3 synergistically activate transcription of the plasminogen activator inhibitor-1 (PAI-1) as well as other genes. We investigated interactions among different bHLHZIP and Smad family proteins, TFE3, TFEB, and Max associated with Smad3 and Smad4 in the absence of DNA and at the PE2.1 element of the PAI-1 promoter. These interactions were mediated by the leucine zipper and MH1 regions of the respective proteins. No interactions were observed with the E47 bHLH family protein. Chimeric proteins, in which leucine zippers from bHLHZIP or bZIP proteins were fused to heterologous bHLH domains, associated with Smad3 proteins in the absence of DNA and at the PE2.1 element. The kinetics of bHLHZIP and Smad protein binding at the PE2.1 element were examined using surface plasmon resonance analysis. TFE3 exhibited cooperative DNA binding with Smad proteins, whereas no cooperativity was observed between E47 and Smads. Max inhibited transcription activation by Smad3 and TGF-\(\beta\) at the PAI-1 promoter, whereas TFE3 and TFEB stimulated transcription activation. These results suggest that Smad family proteins can interact with several bHLHZIP proteins, resulting in different transcriptional outcomes.

Members of the transforming growth factor \(\beta\) (TGF-\(\beta\)) superfamily participate in the regulation of cell proliferation and differentiation (1, 2). The long term responses to TGF-\(\beta\) are mediated at least in part by changes in gene transcription. Signals from the TGF-\(\beta\) receptor are transduced to the nucleus by a family of signal-dependent transcriptional activators called Smads (3–6). In response to ligand stimulation, Smad2 and Smad3 are phosphorylated by the TGF-\(\beta\) receptor complex. This phosphorylation causes the formation of activated hetero-oligomer complexes between receptor-activated Smads and the common mediator Smad4, and results in the translocation of Smads to the nucleus, where they modulate the transcription of many genes (7–10).

Smad family members Smad3 and Smad4 recognize a tetranucleotide (AGAC) sequence element, and therefore have only limited DNA binding selectivity (11–13). This Smad recognition element (SRE) is present in multiple copies in the promoters of many TGF-\(\beta\) responsive genes including the PAI-1 (11, 14), JunB (15, 16), c-Jun (17), type VII collagen (18), and \(\alpha2(I)\) procollagen (19) promoters. Deletion of the SRE selectively reduces TGF-\(\beta\) regulation of some promoters, but has little effect on the activities of other promoters (11, 15–17, 20). Smad3 and Smad4 have also been reported to bind GC-rich sequences (21). Moreover, sequences outside the SREs are likely to contribute to the specificity of transcription activation by TGF-\(\beta\) (3, 5, 6).

Because of the limited sequence specificities of Smads, their transcription regulatory functions are determined in large part through interactions with other transcription factors. Members of many different transcription factor families have been shown to interact with Smads including bZIP family members Fos, Jun (17, 22), and ATF2 (23), and bHLHZIP family members TFE3 (24–28) and Myc (29), as well as members of other transcription factor families including SPI1 (30), FAST (21), VDR (31), Ski (32), SnoN (33), and MyoD (34). These interactions target Smads to different regulatory regions, and thereby contribute to the differential effects of TGF-\(\beta\) on gene expression in different cell types (17–34).

The bHLHZIP family proteins TFE3 and Myc have both been implicated in TGF-\(\beta\)-dependent regulation of gene expression. TFE3 cooperates with Smad3 and Smad4 to activate transcription of the PAI-1 and SMAD7 genes in response to TGF-\(\beta\) stimulation (24, 25, 27, 28). In contrast, Myc inhibits TGF-\(\beta\) activation of the p15\(^{INK4a}\) and p21\(^{Cip1}\) genes (35–38). It is unclear whether the effects of Myc and TFE3 on TGF-\(\beta\) regulation of gene expression are related, and whether other bHLHZIP family members can interact with Smad family proteins.

Several different mechanisms have been implied for the antagonistic effects of Myc and TGF-\(\beta\) on p15\(^{INK4a}\) gene expression (29, 35, 36). Myc and Smads have been suggested to independently regulate the p15\(^{INK4a}\) promoter (35, 36). Myc can also interact directly with Smad2 and Smad3 in vitro and in yeast two-hybrid assays, and the direct interaction has been proposed to mediate Myc repression of p15\(^{INK4a}\) promoter activity (29). Different regions of Myc are involved in the direct and the indirect regulation of promoter activity, and appear to be required for the repression of p15\(^{INK4a}\) transcription under different experimental conditions (29, 36). It is therefore possible that multiple mechanisms mediate the antagonistic effects of Myc and TGF-\(\beta\) in different cell types and the presence of different extracellular stimuli.

