Smads are signal transducers for members of the transforming growth factor-β (TGF-β) superfamily. Upon ligand stimulation, receptor-regulated Smads (R-Smads) are phosphorylated by serine/threonine kinase receptors, form complexes with common-partner Smad, and translocate into the nucleus, where they regulate the transcription of target genes together with other transcription factors. Polymavirus enhancer binding protein 2/core binding factor (PEBP2/CBF) is a transcription factor complex composed of α and β subunits. The α subunits of PEBP2/CBF, which contain the highly conserved Runt domain, play essential roles in hematopoiesis and osteogenesis. Here we show that three mammalian subunits of PEBP2/CBF form complexes with R-Smads that act in TGF-β/activin pathways as well as those acting in bone morphogenetic protein (BMP) pathways. Among them, PEBP2α/CBF3A/AML2 forms a complex with Smad3 and stimulates transcription of the germline Ig Cα promoter in a cooperative manner, for which binding of both factors to their specific binding sites is essential. PEBP2 may thus be a nuclear target of TGF-β/BMP signaling.

Smad proteins are signal transducers for members of the transforming growth factor-β (TGF-β) superfamily, which includes TGF-βs, activins, and bone morphogenetic proteins (BMPs) (1, 2). Smads are classified into three subgroups, i.e. receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads), and inhibitory Smads. Smad2 and Smad3 are R-Smads that transmit TGF-β/activin signals, whereas Smad1, Smad5, and Smad8 act as R-Smads mediating BMP signals. Smad4 is the only Co-Smad identified in mammals. Upon ligand stimulation, R-Smads are phosphorylated by the serine/threonine kinase receptors, form complexes with Co-Smad, and translocate into the nucleus, where they cooperate in the transcription of target genes together with other transcription factors, including Xenopus FAST1 and its mammalian homologues (3–5) and also the c-Jun/c-Fos complex (6, 7). TGF-β is a potent growth inhibitor for most cell types, including hematopoietic cells and lymphocytes. In addition, TGF-β directs class switching to IgA in splenic B cells (8, 9). BMPs play important roles in early embryogenesis and in the induction of bone formation in vivo (10). It is thus important to identify and classify transcription factors that serve as nuclear targets of TGF-β/BMP signals and regulate these biological events.

Polymavirus enhancer binding protein 2/core binding factor (PEBP2/CBF) is a transcription factor complex composed of α and β subunits (11, 12). Three mammalian α subunits have been identified, termed PEBP2αA/CBF1A/AML3 (referred to as αA in this report), PEBP2αB/CBF2A/AML1 (αB), and PEBP2αC/CBF3A/AML2 (αC), whereas only a single β subunit (PEBP2β/CBFB) with several spliced variants is present in mammals. The α subunits of PEBP2, which contain the highly conserved Runt domain, are responsible for binding to DNA and transcription activity. In contrast, the β subunit does not bind to DNA by itself, but it enhances the DNA binding activity of the α subunits by interacting via the Runt domain. PEBP2/CBF plays critical roles in growth and differentiation of cells in certain specific tissues, i.e. αA in bone formation (13–15) and αB in definitive hematopoiesis (16, 17); αC appears to be important in class switching to IgA because of its ability to activate the germline Ig Cα promoter (18). Abnormalities of the PEBP2 genes are linked to human diseases. Mutations in one allele of the human PEBP2αA/CBF1A gene cause human cleidocranial dysplasia syndrome (19, 20), whereas PEBP2αB/AML1 gene is frequently disrupted by chromosomal translocations in several types of human leukemia (11, 12).

PEBP2 has been shown to interact with several transcription factors and co-activators and support context-dependent transcription of target genes (21–23). Because BMPs and αA play critical roles in bone formation, and TGF-β and αC in transcription of germline Ig α transcripts required for IgA class switching, we examined the functional cooperation between the PEBP2α subunits and Smads. Our findings suggest that PEBP2α subunits and Smad proteins cooperate to synergistically activate transcription in both the TGF-β and BMP signaling pathways.
all three mammalian α subunits of PEBP2 interact with R-Smad.

