SMAD Proteins Transactivate the Human ApoCIII Promoter by Interacting Physically and Functionally with Hepatocyte Nuclear Factor 4*

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Cotransfection of HepG2 cells with SMADs established that SMAD3 and SMAD3-SMAD4 transactivated (15–70-fold) the −890/+24 apoCIII promoter and shorter promoter segments, whereas cotransfection with a dominant negative SMAD4 mutant repressed the apoCIII promoter activity by 50%, suggesting that SMAD proteins participate in apoCIII gene regulation. Transactivation required the presence of a hormone response element, despite the fact that SMADs could not bind directly to it. Cotransfection of SMAD3-SMAD4 along with hepatocyte nuclear factor-4 resulted in a strong synergistic transactivation of the −890/+24 apoCIII promoter, proximal promoter segments, or synthetic promoters containing either the apoCIII enhancer or the proximal apoCIII hormone response element. Inhibition of endogenous hepatocyte nuclear factor-4 synthesis by an antisense ribozyme construct reduced the constitutive activity of the apoCIII promoter in HepG2 cells to 10% and abolished the SMAD-mediated transactivation. Co-immunoprecipitation and GST pull-down assays provided evidence for physical interactions between SMAD3, SMAD4, and hepatic nuclear factor-4. Our findings indicate that transforming growth factor β and its signal transducer SMAD proteins can modulate gene transcription by novel mechanisms that involve their physical and functional interaction with hepatocyte nuclear factor-4, suggesting that SMAD proteins may play an important role in apolipoprotein gene expression and lipoprotein metabolism.

Apolipoprotein CIII (apoCIII) is a major component of very low density lipoprotein. ApoCIII has been implicated in modulating the binding of triglyceride-rich lipoproteins to cell receptors and subsequently their catabolism, thus affecting the development of hypertriglyceridemia (1–5). This concept is further supported by in vivo experiments showing that overexpression of the apoCIII gene in transgenic mice is associated with severe hypertriglyceridemia due to the defective clearance of triglyceride-rich lipoprotein remnants (6, 7).

ApoCIII gene expression is tissue-specific (8) and developmentally regulated (9). The human apoCIII gene has been mapped in the long arm of chromosome 11 within a gene cluster that includes the genes for apolipoproteins A-I and A-IV. Previous studies using DNase I footprinting, gel electrophoretic mobility shift assays, and mutagenesis analyses have shown that the apoCIII promoter contains four proximal (A to D) and six distal (E to J) regulatory elements (10). The distal elements F to J (located between nucleotides −592 and −792) comprise a general transcriptional enhancer that potentiates the strength of the proximal apoCIII as well as that of the closely linked apoA-I and apoAI-IV promoters (10–13). This region is also essential for intestinal apoA-I gene expression in vivo (13). Other elements important in apoCIII gene regulation are two hormone response elements (HREs), located in the proximal promoter and enhancer regions, as well as multiple Sp1 binding sites located in the apoCIII enhancer (11–13, 15).

We and others have shown previously that proinflammatory cytokines such as tumor necrosis factor-α and interleukin-1, which stimulate signal transduction pathways leading to the phosphorylation and activation of transcription factors such as NF-κB, c-Jun, activating transcription factor 2, and C/EBPβ, reduced apoCIII mRNA levels and promoter activity in HepG2 cells (16–19). In this report, we examined the effect of the anti-inflammatory cytokine TGF-β and its signal transducers SMAD proteins on the activity of the human apoCIII promoter. TGF-β is expressed constitutively in HepG2 cells in an autocrine fashion and affects hepatic gene expression by binding to type I and type II TGF-β receptors (20). Following TGF-β stimulation, the type II receptor phosphorylates the type I receptor and the latter binds to and phosphorylates the pathway-restricted SMAD2 and -3. The phosphorylated SMAD2 or -3 proteins subsequently bind to the common partner SMAD4, an event that triggers their translocation to the nucleus. There they affect the transcription of target genes that are involved in important biological processes such as cell growth, differentiation, development, apoptosis, and anti-inflammation (21). SMAD proteins have been shown to mediate the transcriptional activation of various TGF-β-responsive genes such as collagen (22), the tissue plasminogen activator inhibitor (23), the JunB protooncogene (24), and the p21/WAF1/Cip1 cell cycle inhibitor (25), among others. Activation may be achieved through direct interactions with DNA sequence elements pres-
ent on the promoters of TGF-β-responsive genes or through interactions with other transcription factors. In this case, SMAD proteins could serve as co-activators. In addition, SMAD3 has been shown to synergize with the universal co-activator CBP/p300, which has been implicated in the transcriptional regulation of an increasing number of genes (26–28). Thus, cross-talk between TGF-β and other signal transduction pathways may affect the transcription rate of a wide range of genes (21).

In the present study using in vitro and in vivo approaches, we show that SMAD3-SMAD4 complexes are transcriptional activators of the human apoCIII gene in HepG2 cells and exert their positive effect on the apoCIII promoter by physical and functional interactions with the orphan nuclear receptor HNF-4.

**EXPERIMENTAL PROCEDURES**

- **Materials**—Reagents were purchased from the following sources: Sequenase version 2 kit from Amersham Pharmacia Biotech/U.S. Biochemical Corp.; kit reagents for cell culture from Life Technologies, Inc.; luciferase assay kit from Promega Corp. (Madison, WI); recombinant human TGF-β1 from R&D Systems and Roche Molecular Biochemicals, respectively; all oligonucleotides from IMBB (Heraklion, Crete, Greece); the monoclonal anti-Myc antibody 9E10 from Sigma; polyclonal anti-HNF-4 antibodies from Santa Cruz Biotechnology, Inc.; monoclonal anti-FLAG antibody from Eastman Kodak Co.; anti-mouse horseradish peroxidase-conjugated secondary antibody from Chemicon. The anti-SMAD3 and -4 rabbit polyclonal antibodies were a generous gift of Dr. Peter ten Dijke (NCI, The Netherlands). Sources for other reagents have been described (12, 18, 25).