Received for publication, November 18, 2002, and in revised form, January 7, 2003Published, JBC Papers in Press, January 27, 2003, DOI 10.1074/jbc.M211734200

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The abbreviations used are: TGF-\(\beta\), transforming growth factor-\(\beta\); bHLHZIP, basic helix-loop-helix leucine zipper; bZIP, basic leucine zipper; SRE, Smad recognition element; GST, glutathione S-transferase; PAI, plasminogen activator inhibitor-1.
The synergistic activation of PAI-1 transcription by TFE3 and Smads requires a promoter region (PE2.1) containing multiple SREs and an E-box recognition sequence for bHLHZIP proteins. The spacing between these elements is critical for synergistic transcription activation and the MH1 region of Smad3 can interact with TFE3 in vitro (24). The direct interaction between Smads and TFE3 has been proposed to mediate the synergistic activation of PAI-1 and Smad7 transcription by these proteins (24, 28).

Synergistic transcription activation by Smads and other transcription regulatory proteins can be mediated by several different mechanisms. Interactions with some transcription factors can recruit Smads to promoters without the requirement for specific Smad-DNA binding (30, 39). At other promoters, DNA binding by both proteins is required for maximal TGF-β responsiveness (21, 24, 28, 40). Finally, in some cases, no direct interactions between Smads and other transcription regulatory proteins appear to be necessary for synergistic activation. The synergy in some of these cases can be mediated by concerted interactions with co-activators or components of the transcriptional machinery (41).

The structural organization of complexes formed by Smad family proteins with other transcription factors has not been characterized in detail. The DNA sequence requirements and the effects of protein interactions on ternary complex formation at composite regulatory elements are largely unexplored. The role of cooperative DNA binding in synergistic transcription activation by Smads and other transcription regulatory proteins remains hypothetical in the majority of cases. We investigated Smad interactions with other transcription factors at the PE2.1 element of the PAI-1 promoter in an effort to better understand the mechanisms of transcriptional synergy. We sought to determine whether other bHLHZIP family proteins can interact with Smads, and whether interactions between different bHLHZIP and Smad family proteins resulted in cooperative DNA binding. We also wanted to identify the protein regions that mediated the interactions and the DNA sequences that were required for cooperative DNA binding. Finally, we wished to compare the functional consequences of Smad interactions with different bHLHZIP family proteins.

**EXPERIMENTAL PROCEDURES**

*Plasmid Constructs*—pGex-Smad3 and pGex-Smad4 as well as pCMV-Smad3 and pCMV-Smad4 were provided by Rick Derynck (University of California, San Francisco) (22). The sequences encoding Smad3/1–145/MH1, Smad3/221–445/MH2, Smad3/2–220/MH1L, Smad4/1–276/MH1, and Smad4/329–552/MH2 were amplified by PCR and inserted into the pGEX2T vector between BamHI and EcoRI sites. GST-TFE3 and GST-TFEB as well as their derivatives were generated by PCR amplification of the respective coding regions from plasmids pSV2a-TFE3 and pSV2a-TFEB provided by Kathryn Calame (Columbia University) (42). Plasmid pCS2-MC47 encoding mouse E47 was provided by David Turner (University of Michigan). The sequences encoding Max (43) or Max381–102 (MaxZIP) were inserted into pCMV-Flag2 (Sigma) to generate the pCMV-Max and pCMV-MaxZIP plasmids.

Genes encoding chimeric bHLHZIP proteins were constructed by PCR and inserted in pGEX2T or pDG56 vectors for bacterial expression. bTFE3 contained the basic, helix-loop-helix, leucine zipper domain of TFE3 (residues 219–304). bE47/TFE3 was truncated after the third leucine residue in the zipper. bTFE3289 contained the basic helix-loop-helix domain of TFE3 (residues 219–289) without the leucine zipper. bTFE3289 contained a substitution of the leucine 289 in the zipper region by a proline residue. Chimera bE47/TFE3 contained the basic region and the helix-loop-helix of TFE3 (residues 282–304). Chimera bTFE3/ZIP(ATF2) contained the basic region and the helix-loop-helix of TFE3 (residues 219–281) and the leucine zipper and COOH-terminal 96 residues of ATF2 (residues 380–505).