Cell Culture and cDNA Transfection—

COS7 cells were transfected with the indicated combinations of cDNAs encoding FLAG-tagged Smads, 6Myc-tagged Mad4, 6Myc-αA, and constitutively active forms of type I receptors (C.A. R-I). Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody followed by immunoblotting (Blot) using anti-Myc antibody. αA and Smad4 co-immunoprecipitated with R-Smads are indicated. Expression levels of 6Myc-Smad4, 6Myc-αA, and FLAG-R-Smads are shown. B, COS7 cells were transfected with the indicated combinations of FLAG-Smad1 or -Smad3, 6Myc-αA, -αB, or -αC, and constitutively active forms of type I receptors. Complex formation between PEBP2α subunits and Smads was detected by anti-FLAG immunoprecipitation followed by anti-Myc immunoblotting. Expression of 6Myc-PEBP2α subunits and FLAG-Smads is indicated.

pathways, thereby regulating the function of cells in specific tissues upon activation by TGF-β-like factors.

**Experimental Procedures**

**Plasmid Construction—**FLAG-pcDEF3 and 6Myc-pcDEF3 containing six tandem copies of the Myc-epitope tag were previously described (24, 25). The constructions of constitutively active forms of TGF-β type I receptor (TβRI(TD)) and BMP-type IB receptor (BMPR-IB(QD)), TβRII, wild-type (WT) Smads, and Smad3(ΔE) were reported (24–26). The constructions of αA, αB, αC, and β2 have been described elsewhere (27–29). Deletion constructs of αC were prepared by a polymerase chain reaction-based approach. For construction of the isolated Ig Cα/TFG-β-responsive element (TβRE) promoter reporter construct (TβRE) and its mutants, three tandemly repeated TβRE promoter (WT or mutant versions) of the Ig Cα promoter were fused to the heterologous c-Fos (30) and luciferase reporters. All of the polymerase chain reaction products were sequenced.

**Cell Culture and cDNA Transfection—**COS7 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics. A20.3 B lymphoma cells (18, 31) were cultured in RPMI 1640 with 10% fetal bovine serum, 50 μg 2-mercaptoethanol, 0.1 M nonessential amino acids, 1 mM sodium pyruvate, 2 M l-glutamine, and antibiotics. P19 murine embryonal carcinoma cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 supplemented with 10% fetal bovine serum and antibiotics (32, 33). For transient transfection, cells were transfected using FuGENE6 (Roche Molecular Biochemicals).

**Immunoprecipitation and Immunoblotting—**COS7 cells were transiently transfected with expression constructs for PEBP2α subunits, Smads and constitutively active forms of type I receptors. Cells were then washed, scraped, and solubilized (25). Immunoprecipitation and immunoblotting using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) were performed as described (25).

**Glutathione S-transferase (GST) Pull-down Assay—**A GST pull-down assay was performed as described previously (22). GST-fusion proteins containing the full-length Smad3 or the Mad homolog (MH1) or MH2 domain of Smad3 were expressed and purified as described (32). In vitro transcription and translation of C-terminal deletion constructs of αC were done using the TNT system (Promega) in the presence of [35S]methionine. GST-Smad3 (full-length), Smad3 (MH1), Smad3 (MH2), or GST bound to glutathione-Sepharose was mixed with αC proteins in 500 μl of Tris-buffered saline, pH 7.4, containing 0.5% Nonidet P-40 for 1 h and washed vigorously three times with 1 ml of the same buffer. After boiling in the SDS-sample buffer, they were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

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Y.-W. Zhang and Y. Ito, unpublished data.
We first tested complex formation between αC and R-Smads activated by BMPR-IB (18), complex formation between αC and Smad3 was studied in detail. The MH1 domain weakly interacts with Smad1, Smad2, and Smad3 (1.8 μg) in combinations as indicated.

**Results**

**Interaction of αC Subunits with Smad3s**—Because αC is predominantly induced by TGF-β in B lymphocytes and is critical for the induction of the promoter for germline Ig Cα transcripts upon TGF-β stimulation (18), complex formation between αC and Smad3 was studied in detail. The αC/Smad3 complex was observed in the presence and absence of TβR-I(TD) (Fig. 2A), and Smad4 interacted with Smad3 upon stimulation by TβR-I(TD). The mode of interaction between αC and Smad3 was studied by GST pull-down assays using deletion constructs of these proteins. When a series of C-terminally truncated constructs of αC was examined, deletion of a C-terminal region (αC (1–283); see Fig. 2B) corresponding to a part of the transcriptional activation domain (AD) identified in αB (27) resulted in a reduction of association with GSSmad3 and interaction became undetectable by deletion of transcriptional AD (αC (1–234)) (Fig. 2B). Smads have highly conserved MH1 and MH2 domains in their N- and C-terminal regions, respectively (1, 2). A GST pull-down assay revealed that the MH2 domain bound to αC (Fig. 2C). In addition, the MH1 domain weakly interacted with αC, but the exact location in αC where MH1 interacts could not be determined unequivocally because of the weakness of the interaction.