**Plasmid Constructions**—The apoCIII promoter plasmids, −890/+24 CAT, −164/+24 CAT, −99/+24 CAT, −55/+24 CAT, −790/−590 CIIm, −790/−590 CIHw, −590/−590 CIHm, −5CII AdmL CAT, −99/−590 CIIm AdML CAT, CIImB AdML CAT, 2XAdmL AdML CAT, and AdML CAT have been described previously (9–12). The plasmid pSMA-24 apoCIII promoter fragment was subcloned into the SmaI site of vector pGL3-Basic (Promega). The expression vectors encoding the FLAG-tagged human SMAD2, SMAD3, SMAD4, and SMAD4–(1–514) proteins were the generous gifts of Dr. Iannis Talianidis (Karolinska Institute, Sweden). The expression vector encoding the FLAG-tagged rat HNF-4 A-(227–455) and rat HNF4-A protein was the generous gift of Dr. Margarita Hadzopoulou-CASSALA, Sweden). The expression vector encoding the FLAG-tagged mouse SMAD2 and SMAD4 proteins; the GST, GST-SMAD3, GST-SMAD4 fusion plasmids; and the CA-ALK5 plasmid were the generous gift of Dr. Aristidis Moustakas (LICR, Upsala, Sweden). The expression vector encoding the FLAG-tagged rat HNF4-A protein was the generous gift of Dr Margarita Hadzopoulou-Cassalas (Aristotelian University of Thessaloniki, Greece) (29). The expression vector encoding the rat HNF4-A2/42 (42–455) and rat HNF4-A3/42 (42–455) proteins was the generous gift of Dr. Ivanis Talalidou (IMBB-FORTH, Heraklion). The design of the antisense ribozyme construct pCDA1–HNF4-RZ was described previously (30, 31). The expression vector encoding the 6X Myc-tagged human HNF4 protein (pCDNA1–6X-Myc-HNF4) was constructed by subcloning the full-length human HNF4 cDNA from pMT2-2-HNF4 (15) to the EcoRI site of pCDA1–6X-Myc vector. The latter was constructed by inserting a hexamerized c-Myc epitope tag into the BamHI/EcoRI sites of pCDA1-amp vector (In VivoGen Corp.).

**Cell Cultures, Transient Transfections, and CAT and Luciferase Assays**—Human hepatoma HepG2 cells and monkey kidney COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37 °C in a 5% CO2 atmosphere. Transient transfections were performed using the Ca3(PO4)2 co-precipitation method (32). Chloramphenicol acetyltransferase, β-galactosidase assays were performed as described previously (33, 34). The luciferase assay was performed using the luciferase assay kit from Promega according to the manufacturer’s instructions.

**Gel Electrophoresis Mobility Shift Assay**—Gel electrophoresis mobility shift assays were performed as described previously (35). Sense and antisense oligonucleotides were annealed to generate the double-stranded oligonucleotide probe and labeled with Klenow fragment of DNA polymerase I and [α-32P]dCTP as described (10). The sequence of the sense oligonucleotides used as probes is as follows: (a) ApoCIII (−92/−67), 5’-GGTT CAG CAG GTG ACC TTT GCC CAG CG-3’; (b) 2X CAGA sense, 5’-GCA GAC AGT CAG ACA GTC-3’. An expression of Proteins in COS-7 Cells and in E. coli—Expression of recombinant proteins in COS-7 cells and preparation of total cell extracts for gel electrophoretic mobility shift assays was performed as described previously (15). The GST and GST-SMAD4 fusion proteins were expressed in *Escherichia coli* strain DH-10B. Bacteria were grown to an OD600 of 0.8, diluted 1:25, and induced with 0.1 mM IPTG. The cultures were incubated with 250 μl isopropyl-β-D-thiogalactopyranoside for 4 h at 37 °C. Bacteria were then harvested, resuspended in 0.03 volume of phosphate-buffered saline (PBS), sonicated for 1 min in PBS on ice, lysed by the addition of Triton X-100 to a final concentration of 1%, and cleared by centrifugation at 10,000 rpm for 4°C for 10 min, resulting in a first supernatant that was enriched in GST fusion protein. The pellets were dissolved in solubilization buffer (1 mM EDTA, 25 mM triethanolamine, 1.5% N-laurylsarcosine) at 4°C for 30 min, with gentle agitation. Triton X-100 to a final concentration 2% and CaCl2 to a final concentration 1 mM were added, and the lysates were cleared by centrifugation at 10,000 rpm at 4°C for 10 min, resulting in a second supernatant also enriched in GST-fusion proteins. Both supernatants were used for the GST-protein-protein interaction experiments or the affinity purification. The solubilization of the expressed proteins was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining.

**GST Protein Interaction Assay**—Glutathione-Sepharose 4B beads were equilibrated in PBS and mixed with 1 volume of bacterially expressed and purified GST fusion proteins. The bound proteins were washed three times with 10 volumes of PBS and equilibrated in washing buffer (20 mM Hepes (pH 7.9), 100 mM KCl, 5 mM MgCl2, 0.2% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). Fifty microliters of 1.1 bead slurry in washing buffer was combined with 5–10 μl of COS-7 whole cell extracts in a final volume of 500 μl of washing buffer, 10% glycerol (interacting buffer) on a rotor shaker for 90 min at 4°C. The beads were then washed five times with 20 volumes of washing buffer, and the bound proteins were eluted by boiling in Laemmli SDS-PAGE loading buffer and subjected to SDS-PAGE. Protein bands were visualized by Western blotting.