**Protein Purification**—Echerichia coli BL21 containing pGex-Smad3, pGex-Smad4, pGex-TFE3, pGex-TFEB, or mutant variants were grown in LB media supplemented with 50 μg/ml ampicillin at 30 °C. The protein expression was induced by addition of 0.3 mM isopropyl-1-thio-

β-D-galactopyranoside. After 5 h, the cells were harvested by centrifugation at 5,000 × g for 10 min. The cells were resuspended in phosphate-buffered saline, 0.1% Triton X-100 with protease inhibitors and leupeptin, incubated for 30 min on ice, and sonicated. The lysate was centrifuged at 15,000 × g for 20 min. The supernatant was incubated with 0.5 μl of glutathione-Sepharose beads per liter of culture for 30 min at room temperature. The beads were washed 3 times with phosphate-buffered saline and 2 times with phosphate-buffered saline + 0.1% Triton, and the fusion proteins were eluted with 20 μg glutathione-conjugated proteins containing Max, Max zinc finger mutants thereof, or chimeric proteins bE47/ZIP(TFE3) and bTFE3/ZIP(ATF2) were expressed in E. coli and purified in the presence of 6 μg guanidine HCl using nickel chelate chromatography as described (44). Protein concentrations were measured using the Bradford assay with bovine serum albumin as a standard. The purified proteins were analyzed by SDS-PAGE and detected using Coomassie Brilliant Blue (Supplemental Fig. 1). All protein preparations contained greater than 90% of the expressed proteins, although some preparations contained a protein with the mobility of GST lacking a fusion. The fraction of active molecules was measured by oligonucleotide titration. All preparations tested had between 70 and 100% active molecules. The identities of the proteins were confirmed by Western blotting using antibodies Smad3(1–445), Smad4(N-16) (Santa Cruz), and TFE3–15451A (Pharmingen).

**Electrophoretic Mobility Gel Shift Analysis**—For electrophoretic mobility shift analysis oligonucleotides were radioactively labeled with [γ-32P]ATP using T4 polynucleotide kinase, annealed with the complementary strand and the duplexes were purified using 5% PAGE. DNA binding assays were carried out in a buffer containing 20 mM HEPES, pH 7.7, 100 mM KCl, 5% glycerol, 1 mM EDTA, 5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin in the presence of 50 μg/ml poly(dI-dC). The complexes were resolved in 5% TBE gels at 250 mV for 2.5–3.5 h.

**In Vitro Protein Binding Assays** (GST Pull-downs)—In vitro protein binding assays were carried out to test the ability of bHLHZIP proteins to interact with Smads. Briefly, 50–60 μg of bead were washed three times with binding buffer (20 mM HEPES, 100 mM KCl, 5% glycerol, 1 mM EDTA, 5 mM dithiothreitol, 0.2% Nonidet P-40, 0.1% bovine serum albumin, pH 7.7). GST fusion proteins (5 μg) were incubated with beads in a 200-μl volume for 1 h at room temperature. The beads were collected by centrifugation, washed three times, and resuspended in 200 μl of binding buffer. Different bHLHZIP proteins and mutants (500 ng of the wild type and equimolar concentrations of the mutants) were added to the beads and incubated for 1 h at room temperature. The beads were collected by centrifugation (500 × g for 5 min in a microcentrifuge) and washed three times with binding buffer without bovine serum albumin as a standard. The purified proteins were analyzed by SDS-PAGE, transferred onto polyvinylidine difluoride membrane, and visualized using anti-His tag antibody (Amersham Biosciences).

**Transient Transfections**—For transient transfections the mammalian expression vectors pCMV-Smad3, pCMV-Max, pCMV-MaxZIP, pSV2a-TFE3S, and pSV2a-TFEB were used. HEF20 or HEF3B cells were transfected with the expression vectors, incubated together with a reporter construct controlled by the PAI-1 (–800/+24) promoter (14). After 24 h, the cells were transferred into medium with 0.5% fetal calf serum for 6 h and TGF-β was added. After 16–20 h, the cells were lysed and β-galactosidase and luciferase activities were measured using o-nitrophenyl-p-b-galactopyranoside hydrolysis and luciferase assays (Promega). The mean ± S.D. were determined from at least three transfections for each construct.

**Surface Plasmon Resonance Analysis**—Oligonucleotides containing the PE2.1 element from the PAI promoter and variants thereof were synthesized using standard phosphoramidite chemistry, and biotin was added on the 5′ end of one strand. Equimolar amounts of complementary oligonucleotides were annealed at 55°C and renatured in 50 mM HCl, 50 mM Tris-HCl (pH 8.0), and the duplexes were purified by PAGE in an 8% native gel. Binding kinetics was analyzed using a Biacore 3000 instrument (Biacore, Inc., Piscataway, NJ). 400 Resonance units of PE2.1 oligonucleotide was immobilized on a SM5 sensor chip. A flow cell without DNA was used as a blank reference control. HBS-EP, used as a Biacore conjugation buffer contained 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% polysorbate 20 (v/v). Buffer passed over the chip at 10 μl/min for 18 h resulted in a baseline drift of less than 5 resonance units/h, indicating that the immobilized DNA was stable over the course of the experiment.