**Transcriptional Activation of the Germline Ig Cα Promoter**—We next studied the functional consequence of R-Smad/PEBP2α interaction using the mouse Ig Cα promoter. The promoter for mouse germline Ig Cα transcripts has been shown to contain a TGF-β-responsive element, TβRE (34), in which two PEBP2α binding sites have recently been identified (18).
The human germline Ig Ca promoter was also shown to contain PEBP2α binding sites in its TβRE (35). In addition, two potential Smad binding motifs (36–38) are found in the TβRE (Fig. 3A). Moreover, an additional PEBP2α binding site and one Smad binding motif are observed between the TβRE and the transcription initiation site. To determine the functional importance of these binding motifs, nucleotide mutations were introduced into the promoter, and a transcriptional response assay was performed using A20.3 B lymphocytes. As previously reported (18), TGF-β activates the promoter, which is further enhanced by the presence of αC. Mutations in the Smad binding motifs in the TβRE (TβRE-mS) and those in the PEBP2α binding sites (TβRE-mP) result in dramatic decreases in transcriptional activity (Fig. 3B). A complete loss of response was observed in the mSP mutant with mutations in all PEBP2α and Smad binding motifs, indicating that both of these binding motifs are essential for transcriptional activation.

A dominant negative form of Smad3, Smad3(ΔE), which prevents the activation of both Smad2 and Smad3 by TβR-I(TD) (26), inhibited the transcription induced by TβR-I(TD) and αC (Fig. 3C). This finding suggests that transcription may be induced by the endogenous R-Smads activated by TβR-I(TD). Moreover, co-transfection of Smad3 with αC strongly induced transcription from the Ig Ca promoter (Fig. 3D) but not from the Ig Ca promoter containing mutations in the TβRE, as shown in Fig. 3A (data not shown). Interestingly, Smad2 did not significantly induce the transcription, probably because Smad2 is unable to bind to the Smad binding motifs (36–39).

Transcriptional Activation through TβRE by αC and Smad3/4—To further study the roles of αC and Smad3/4 in activating transcription, three tandemly repeated TβREs (WT or mutant versions) of the Ig Ca promoter were fused to the heterologous c-Fos promoter, and transcriptional activity was determined using transfected P19 embryonal carcinoma cells, which have very low levels of endogenous PEBP2α activity (32, 33). Similar to the results obtained with the natural Ig Ca promoter using A20.3 B lymphocytes, transcriptional activity of (TβRE-WT)3-Lux was mildly induced by Smad3 and -4, whereas the addition of Smad3/4 and αC in cells activated by TβR-I(TD) greatly induced transcription (Fig. 4A). In contrast, mutant versions of (TβRE)p-Lux, i.e., (TβRE-mP)p-Lux and (TβRE-mS)p-Lux, which have mutations in the two PEBP2 binding sites and two Smad binding motifs, respectively, did not respond to TβR-I(TD), Smad3/4, or αC, indicating that both

FIG. 5. The presence of Smad3 and αC/β2 in the same complex bound to the Ig Ca-TβRE. A, lanes 1–12, COS7 cells were separately transfected with a mixture of expression plasmids encoding TβR-I(TD) (0.4 μg), TβR-II (0.2 μg), and FLAG-Smad3 (0.8 μg) or that containing 6Myc-αC (0.2 μg) and β2 (0.2 μg), or the cells were mock-transfected with an empty plasmid. Whole-cell extracts were mixed in vitro in combinations as indicated and used for EMSA with a 32P-labeled TβRE. The total amount of extract was kept constant using the mock extract. For lanes 13–15, a whole-cell extract obtained from cells co-transfected with all of the above expression plasmids was used. The positions of Smad3, αC/β2, and Smad3/αC/β2 complexes are indicated on the left. Complexes super-shifted (SS) by the addition of anti-FLAG (F), anti-β (β), or anti-Myc (M) antibody are indicated on the right. B, COS7 cells were transfected with expression plasmids encoding TβR-I(TD) (0.4 μg), TβR-II (0.2 μg), FLAG-Smad3 (0.8 μg), 6Myc-αC (0.2 μg), and β2 (0.2 μg) in the indicated combinations. Whole-cell extracts were subjected to EMSA using the 32P-labeled wild-type TβRE or 32P-labeled oligonucleotides in which mutations (M) were introduced as indicated. The mutations correspond to those in TβRE-mP and TβRE-mS in Fig. 3A.
of these binding motifs are essential for transcriptional activation by the Smad3/αC complex.