**Co-immunoprecipitation/Western Blotting Assays**—For co-immunoprecipitation assays of Myc-tagged SMAD3 and SMAD4 and FLAG-tagged human SMAD2 and SMAD4 proteins, the GST, GST-SMAD3, GST-SMAD4 fusion plasmids were expressed in E. coli strain DH-10B, bacteria were prepared by lysing the cells in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin at 4°C. The cell extract was clarified into the insoluble material by a brief centrifugation at 10,000 rpm and was precleared by incubation with protein A-Sepharose at 4°C for 30 min. The precleared extract was incubated with the FLAG antibody at 4°C for 2 h in the presence of fragmented GST-SMAD3 and SMAD4 and the antibody was washed with lysis buffer four times, and dissolved in Laemmli SDS-PAGE loading buffer. Similarly, we prepared and analyzed extracts from HepG2 cells and the HepG2 anti-HNF-4 ribozyme cell line. After 7% SDS-PAGE, the resolved proteins were transferred to Hybond-C extra nitrocellulose (Amersham Pharmacia Biotech), and the relevant proteins were detected after incubation with the anti-Myc (9E10) antibody followed by anti-mouse horseradish peroxidase-conjugated secondary antibody and home-made enhanced chemiluminescent assay on x-ray film (Fuji).

**RT-PCR Analysis**—RT-PCR analysis was performed using the Superscript One Step RT-PCR system from Life Technologies, Inc. In each reaction, 1 μg of total RNA was incubated with 40 pmol of each 5’ and 3’ primer and 1 μl of Superscript II RT/Taq mix in a final volume of 50 μl. The reverse transcription reaction was performed at 50 °C for 30 min followed by a 2-min, 84 °C denaturation step. Following the RT step, PCRs were performed as follows: 1 min at 94 °C, 30 s at 60 °C, and 2 min at 72 °C for a total of 40 cycles followed by a 10-min final extension step at 72 °C. The same conditions were used for the PCR amplifications. The following primers were used in the PCRs and RT-PCRs: 5’ primer, 5’-AGA GAA TTC AGC ACT CGA AGG TGA AGC TAC TAT 3’; 3’ primer, 5’-CCT GAT GGC TCG ACC AGG AAA 3’. The 3’ primer is complementary to the catalytic domain of the hammerhead ribozymes.

**RESULTS**

To investigate the potential role of the TGF-β signal transducers, the SMAD proteins, on the transcriptional regulation of the apoCIII gene in hepatocytes, HepG2 cells were transiently co-transfected with a reporter construct containing the human −890/+24 apoCIII promoter fused with the CAT reporter gene along with various wild type or mutant SMAD protein forms (Fig. 1E) in the presence and absence of 400 pm TGF-β. As
Transactivation of Human ApoCIII Promoter by SMAD Proteins

Fig. 1. A–D, cotransfection experiments showing transactivation of the human apoCIII promoter by SMAD2, SMAD3, SMAD4 proteins, and a dominant negative SMAD4 protein in HepG2 cells. A shows that a dominant negative form of SMAD4 protein represses the -890/+24 apoCIII promoter activity in the presence or absence of TGF-β. HepG2 cells were co-transfected with the human -890/+24 apoCIII promoter-CAT construct (2 μg) in the absence (-) or presence (4 μg) of an expression vector for a dominant negative SMAD4 form (SMAD4-(1–514)) and the CMV-β galactosidase plasmid (1 μg). Experiments were performed in the presence and absence of 400 pM TGF-β. CAT activity was determined as described under “Experimental Procedures.” The normalized, relative CAT activity (mean ± S.E.) of at least two independent experiments performed in duplicate are shown in the form of a bar graph. B, transactivation of the human -890/+24 apoCIII promoter by SMAD proteins. HepG2 cells were co-transfected with the human -890/+24 apoCIII promoter-CAT construct (2 μg) along with expression vectors for the indicated human SMAD proteins (1 μg) and the CMV-β galactosidase plasmid (1 μg) in the presence and absence of 400 pM TGF-β. CAT activity was determined as described under “Experimental Procedures.” The normalized, relative CAT activity (mean ± S.E.) of at least two independent experiments performed in duplicate are shown in the form of a bar graph. C, transactivation of the TGF-β-responsive synthetic promoter containing 12× CAGA motifs in front of the minimal AdML promoter by SMAD3-SMAD4 in the presence and absence of TGF-β. HepG2 cells were co-transfected with the 12× CAGA luciferase construct (2 μg), the CMV-β galactosidase plasmid (1 μg), along with an expression vector for the human SMAD3 and SMAD4 proteins (1 μg) in the presence and absence of 400 pM TGF-β. Luciferase activity was determined as described under “Experimental Procedures.” The normalized, relative luciferase activity (mean ± S.E.) of at least two independent experiments performed in duplicate are shown in the form of a bar graph. D shows that SMAD3 and SMAD4 proteins are constitutively expressed in HepG2 cells. Total cell extracts from HepG2 (5, 10, and 20 μg) or COS-1 cells (5 μg) were analyzed by SDS-PAGE followed by Western blotting using polyclonal antibodies specific for human SMAD3 and SMAD4 proteins. The arrows indicate the position of the SMAD proteins. The position and the size (M) of the molecular mass protein markers are shown on the left. E, a schematic representation of the human SMAD proteins utilized in the transactivation experiments presented in A–C. MH1 and MH2 are the two SMAD homology domains shared by all SMAD family members.

shown in Fig. 1A, the -890/+24 apoCIII promoter activity was reduced by 50% in the presence of the dominant negative SMAD4-(1–514) mutant in HepG2 cells, whereas wild-type SMAD4 caused only a small (1.2-fold) increase. These findings indicate that TGF-β and the endogenous SMAD proteins contribute to the constitutive apoCIII promoter activity in hepatic cells. Western blotting analysis of HepG2 total cell extracts using antibodies specific for SMAD3 and SMAD4 showed that SMAD3 and SMAD4 proteins are expressed constitutively in HepG2 cells (Fig. 1D).