D. Turner, personal communication.
To examine the kinetics of Smad and TFE3 binding, proteins were injected using the KINJECT procedure for 600 s at 8, 16, 32, 64, 128, 160, 256, and 512 nM concentrations in HBS-EP buffer at a flow rate 10 or 30 μl/min. No effect of the flow rate on the binding kinetics was observed. Any change in surface plasmon resonance signal of the sample cell compared with the reference cell was interpreted to reflect protein binding. Dissociation of the complex was monitored by passing HBS-EP buffer over the chip for 600 s. The sensor chip was regenerated by a 60-s injection of 0.03% SDS and 3.3 mM EDTA, which restored the original resonance signal of the surface. Duplicate injections (the variation between the replicates was less than 1%) of each ligand concentration were analyzed in random order, with buffer blanks injected periodically for double referencing. To obtain kinetic rate constants, corrected response data were fitted with Biacore™ 3.1 software. Apparent kinetic constants were calculated by analyzing binding data from the early parts of the association and dissociation phases. The fit was satisfactory to a simple bimolecular model (χ² < 5). Dissociation kinetics showed no dependence on concentration, whereas the association rate increased with the concentration of the protein. The dissociation rate constants reported here are the averages of kₒ values determined at the different concentrations.

RESULTS

The Sequences Required for Smad3 and Smad4 Binding to the PE2.1 Element Together with TFE3 Are Distinct—The PE2.1 element is the primary target for synergistic activation of the PAI-1 promoter by Smad3, Smad4, and TFE3 in response to TGF-β (24, 25). To investigate the interactions of Smad3 and Smad4 with TFE3 at the PE2.1 element, we examined binding of the purified proteins to oligonucleotides using an electrophoretic mobility shift assay (Fig. 1A). Homogeneous, bacterially expressed TFE3 produced two bands. Smad3 also produced two closely migrating bands. These may represent different conformations or oligomeric states of the proteins, or they may result from proteolysis during the DNA binding assay. Co-incubation of TFE3 and Smad3 with DNA produced an additional band with a lower electrophoretic mobility than complexes formed by Smad3 or TFE3 alone (Fig. 1A, upper panel, lane 3). Similarly, co-incubation of TFE3 and Smad4 with DNA produced an additional band with a lower electrophoretic mobility than complexes formed by Smad4 or TFE3 alone (Fig. 1A, lower panel, lane 3). These higher order complexes formed more efficiently than would have been the case if TFE3 and Smads bound independently to the oligonucleotide. The amount of higher order complexes formed was generally greater than the sum of the complexes formed by TFE3 and Smads independently. Independent binding by TFE3 and Smads shifted less than 10% of the oligonucleotide in the binding reaction, but TFE3 and Smads together favored binding to the same oligonucleotide rather than independent binding to separate oligonucleotides (Supplemental Fig. 2). Thus, both Smad3 and TFE3 as well as Smad4 and TFE3 facilitated binding by each other at the PE2.1 element in the electrophoretic mobility shift assay.

The PE2.1 element contains three SREs and an E-box (24) (Fig. 1A). We compared the DNA sequence requirements for Smad3 and Smad4 binding to this element in the presence and absence of TFE3 by incubating the proteins with mutated variants of the element (Fig. 1A). Smad3 binding and formation of the higher order complex with TFE3 was virtually eliminated by mutation of SRE-3, whereas mutation of SRE-1 or SRE-2 individually or in combination had little effect on Smad3 and TFE3 binding. In contrast, mutation of any one of the SREs (SRE-1, SRE-2, or SRE-3) did not abolish binding or higher order complex formation by Smad4 and TFE3. Simultaneous mutation of any two SREs (SRE-1 and -2, SRE-2 and -3, or SRE-1 and -3) eliminated Smad4 binding and formation of the Smad4-TFE3 complex. Thus, whereas formation of the Smad3-TFE3 complex specifically required SRE-3, formation of the Smad4-TFE3 complex required two SREs, without certain specificity, within this element.

The spacing between the SREs and the E-box influences the synergistic activation of PAI-1 and Smad7 transcription by Smad3 and TFE3 in response to TGF-β (24, 28). We examined the effect of the spacing between the SREs and the E-box on formation of the Smad3-TFE3 and Smad4-TFE3 ternary complexes at the PE2.1 element (Fig. 1B). Insertion of one or two base pairs between SRE-3 and the E-box reduced Smad3-TFE3 complex formation, and deletion of one base pair virtually eliminated formation of the ternary complex. These results correlate with the loss of TGF-β responsive transcription activation of a reporter gene fused to a PE2.1 element in which the distance between the SREs and the E-box was altered (24). In contrast, these mutations had no significant effect on formation of Smad4-TFE3 complexes (data not shown). The mutations also had no detectable effect on DNA binding by Smad3, Smad4, or TFE3 alone. Hence, the spacing between the SREs and the E-box was critical for TFE3 interactions with Smad3 but not for interactions with Smad4.