To determine the domain(s) in αC critical in the transcriptional activation in concert with Smads, a series of C-terminal deletions of αC was tested for transcription activity. αC mutants containing the transcriptional AD increased transcriptional activation in the presence of TβRI-T(TD) and Smad3/4; however, deletion of one-half of the AD resulted in a significant decrease in transcriptional response; complete loss of response was obtained with the mutants lacking the entire AD (Fig. 4B).

This result indicates that the physical interaction between Smad3 and αC may be critical for the transcriptional activation through TβRI (see Fig. 2B).

DNA Binding of the αC, β2, and Smad3 Complex—The formation of DNA-binding complexes containing αC and Smad3 on the germline Cα TβRI DNA was studied by EMSA. The β subunit of PEBP2 (β2 isoform) was included in this assay to enhance the DNA binding of PEBP2. Smad3 activated by TβRI-T(TD) and αC independently formed DNA-binding complexes, which could be detected as slowly migrating complexes in EMSA (Fig. 5A, lanes 2 and 3, and B, lanes 3 and 4). In the presence of activated Smad3 and αC/β2, a more slowly migrating complex was formed both in vitro and in vivo (Fig. 5A, lanes 4 and 13, and B, lane 5). These complexes were super-shifted in the presence of corresponding antibodies to the epitope tags or an antibody to the β subunit, indicating that αC/β2 and Smad3 can concomitantly bind to DNA as a multimeric Smad3/αC/β2 complex.

Mutations in the Smad binding motifs S1 or S2 resulted in the decrease or loss, respectively, of Smad3 and Smad3/αC/β2 bindings, but the binding of αC/β2 still remained (Fig. 5B). When the PEBP2α sites were mutated, a mutation in P2, but not in P1, disrupted the bindings of αC/β2 and Smad3/αC/β2, but binding of Smad3 was still detected. The Smad binding motifs and PEBP2α binding sites thus appear to be specific and sufficient for the binding of corresponding proteins, but both are required for the binding of the Smad3/αC/β2 complex to the TβRIE and for activation of the promoter by αC and Smad3.

DISCUSSION

The findings shown in the present study revealed that PEBP2α subunits and R-Smads specific for both TGF-β and BMP signaling pathways form complexes together with Smad4 and that the complex formation appears to be critical for efficient transcriptional activation of target genes, including the germline Ig Cα promoter. Our findings suggest that PEBP2 may function as a nuclear target of Smads tested in the present study. Recently, SIP1 has been shown to interact with all R-Smads; in contrast to the PEBP2 α subunits, however, SIP1 is a transcriptional repressor, and interaction with R-Smads may lead to relief of repression of target genes by SIP1 (44).

Smad3 interacts with αC mainly through the MH2 domain, whereas the MH1 domain binds weakly to αC. Analysis by C-terminal deletion of αC revealed that the C-terminal region, including the transcriptional AD of αC, is required for efficient interaction with the MH2 domain of Smad3.

PEBP2 is a context-dependent transcription factor, requiring interacting partners for transcriptional activation, including Etς-1 (21, 22). In the germline Ig Cα promoter, both PEBP2 and Smad binding sites are essential for transcriptional activation. In contrast, FAST1 binds to the Mix.2 gene promoter with high affinity, and therefore direct binding of Smads to DNA may be less important than in the Ig Cα promoter (39). Thus, in certain other promoters to which PEBP2 binds with a high affinity together with other transcription factors, direct DNA binding of Smads may not be critical for cooperative transcriptional activation by PEBP2 and Smads.

Our present study revealed that PEBP2α subunits interact with R-Smads activated by TGF-β/activin, as well as with those activated by BMPs, and that functional cooperation between αC and Smad3 is required for transcription driven by the germline Cα promoter. Germline Ig α transcripts are required for Igκ class switching (45). Because members of the TGF-β superfamily exhibit a wide variety of biological effects, it will be very important to examine whether PEBP2 is involved in these biological events as a nuclear target of Smads.

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