To investigate the ability of different SMAD proteins to transactivate the apoCIII promoter, we performed transient transfection experiments in HepG2 cells using the -890/+24 apoCIII promoter CAT construct along with expression vectors for human SMAD2, -3, and -4 proteins (Fig. 1E) independently or in combinations in the presence and absence of 400 pM TGF-β. As shown in Fig. 1B, among the various SMAD proteins tested, SMAD3 showed the strongest transactivation potential (14-fold when transfected alone and 26-fold when co-transfected along with SMAD4). SMAD2, SMAD4, and SMAD2–SMAD4 had a minor effect. A SMAD3 mutant lacking the transactivation domain MH2 and part of the linker region (human SMAD3-(1–220), Fig. 1E) was unable to transactivate the apoCIII promoter even when co-transfected with SMAD4 (Fig. 1B). The addition of 400 pM TGF-β in the culture medium for 24 h did not affect significantly the TGF-β-mediated transactivation of the apoCIII promoter. Furthermore, the combination of TGF-β and SMAD3-SMAD4 did not affect the transactivation of a synthetic TGF-β-responsive promoter containing 12 CAGA sequences in front of a minimal AdML promoter (Fig. 1C). The SMAD3-SMAD4 proteins that had the strongest transactivation potential on the apoCIII promoter in HepG2 cells were subsequently used in all experiments.

The Proximal and Distal HREs Present on the ApoCIII Promoter/Enhancer May Contribute Independently to the TGF-β-Mediated Transactivation—The observation that SMAD3–SMAD4 could act as transcriptional activators of the apoCIII promoter (Fig. 1B) prompted us to identify SMAD-responsive elements in this promoter. For this purpose, a series of apoCIII promoter deletion fragments fused with the CAT reporter gene (Fig. 2A) were transiently co-transfected into HepG2 cells along with expression vectors for SMAD3 and SMAD4 proteins. This analysis showed that a deletion of the apoCIII promoter extending to nucleotide -163 resulted in a reduction in both the constitutive and SMAD3-SMAD4-inducible apoCIII promoter activity. The -fold transactivation of the apoCIII promoter by SMADs, however, was similar to that of the wild type (-890/+24) apoCIII promoter (20–versus 26-fold). This finding indicates that the proximal -163/+24 apoCIII promoter contains SMAD-responsive element(s). A stronger transactivation by SMAD3-SMAD4 (50-fold) was achieved when the apoCIII promoter was further deleted to nucleotide -59. In contrast, an apoCIII promoter fragment extending to nucleotide -55 was transactivated by SMAD3-SMAD4 proteins only 5-fold, suggesting that a putative SMAD3-SMAD4-responsive element is localized within the -99–55 apoCIII promoter region. This region contains a previously identified and characterized HRE (element CIIB between nucleotides -92 and -67) that serves as the binding site for various orphan and ligand-dependent nuclear receptors such as HNF-4, ARP-1, EAR-2, homodimers of RXRα, and heterodimers of RXRs with RARα, T3Rβ, and human peroxisome proliferator-activated receptor α among others (11, 12, 15, 37).

To test the role of the apoCIII enhancer (-790–590) in SMAD3-SMAD4-mediated transactivation of the apoCIII promoter, a synthetic reporter construct consisting of the apoCIII
enhancer fused with the heterologous AdML minimal promoter (−44/+1) (Fig. 2A) was transiently transfected into HepG2 cells along with expression vectors for SMAD3 and SMAD4. As shown in Fig. 2B, SMAD3-SMAD4 transactivated the apoCIII enhancer/AdML CAT construct 5-fold, whereas they caused a small 1.8-fold transactivation on the AdML-CAT construct that was used as a control. It was shown previously that the strong positive effect of the apoCIII enhancer in HepG2 cells on the activity of the homologous apoCIII promoter or the heterologous apoA-I promoter is diminished by point mutations at an HRE (element 14) localized between nucleotides −736 and −714 of the apoCIII enhancer (12, 37). Mutations in the HRE did not affect the activity of the apoCIII enhancer/minimal AdML promoter construct, but they diminished its transactivation by SMAD3-SMAD4 (1.8-fold for the mutant versus 5-fold for the wild type enhancer minimal AdML promoter construct).

The overall findings of Fig. 2, A and B, indicate that the proximal and distal HREs present on the apoCIII enhancer/SMAD3-SMAD4-mediated transactivation of the apoCIII promoter/enhancer region.

**SMAD Proteins Do Not Bind to the ApoCIII HREs**—The observation that HREs play an essential role for the SMAD3-SMAD4-mediated transactivation of the apoCIII promoter/enhancer suggested that SMAD proteins could either bind directly to the apoCIII HREs or cooperate functionally with nuclear hormone receptors bound to these sites.