Smad3 and Smad4 Can Form a Quaternary Complex with TFE3 at the PE2.1 Element—Because the PE2.1 fragment of the PAI-1 promoter contains three potential Smad binding sites, we examined if Smad3 and Smad4 could simultaneously participate in a complex with TFE3 at this element. Incubation of TFE3 with a mixture of Smad3 and Smad4 resulted in formation of a complex with a mobility distinct from those of Smad3-TFE3 or Smad4-TFE3 (Fig. 1C). Anti-Smad4 antibody supershifted both the Smad4-TFE3 complex as well as the complex formed in the presence of Smad3, Smad4, and TFE3 (data not shown). Smad3 and Smad4 favored formation of the quaternary complex with TFE3 over formation of Smad3-TFE3 and Smad4-TFE3 ternary complexes (Supplemental Fig. 4). The quaternary complex was therefore preferred over the two ternary complexes, and the ternary complexes were preferred over the respective binary complexes. Incubation of TFE3 with higher concentrations of Smad3 or Smad4 in the absence of the second Smad did not produce additional bands. Insertion of one or two base pairs between SRE-3 and the E-box, or mutation of SRE-3 eliminated formation of the Smad3-Smad4-TFE3 complex (data not shown). Thus, Smad3, Smad4, and TFE3 formed a specific quaternary complex at the PE2.1 element of the PAI-1 promoter.

Complex Formation at the PE2.1 Element Is Mediated by the bHLHZIP Domain of TFE3 and the MH1 Domains of Smad3 and Smad4—To identify the regions of Smad3 and Smad4 involved in their interactions with TFE3 at the PE2.1 element, we compared DNA binding by truncated Smad3 and Smad4 proteins in the presence and absence of TFE3 (Fig. 2A). Fragments encompassing the MH1 regions of either Smad3 or Smad4 formed complexes with TFE3 at the PE2.1 element. Both of these fragments favored binding to the PE2.1 element together with TFE3 over binding to free DNA. These results suggest that the MH2 regions of Smad3 or Smad4 were not required for facilitated binding to the PE2.1 element in the presence of TFE3. The purified MH2 domains of Smad3 or Smad4 did not bind DNA, and did not alter the mobility of TFE3 complexes on DNA. Thus, the MH1 regions of Smad3 and Smad4 were sufficient for ternary complex formation with TFE3 at the PE2.1 element.

To identify the region within TFE3 that mediates the interaction with Smads, we performed electrophoretic mobility shift assays using a truncated protein containing only the bHLHZIP domain (bTFE3). bTFE3 formed complexes with Smad3MH1L at the PE2.1 element (Fig. 2B). bTFE3 also formed complexes with full-length Smad3 and Smad4 as well as Smad4MH1 (data not shown). Thus, the regions outside the bHLHZIP motif
were not required for TFE3 interactions with Smad3 or Smad4 at the PE2.1 element.

Other bHLHZIP Family Members Can Interact with Smad Proteins—The bHLHZIP motif is conserved among a large number of transcription regulatory proteins with diverse biological functions (45–47). To determine whether other bHLHZIP proteins can bind to the PE2.1 element together with Smads, we examined binding by the closely related TFE3 pro...
tein as well as the distantly related Max protein. Electrophoretic mobility shift assay experiments demonstrated that TFE3 (data not shown) as well as Max (Fig. 3A) can bind to the PE2.1 element with either full-length Smad3 or Smad4, or with the MH1L regions of Smad3 or Smad4. Similar to the complexes formed by Smads with TFE3, SRE-3 was required for complex formation by Smad3MH1L with Max or TFE3. Deletion or insertion of base pairs between the SREs and the E-box eliminated formation of the Smad3-Max and Smad3-TFEB complexes. We also tested the bHLH fragment of E47, which shares 45% sequence similarity with TFE3 in the basic and helix-loop-helix regions, but does not contain a leucine zipper. Incubation of Smad3MH1L (Fig. 3B) or Smad3 (data not shown) with bE47 did not produce any complexes at the PE2.1 element other than those produced by Smad3MH1L and bE47 alone. A slight inhibition of Smad3MH1L binding to DNA was observed in the presence of bE47. Interestingly, no effect of TGF-β-Smad signaling was observed on the transcriptional activity of E47 in C3H10T1/2 fibroblasts (34). Thus, TFE3, TFEB, and Max, but not bE47 can interact with Smad proteins at the PE2.1 element.

The Leucine Zipper Mediates Complex Formation with Smads at the PE2.1 Element—The difference between Smad interactions with TFE3, TFEB, and Max versus E47 suggested that TFE3, TFEB, and Max possess some common structural element absent in E47 that mediates their association with Smads. Whereas all of these proteins contain structurally related helix-loop-helix dimerization interfaces and basic DNA binding regions, E47 lacks a leucine zipper. To determine whether the bHLH region of TFE3 was sufficient for the interactions with Smads we tested a truncated bTFE3ΔZIP protein lacking the leucine zipper. bTFE3ΔZIP protein showed significantly reduced DNA binding affinity compared with bTFE3. bTFE3ΔZIP did not produce ternary complexes with Smad3MH1L (Fig. 4A). To confirm that the lack of ternary complex formation was not caused by the reduced binding by bTFE3ΔZIP, we used higher concentrations of bTFE3ΔZIP to produce binding equivalent to that observed by bTFE3. No ternary complex formation was observed under any condition examined. Thus, the bHLH motif of TFE3 was not sufficient for TFE3-Smad complex formation at the PE2.1 element.

To identify the part of the leucine zipper required for complex formation by TFE3 with Smads, we tested mutants of bTFE3 containing truncated (bTFE3219–297) or mutated (bTFE3L289P) leucine zippers. These bTFE3 mutants specifically recognized the PE2.1 element, and exhibited binding comparable with that observed by the bTFE3ΔZIP protein. Truncation of the leucine zipper after the third leucine (bTFE3219–297) reduced, but did not eliminate complex formation with Smad, suggesting that the carboxyl-terminal end of the leucine zipper was not required for the interactions. In contrast, substitution of the second leucine by a proline (bTFE3L289P) eliminated all detectable complex formation (Fig. 4A). Thus, the amino-terminal end of the TFE3 leucine zipper was critical for complex formation with Smad3.

To examine the ability of the leucine zipper of TFE3 to mediate bHLHZIP protein interactions with Smads in the context of a heterologous bHLH domain, we constructed a chimeric protein in which the bHLH region of E47 was fused to the leucine zipper of TFE3 (bE47/ZIP(TFE3)). bE47/ZIP(TFE3) produced a ternary complex with Smad3MH1L at the PE2.1 element (Fig. 4B). It is therefore likely that the leucine zipper of TFE3 interacts with Smads independent of other protein domains.

The bZIP proteins Jun and ATF2 cooperate with Smads in the regulation of transcription at several promoters (18). To determine whether the leucine zipper of a bZIP protein could also mediate interactions with Smads, we constructed a chimeric protein in which the bHLH region of TFE3 was fused to the leucine zipper of ATF2 (bTFE3ZIP(ATF2)). bTFE3/ZIP(ATF2) exhibited complex formation with Smad3MH1L at the PE2.1 element (Fig. 4B). These results show that a heterologous leucine zipper can substitute for the TFE3 zipper to mediate association with Smads. The leucine zipper may therefore represent a generic interface for interactions with Smads in both bHLHZIP and bZIP family proteins.

bHLHZIP Proteins and Smads Can Interact in Vitro in the Absence of DNA—To determine whether Smads and bHLHZIP proteins can interact in the absence of DNA, we examined the ability of immobilized GST-Smad fusion proteins to capture bHLHZIP and bHLH proteins from solution. TFE3 (24) and Max were retrieved by both GST-Smad3 as well as GST-
Smad4, whereas bE47 was not captured (Fig. 5, A–C). GST alone used as a control did not capture any of the bHLHZIP proteins (data not shown). The MH1 regions of Smad3 and Smad4 retrieved Max from solution with the same efficiency as the full-length proteins (Fig. 5D). In contrast no capture was observed when the MH2 domain of either Smad3 or Smad4 was used. Thus, the MH1 domains of Smads can interact directly with bHLHZIP proteins in the absence of DNA.

We examined the effect of the leucine zipper on the association between TFE3 and Smads (Fig. 5, A–C). The association in the absence of DNA was lost when the entire leucine zipper was deleted (bTFE3ZIP). Likewise, the single amino acid substitution in bTFE3L289P eliminated TFE3 association with Smad3 and Smad4. The chimeric bE47ZIP(TFE3) and bTFE3ZIP(ATF2) proteins were retrieved from solution by both Smad3 and Smad4. Thus, the leucine zipper can mediate interactions with Smad family proteins both at specific DNA regulatory elements as well as in the absence of DNA.