To test whether SMAD proteins can bind directly to the proximal apoCIII HRE, a gel electrophoretic mobility shift assay was performed using a synthetic double-stranded oligonucleotide containing the proximal apoCIII promoter region −92 to −67 as a probe (CIII (−92/−67) (Fig. 3A) and either HNF-4 expressed in COS-7 cells or a GST-SMAD3 fusion protein expressed in bacteria and purified by affinity chromatography. As shown in Fig. 3B (lane 2), HNF-4 bound efficiently to this oligonucleotide in accordance with our previous findings (15), whereas no binding by GST-SMAD3 to this probe was observed (Fig. 3B, lane 4). To ensure that the bacterially expressed GST-SMAD3 protein retained DNA binding capacity, a synthetic oligonucleotide containing a dimerized SMAD binding element (2 × CAGA) (Fig. 3A) shown previously to bind SMAD3 proteins (24) was used as probe in DNA binding. As shown in Fig. 3B (lane 3), GST-SMAD3 bound efficiently to the dimerized CAGA probe, whereas HNF-4 did not bind to this probe (Fig. 3B, lane 1). These findings establish that SMAD3 cannot bind to the proximal apoCIII HRE, and thus, DNA binding cannot explain the ability of SMAD3 protein to transactivate the −99/+24 apoCIII promoter.

**Synergistic Transactivation of the ApoCIII Promoter by HNF-4 and SMAD Proteins**—To investigate the possibility that SMAD3-SMAD4 proteins transactivate the apoCIII promoter.

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**Fig. 2.** A and B, cotransfection experiments showing transactivation of different apoCIII promoter or enhancer segments containing HREs by SMAD3-4. A, a schematic representation of apoCIII promoter-CAT reporter plasmids used in transactivation experiments. The position of the HREs present on the distal enhancer and proximal apoCIII promoter is indicated. AdML indicates the minimal adenovirus major late promoter (−44/+1) used for the construction of chimeric promoters. B, transactivation of different apoCIII promoter fragments by SMAD3 and SMAD4 proteins. HepG2 cells were co-transfected with the indicated apoCIII promoter-CAT constructs (2 μg) either alone or in combination with expression vectors for human SMAD3 and SMAD4 proteins (1 μg) and the CMV-β galactosidase plasmid (1 μg). CAT activity was determined as described under “Experimental Procedures.” The normalized, relative CAT activity (mean ± S.E.) of at least two independent experiments performed in duplicate is shown in the form of a bar graph.

**Fig. 3.** A and B, gel electrophoretic mobility shift assays showing that SMAD3 cannot bind to the proximal apoCIII HRE. B, shows gel electrophoretic mobility shift assays using HNF-4 expressed in COS-7 cells and GST-SMAD3 fusion protein expressed in bacteria using the oligonucleotides CIIIB (−92/−67) or 2 × CAGA shown in A as probes. The probes indicated at the top of the autoradiogram were labeled with [γ-32P]dCTP using the Klenow fragment of DNA polymerase I and incubated with total cell extracts from COS-7 cells transfected with human HNF-4 (1 μg) or affinity-purified GST-SMAD3 protein as described under “Experimental Procedures.” A, shows the sequence of the oligonucleotides used as probes in the gel electrophoretic mobility shift assay shown in B. The arrows above the apoCIII/−92/−67 promoter sequence indicate the location and orientation of the two half-repeats of the HRE present in this region. The arrows above the 2 × CAGA sequence indicate the location and orientation of the two CAGA elements present in this synthetic oligonucleotide. The position of the protein-DNA complexes is shown with arrows.
Fig. 4. A–D, cotransfection and control experiments showing synergistic interactions between HNF-4 and SMAD proteins bound on the apoCIII HRE −92/−63. A, HepG2 cells were co-transfected with the apoCIII promoter-CAT constructs indicated below the bar graphs (2 µg) either alone or in combination with expression vectors for human HNF-4, SMAD3, and SMAD4 proteins (1 µg) and the CMV-β galactosidase plasmid (1 µg). CAT activity was determined as described under “Experimental Procedures.” The normalized, relative CAT activity (mean ± S.E.) of at least two independent experiments performed in duplicate is shown in the form of a bar graph. The apoCIII promoters used are −99/−24 apoCIII, −55/−24 apoCIII, and −92/−63 apoCIII AdML. B, HepG2 cells were co-transfected with the wild type or mutated apoCIII enhancer AdML constructs indicated below the bar graphs (2 µg) either alone or in combination with expression vectors for human HNF-4, SMAD3, and SMAD4 proteins (1 µg) and the CMV-β galactosidase plasmid (1 µg). CAT activity was determined as described under “Experimental Procedures.” The normalized, relative CAT activity (mean ± S.E.) of at least two independent experiments performed in duplicate is shown in the form of a bar graph. The apoCIII enhancer constructs used are −790/−590 AdML, −790/−590 I4Mut (AdML). C, HepG2 cells were co-transfected with the 2× AID AdML promoter-CAT construct (2 µg) either alone or in combination with expression vectors for human HNF-4, SMAD3, and SMAD4 proteins (1 µg) and the CMV-β galactosidase plasmid (1 µg). CAT activity was determined as described under “Experimental Procedures.” The normalized, relative CAT activity (mean ± S.E.) of at least two independent experiments performed in duplicate are shown in the form of a bar graph. D, Western blotting (WB) analysis of HNF-4 and SMAD3 proteins expressed in COS-7 cells. COS-7 cells were co-transfected with the indicated expression vectors. 48 h following transfection, cells were lysed, and total cell extracts were analyzed by SDS-PAGE and Western blotting using the indicated antibodies as shown in the figure and described under “Experimental Procedures.”
Transactivation by SMAD3-SMAD4 alone transactivated the -99/+24 apoCIII promoter 2.5- and 7.5-fold, respectively, for a total of 10-fold additive transactivation. However, the combination of HNF-4 and SMAD3-SMAD4 transactivated the promoter 69-fold. The observed 69-fold transactivation is not additive but synergistic. Deletion of the apoCIII HRE in the -55/+24 apoCIII CAT construct abolished the synergistic transactivation of this promoter by the combination of HNF-4 and SMAD3-SMAD4 proteins. When a single copy of the proximal apoCIII HRE (CIIB, -92/-63) fused to a heterologous AdML promoter was used, we also observed comparable (28-fold) synergistic transactivation of this heterologous promoter by the combination of SMADs and HNF-4 (Fig. 4A). A 2-fold synergistic transactivation was observed when HFN-4 and SMAD3-SMAD4 were co-transfected into HepG2 cells along with a reporter construct containing the -790/-590 apoCIII enhancer in front of the minimal AdML promoter (Fig. 4B). This synergistic transactivation was abolished by a mutation that prevents the binding of HNF-4 to its cognate HRE on the element I4 of the apoCIII enhancer (Fig. 4B). Finally, a strong (50-fold) synergistic transactivation was observed when HNF-4 and SMAD3-SMAD4 were co-transfected into HepG2 cells along with a reporter construct containing two copies of the HRE on element AID of the apoA-I promoter in front of the minimal AdML promoter (Fig. 4C). The findings indicate that functional HNF-4 SMAD interactions can occur between SMAD3-SMAD4 proteins and HNF-4 bound to the proximal promoter as well as to the apoCIII enhancer HREs. These interactions may not be restricted to the apoCIII promoter but may apply to other promoters that contain HNF-4 binding sites. To exclude the possibility that the SMAD-mediated synergistic transactivation of the apoCIII promoter is due to a positive effect of SMAD3-SMAD4 proteins on the CMV promoter that controls the expression of transfected HNF-4 and SMAD3-SMAD4 proteins, total cell extracts from transfected cells were analyzed for HNF-4 and SMAD3-SMAD4 protein expression by SDS-PAGE and Western blotting. As shown in Fig. 4D, the expression of transfected HNF-4 was not increased by co-expressed SMAD3 protein even under conditions of SMAD activation by a constitutively active type I TGF-β receptor (CA-ALK5 lane) but rather decreased to approxi-
mately 50%. This finding confirmed that the observed synergism between SMADs and HNF-4 is due to a functional cooperation among these factors in HepG2 nuclei.

The overall findings of Fig. 4 support the hypothesis that SMAD3-SMAD4 proteins transactivate the apoCIII promoter via synergistic interactions with HNF-4 and possibly other orphan or ligand-dependent nuclear receptors bound to the proximal as well as to the distal HRE.

Antisense Inhibition Experiments Indicate That HNF-4 Levels May Modulate the SMAD-mediated Transactivation of the ApoCIII Promoter in HepG2 Cells—In a second experimental approach, the transactivation potential of SMAD3-SMAD4 proteins on the −890/+24 apoCIII promoter was tested under conditions of reduction of the endogenous HNF-4 in HepG2 cells. For this purpose, a permanent HepG2 cell line was generated that expresses antisense HNF-4 sequences corresponding to amino acids 129–394 of the HNF-4 protein fused with the catalytic domain of hammerhead ribozymes (Fig. 5B). Quantitative RT-PCR showed expression of the antisense HNF-4 RNA (Fig. 5C). Western blotting showed that in the cell line expressing the antisense ribozyme, the expression of HNF-4 was greatly reduced (Fig. 5D). This HepG2 cell line was transiently co-transfected with the −890/+24 apoCIII promoter CAT construct along with expression vectors for HNF-4, SMAD3-SMAD4, or the two in combination. In each case, the activity of the −890/+24 apoCIII promoter was measured and compared with the constitutive activity of this promoter in normal HepG2 cells. In the HepG2 cell line expressing the anti-HNF-4 ribozyme construct, the −890/+24 apoCIII promoter activity was reduced to 10% of the control as compared with the parent HepG2 cell line. The apoCIII promoter was transactivated by either HNF-4 or SMAD3-SMAD4 to a minor extent (3–4-fold) compared with the transactivation of this promoter by the same factors in normal HepG2 cells (8-fold by HNF-4 (11) and 26-fold by SMAD3-SMAD4) (Figs. 1 and 2). However, co-expression of both HNF-4 and SMAD3-SMAD4 in the anti-HNF-4 Rz HepG2 cells resulted in a 39-fold transactivation. The findings suggest that in the anti-HNF-4 Rz HepG2 cells, HNF-4 is rate-limiting for the activity of the apoCIII promoter. The promoter activity can be restored by increasing the concentration of HNF-4 and SMAD3 in the cell nucleus. Overall, the findings of Figs. 4, A–D, and 5, A–D, indicate that the activity of the apoCIII promoter in HepG2 cells can be modulated by the relative abundance of HNF-4 and SMAD3-SMAD4.

Physical Interactions between SMADs and HNF-4—The functional cooperation between HNF-4 and SMAD3-SMAD4 proteins on the human apoCIII HRE suggested potential physical interactions among these factors. Physical interactions between SMADs and HNF-4 were examined by using co-immunoprecipitation assays. First, COS-7 cells were co-transfected with expression vectors for Myc-tagged SMAD3 and SMAD4 and FLAG-tagged HNF-4 protein. In each transfection, an expression vector for a constitutively active TGF-β type I receptor (CA-ALK5) was included (38) to allow activation of SMADs in COS-7 cells that do not respond to TGF-β. Co-transfections in the absence of CA-ALK5 were also performed. Expression of different proteins was monitored by Western blotting of total COS-7 cell extracts using anti-Myc (Fig. 6A, lanes 1–3) or anti-FLAG (Fig. 6A, lanes 4–6) monoclonal antibodies. To monitor the efficiency of the interaction assay, a co-transfection experiment was performed using Myc-tagged SMAD3 and FLAG-tagged SMAD4 proteins. As expected, efficient interaction between SMAD3 and SMAD4 proteins was observed (lane 7). To test potential interactions between HNF-4 and SMAD4 proteins, total cell extracts from COS-7 cells co-transfected with Myc-tagged SMAD3 (lane 8) or Myc-tagged SMAD3 and SMAD4 proteins (lane 9) along with FLAG-tagged HNF-4 were subjected to immunoprecipitation with an anti-FLAG monoclonal antibody followed by Western blotting analysis of the immunoprecipitated proteins with an anti-Myc monoclonal antibody that detects SMAD proteins. As shown in Fig. 6A (lanes 8 and 9), both SMAD3 and SMAD4 were co-immunoprecipitated with HNF-4 in this assay, whereas no interaction between these proteins could be observed using an unrelated antiserum instead of anti-FLAG in the immunoprecipitation reaction (data not shown). The efficiency of the immunoprecipitation reaction was monitored by Western blotting analysis of the same blot shown in lanes 7–9 using the anti-FLAG antibody that detects HNF-4 and SMAD4 (lanes 10–12). The control co-immunoprecipitation assays in COS-7 cells in the absence of the constitutively active TGF-β type I receptor (CA-ALK5) showed that SMADs can bind to HNF-4 even in the absence of the receptor and that in the presence of the receptor, binding is enhanced (data not shown).