Surface Plasmon Resonance Analysis of the Dynamics of Smad-TFE3 Complexes at the PE2.1 Element—The electrophoretic mobility shift assay does not allow direct measurement of the kinetics of DNA binding by Smads and TFE3. We used surface plasmon resonance (48, 49) to investigate the dynamics of Smad-TFE3 complex formation and dissociation at the PE2.1 element. To examine the dynamics of Smad binding to DNA, a biotinylated oligonucleotide containing the PE2.1 element was immobilized on a streptavidin-coated sensor chip and different concentrations of Smad3 or Smad4 were applied to the flow chamber (Fig. 6). The rates of Smad3 and Smad4 binding and dissociation were determined based on time-dependent changes in the resonance signal, which reflect changes in the mass of molecules bound to the chip. No Smad binding was observed to a surface with mutated binding site Sm1,2,3Em (Fig. 6A, dashed line), or to a surface with the intact PE2.1 element in the presence of an excess of specific competitor oligonucleotide in the solution (data not shown). Thus, the mass change on the surface of the sensor chip was because of specific DNA binding by Smads.

We compared the dynamics of Smad3 and Smad4 interac-
Interactions between Smads and bHLHZIP Proteins

Fig. 5. bHLHZIP but not bHLH family proteins can associate with Smad3 and Smad4 in vitro. Effects of mutations in the leucine zipper on interactions between TFE3 and Smads in the absence of DNA. Max, bE47, TFE3 variants bTFE3, bTFE3LZIP, bTFE3LSP9 as well as fusion proteins bTFE3/ZIP(ATF2) or bE47/ZIP(TFE3) were incubated with GST-Smad3 (panel A) or GST-Smad4 (panel B) immobilized on glutathione-Sepharose beads as described under “Experimental Procedures.” Bound proteins were resolved by SDS-PAGE and visualized by immunoblotting. C, schematic diagrams of the TFE3 mutants and chimeras used in GST pull-down experiments and summary of the results. BR, basic region; LZ, leucine zipper. D, analysis of the region of Smads required for interactions with Max in the absence of DNA. The proteins indicated above the lanes were fused to GST and immobilized on glutathione-Sepharose beads. The beads were incubated with equal amounts of Max and the Max bound to the beads was analyzed by Western blotting as described under “Experimental Procedures.”

The observed kinetics of binding and dissociation for Smad4 and TFE3 together were compared with the sum of the kinetics of binding by Smad4 and TFE3 separately (Fig. 6D). The half-time for complex association was 1.8 times faster and the half-time for complex dissociation was 2.5 times slower than those predicted by independent binding of the proteins. Smad3 and TFE3 exhibited 2 times slower dissociation from DNA together than separately (data not shown), and with a comparable rate of complex formation. The increased stability of Smad-TFE3 complexes compared with complexes formed by the individual proteins was also observed when the rates of dissociation were measured using electrophoretic mobility shift analysis following the addition of competitor oligonucleotide to the complexes (data not shown). Thus, TFE3 binds cooperatively with both Smad3 and Smad4 to the PE2.1 element.

To examine the specificity of the effects of Smad and TFE3 on the kinetics of DNA binding by each other, we compared the kinetics of binding by bE47 and Smad4 separately with the binding kinetics of a mixture of bE47 and Smad4 at the PE2.1 oligonucleotide. bE47 exhibited fast association ($k_0 = 1.1 \pm 0.04 \times 10^5 \text{M}^{-1} \text{s}^{-1}$) and dissociation ($k_1 = 1.1 \pm 0.03 \times 10^{-2} \text{M}^{-1} \text{s}^{-1}$) at the PE2.1 element. The kinetics of binding by the mixture closely reflected the sum of the kinetics of binding of bE47 and Smad4 separately (Fig. 6E). No effects of bE47 and Smad4 were observed on the association or dissociation rates of each other at any ratio tested (1:2 to 4:1). Thus, the kinetics of TFE3 and Smad binding at the PE2.1 element reflected a specific interaction between the proteins.

Effects of Smad Interactions with bHLHZIP Proteins on Transcription—Smad and TFE3 can cooperatively activate the PAI-1 promoter (24, 25). To investigate if the interaction between Smads and other bHLHZIP family members affect the transcriptional activity of the PAI-1 promoter, we co-transfected expression vectors encoding different bHLHZIP family members with Smad3 into HEPG2 or HEP3B cells and examined the expression of a PAI-1 reporter gene in the presence and absence of TGF-$\beta$ (Fig. 7). The PAI-1 promoter was activated by TGF-$\beta$ treatment and Smad3 expression as shown previously (25). Expression of Max did not affect the basal activity of the PAI-1 promoter. However, co-expression of Max with Smad3 inhibited activation of the promoter by Smad3 in the presence and absence of TGF-$\beta$ (Fig. 7A). No inhibition was observed by MaxZIP, in which the leucine zipper was deleted.