The results of the co-immunoprecipitation experiment of Fig.
A strongly supported physical interactions between HNF-4 and SMAD3-SMAD4 proteins in vivo. Physical interactions between these factors could also be demonstrated in vitro by using the glutathione S-transferase (GST) pull-down assay. Specifically, fusion proteins consisting of human SMAD3 or SMAD4 and GST (GST-SMAD3, GST-SMAD4) or the GST portion alone were expressed in bacteria and coupled to glutathione-Sepharose affinity beads (Fig. 6B, right). GST- and GST-SMAD-coupled beads were incubated with total cell extracts of COS-7 cells transfected with a vector expressing Myc-tagged HNF-4 were incubated with GST or the indicated GST-SMAD-coupled Sepharose beads as described under “Experimental Procedures.” Bound proteins were analyzed by SDS-PAGE and Western blotting using an anti-Myc antibody. INPUT represents 20% of the initial cell extract used in the binding experiments. Right, bacterial expression of GST or GST-SMAD fusion proteins. Following coupling of GST fusion proteins to the glutathione-Sepharose-beads, an aliquot of the affinity beads was analyzed for coupling efficiency by SDS-PAGE and Coomasie Brilliant Blue staining. M indicates molecular mass standards. The arrows indicate the positions of the GST fusion proteins.

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Domains in HNF-4 Protein Required for Functional Cooperation with SMADs—In an attempt to identify domains in HNF-4 protein required for functional cooperation with SMADs, HepG2 cells were co-transfected with the (-890/+24) apoCIII promoter luciferase reporter construct along with expression vectors for SMAD3-SMAD4 and wild type or mutated HNF-4 proteins. Two mutated HNF-4 forms were used (Fig. 7B). The first, HNF-4-(227–455), lacks domain A (transactivation), domain B/C (DNA binding), domain D (linker), and part of domain E (ligand binding). The second mutant, HNF-4-(227–354), contains an internal deletion in the putative ligand binding domain of HNF-4 (domain E) (39). Both mutated HNF-4 forms are unable to transactivate HRE-dependent promoters (39). As shown in Fig. 7A, SMAD3-SMAD4 proteins increased the HNF-4-mediated transactivation of the apoCIII promoter 5-fold (30–versus 150-fold). In contrast, both HNF-4 mutants were unable to transactivate the apoCIII promoter either alone or synergistically with SMAD3-SMAD4 proteins. These findings indicate that nuclear localization and DNA binding, as well as the presence of the 272–353 region of HNF-4, are essential for the functional cooperation of HNF-4 with SMADs.

DISCUSSION

Previous studies have demonstrated that apoCIII is a negatively regulated acute phase-responsive gene and that its expression is inhibited by the preinflammatory cytokines tumor necrosis factor-α and interleukin-1 (16, 17). Certain transcription factors were found to mediate this process including activation protein-1 proteins, activating transcription factor 2, NF-κB, and C/EBPδ (16–19).

Natural extinguishing of the acute phase response occurs in part due to the production of anti-inflammatory cytokines such as interleukin-10, interleukin-13, and TGF-β. TGF-β is a potent anti-inflammatory cytokine (40). Following binding of TGF-β ligands, the receptor phosphorylates specific SMAD2 and SMAD3 proteins, which then associate with the common
partner SMAD4/DPC4, and the complex translocates to the nucleus and modulates the transcription of target genes (20). Thus, one potential pathway through which TGF-β could exert its effect on the apoCIII promoter could be mobilization of specific SMAD proteins and sequestration on the apoCIII promoter.

Another point of interest is the specificity of SMAD proteins in the transactivation of the apoCIII promoter. SMAD3 and SMAD3-SMAD4 complexes strongly transactivated the apoCIII promoter, whereas SMAD2 or SMAD2-SMAD4 complexes could not (Fig. 1B). Despite the high degree of similarity between SMAD2 and SMAD3 (~90%), these proteins differ in certain regions within the N-terminal MAD homology domain 1 (MH1) (20). This SMAD domain has been shown to be important for DNA binding (41) and interaction with other transcription factors such as c-Jun (42) and Sp1 (25, 43). The data of the current study (Figs. 2 and 3) establish that transactivation of the apoCIII promoter by SMAD3-SMAD4 requires the presence of an HRE and does not involve binding of SMAD proteins to this site. The role of the N-terminal domain of SMAD proteins in the transactivation of the apoCIII promoter has not been determined. Deletion of the C-terminal MH2 domain of SMAD3 that contains the dimerization and transactivation function of this protein abolished its transactivation potential on the apoCIII promoter (Fig. 1, B and E) in accordance with a number of previous reports (20).

HNF-4 Is Required for the SMAD3-SMAD4-mediated Activation of the ApoCIII Promoter—Analysis of the apoCIII promoter in cell cultures localized 10 regulatory elements (A–J) between nucleotides −890 and +24 that are required for hepatic and intestinal transcription (10–12). The regulatory elements B (nucleotides 87–63), I4 (nucleotides −736 to −714) and G (nucleotides −669 to −648) of the apoCIII promoter contain HREs that are recognized by various combinations of orphan and ligand-dependent nuclear receptors (10–12, 37). Regarding the nuclear receptor specificity of the HREs, it was shown previously that element B, which contains a DR1 site, binds strongly to HNF-4, ARP-1, EAR-2, and EAR-3; heterodimers of RXR with RARα and, less efficiently, homodimers of RARα and heterodimers of RARα with T3Rβ or human peroxisome proliferator-activated receptor α (11, 37). Element G, which contains both DR0 and DR5 sites, binds strongly on the DR0 site ARP-1 and EAR-3 (11). Element G also binds strongly, on the DR5 site, heterodimers of RARα with either RARα or T3Rβ and does not bind HNF-4 (37). Finally, element I4, which contains a DR1 site, binds strongly HNF-4, ARP-1, EAR-3, and RXRα/RARα heterodimers and, less efficiently, to RXRα/T3Rβ heterodimers (12, 37). Cotransfection experiments have shown that HNF-4 and RXRα heterodimers in the presence of 9-cis-retinoic acid act as positive regulators of the apoCIII promoter (15, 37). Elements C and D bind C/EBP factors (Fig. 8). The distal regulatory region, in addition to the two HREs on elements G and I4, contains three binding sites for the ubiquitous transcription factor Sp1 (11) on elements F, H, and I, respectively (Fig. 8).

The current study, using apoCIII promoter deletions as well as synthetic heterologous promoters driven by specific regions or specific regulatory elements of the apoCIII promoter, establishes that the SMAD-mediated transactivation of the apoCIII promoter requires one or both HREs on element B and I4 that bind HNF-4 (Figs. 2B and 4, A and B). SMAD proteins transactivated the entire apoCIII promoter, the apoCIII enhancer, or constructs containing at least one HRE that binds HNF-4 (Fig. 4, A–C). Our findings suggest that an optimal stoichiometry of HNF-4 and SMAD3-SMAD4 proteins in HepG2 cells is required to achieve maximal levels of transactivation of the apoCIII promoter. Under the experimental conditions used, it appeared that the steady-state concentration of the SMAD3 and/or SMAD3-SMAD4 proteins was limiting, since overexpression of SMAD3-SMAD4 proteins alone could transactivate the apoCIII promoter by 26-fold (Fig. 1B). Increases in the concentrations of both HNF-4 and SMAD3-SMAD4 could transactivate different apoCIII promoter segments by 28–69-fold, as determined by CAT or luciferase assays (Fig. 4A).

More evidence that HNF-4 is crucial for the SMAD-mediated transactivation of the apoCIII promoter comes from utilization of a HepG2 cell line that expresses an antisense HNF-4 ribozyme construct (Fig. 5, A–C) and has reduced endogenous HNF-4 levels (Fig. 5D). In this cell line, the apoCIII promoter activity was greatly reduced (10% relative to its activity in normal HepG2 cells) and could not be transactivated by SMAD3-SMAD4 proteins. Transactivation of this cell line could only be achieved via a combination of SMAD3-SMAD4 and HNF-4 (Fig. 5A).

**Physical Interactions between SMAD and HNF-4 Explain Their Transcriptional Synergy on Promoters That Contain HNF-4 Binding Sites**—Although HNF-4 binding sites are required for the transcriptional synergy between SMAD3-SMAD4 and HNF-4, SMAD3 does not bind to the regulatory elements B or I4, which contain the HNF-4 binding sites (Fig. 3). This is in agreement with the absence of CAGA elements in these regions. Co-immunoprecipitation assays in COS-7 cells and GST pull-down assays established direct physical interactions between HNF-4 and SMAD3 (Fig. 6, A and B). Physical interactions between these factors could be responsible for their functional interaction on the apoCIII promoter. Most importantly, these interactions were abolished by deletion of the N-terminal residues 1–226 or the 271–354 region of HNF-4. The first HNF-4 mutant lacks the transactivation domain, the
DNA binding domain, the linker region, and part of the ligand binding domain and cannot be transported into the nucleus. The second HNF-4 mutant lacks part of the ligand binding domain. Our findings suggest that specific domains of HNF-4 bound to the HREs of the apoCIII promoter interact with SMAD3-SMAD4 complexes in the nucleus. These specific protein-protein interactions may in turn potentiate the interaction of HNF-4 with the proteins of the basal transcription machinery, thus leading to the activation of the apoCIII promoter and other promoters that contain HNF-4 binding sites (Fig. 8).

Physical and functional interactions in the TGF-β/SMAD signaling pathway have also been described for other members of the nuclear receptor superfamily. It was recently shown, for instance, that SMAD3 acts as a coactivator of the vitamin D receptor in COS-1 cells (44). The domains in the two proteins required for physical and functional interactions were mapped within the MIH domain of SMAD3 and part of the ligand binding domain of VitDR (44). SMAD3 was also shown to form complexes with SMAD4-SMAD5, which may mediate the binding domain of VitDR (44). SMAD3 was also shown to form multimers with SMAD2 and SMAD3, which may mediate the binding domain of SMAD4 (44). SMAD3 was also shown to form complexes with SMAD2 and SMAD3, which may mediate the binding domain of SMAD4 (44).

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