The level of MaxZIP protein expression was indistinguishable from that of wild type Max as determined by Western blot analysis (data not shown). In contrast, co-expression of either TFEB (Fig. 7B) or TFE3 (Fig. 7C) stimulated transcription from the PAI-1 promoter alone and in combination with Smad3. The opposite effects of Max and TFE3 or TFEB on Smad3 activation of PAI-1 transcription were observed in both HEPG2 and HEP3B cells. Consequently, different bHLHZIP proteins have opposite effects on the activation of PAI-1 transcription by Smad3 and TGF-$\beta$.

**DISCUSSION**

Smad family transcription factors participate in a broad range of biological functions regulated by TGF-$\beta$ family cyto-
The functional interactions between Smads and bHLHZIP family transcription factors can be mediated by cooperative binding at composite regulatory elements within the promoter regions of target genes. We found that both Smad3 and Smad4 can interact with several other members of the bHLHZIP family of transcription factors. The bHLHZIP proteins participate in the control of cell growth and differentiation, and many members of this family are produced in specific cell types and in response to particular extracellular stimuli (43–45). Thus, interactions between Smad and bHLHZIP transcription factor families can contribute to context-dependent responses to TGF-β family cytokines (4).

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Smad binding to the PE2.1 element of the PAI-1 promoter by Smad3.

The data shown represent the averages and standard deviations from triplicate experiments.

The conserved MH1 and leucine zipper domains mediated cooperative DNA binding by Smads and TFE3 at the PE2.1 element. These same protein regions were able to interact with each other in the absence of DNA, suggesting that cooperative DNA binding was mediated by direct interactions between the proteins on DNA. These results are consistent with stabilization of the complex by simultaneous protein-protein and protein-DNA interactions at the PE2.1 element. Fusion proteins on DNA. These results are consistent with stabilization of the complex by simultaneous protein-protein and protein-DNA interactions at the PE2.1 element. Fusion of the leucine zipper of TFE3 associated with Smad3 and Smad4 in the absence of DNA, and formed complexes with Smads at the PE2.1 element. Hence, the interactions of Smads with bHLHZIP and bZIP family proteins may be mediated by a common structural interface. Interestingly, Smad3 was shown to physically interact with the bHLH domain of MyoD, a protein, which does not contain a leucine zipper (34). In the case of TFE3 the bHLH domain was not sufficient for interactions with Smads. However, we do not exclude a possibility that the bHLH domain of bHLHZIP proteins might serve as a secondary interaction interface for Smad proteins or contribute to the stability of the ternary complex.

The rate of Smad and TFE3 binding to the PE2.1 element was faster when the proteins were present together than when they were analyzed separately. This higher rate of binding is consistent with the interaction of Smads and TFE3 in the absence of DNA, and suggests that this interaction facilitates DNA binding by the proteins. The increased rate of association may be because of changes in the conformation of one or both interaction partners that facilitate DNA binding. The lower rate of dissociation of the complexes by Smads and TFE3 at the PE2.1 element relative to the rates of dissociation of complexes formed by the individual proteins indicates that Smads and TFE3 stabilize DNA binding by each other. The kinetic analysis corroborated the results of electrophoretic mobility shift analysis and quantitatively demonstrated that Smads and TFE3 bind cooperatively to the PE2.1 element. Additional cooperativity may be achieved by phosphorylated Smad3, which has an increased affinity for TFE3 (24).

TFE3 and Smad3 can synergistically activate the transcription of PAI-1, Smad7, and laminin γ1 chain genes (25, 26, 28, 60). The present results demonstrate that other bHLZHIP proteins, including TFE3 and Max, can also interact with Smads and influence their transcriptional activities. Because TFE3 and Max are distantly related within the bHLZZIP family, it is likely that Smads can interact with many, and perhaps all bHLZZIP family members. It was recently suggested that the laminin promoter can be regulated by Smad interactions with both TFE3 and USF1 (60). Smads have also been shown to interact with Myc, although the interaction interface identified in those studies is outside the conserved bHLZZIP region (29). The interactions between Smad and bHLZZIP family proteins, both at composite regulatory elements as well as in the absence of DNA, provide a flexible mechanism for the integration of signals from multiple pathways at shared regulatory targets.

The opposite effects of TFE3 and Max on Smad3 activation of the PAI-1 promoter show that interactions involving the same contact interface can both stimulate and inhibit transcriptional activity. This phenomenon might be because of the recruitment of a distinct set of co-regulatory proteins to the promoter. TFE3 can interact with the p300 co-activator (61), whereas complexes...
formed by Mad with Mad family proteins can interact with mSin3 and N-CoR co-repressors (62, 63). Thus, the antagonistic effects of different bHLHZIP proteins on Smad3 transcriptional activity provide a potential mechanism for modulation of responses to TGF-β signaling in different cell types and in the presence of other extracellular stimuli.

Acknowledgments—We are grateful to members of Kerppola laboratory for helpful discussions.

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