Differential, Tissue-specific, Transcriptional Regulation of Apolipoprotein B Secretion by Transforming Growth Factor β*

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Apolipoprotein B (apoB) is required for the assembly and secretion of triglyceride-rich lipoproteins. ApoB synthesis is constitutive, and post-translational mechanisms modulate its secretion. Transforming growth factor β (TGF-β) increased apoB secretion in both differentiated and nondifferentiated Caco-2 cells and decreased secretion in HepG2 cells without affecting apolipoprotein A-I secretion. TGF-β altered apoB secretion by changing steady-state mRNA levels and protein synthesis. Expression of SMAD3 and SMAD4 differentially regulated apoB secretion in these cells. Thus, SMADs mediate dissimilar secretion of apoB in both the cell lines by affecting gene transcription. We identified a 485-bp element, 55 kb upstream of the apoB gene that contains a SMAD binding motif. This motif increased the expression of chloramphenicol acetyltransferase in Caco-2 cells treated with TGF-β or transfected with SMADs. Hence, TGF-β activates SMADs that bind to the 485-bp intestinal enhancer element in the apoB gene and increase its transcription and secretion in Caco-2 cells. This is the first example showing differential transcriptional regulation of the apoB gene by cytokines and dissimilar regulation of one gene in two different cell lines by TGF-β. In this regulation, the presence of cytokine-responsive motif in the tissue-specific enhancer element confers cell-specific response.

The B apolipoproteins, apoB-100 and apoB-48, are necessary for triglyceride-rich lipoprotein assembly and neutral lipid transport in the body (1). There is only one apoB gene in the human genome, and its expression is limited to the liver, intestine, and heart (2–6). The gene consists of 29 exons and 28 introns and exists as a 47.5-kb DNase-sensitive domain (7, 8). The presence of proximal 5-kb and distal 1.5-kb sequences is sufficient for the expression of the apoB gene in the liver and heart of mice (9, 10). However, elements required for the transcriptional expression of the apoB gene in the intestine are located between 54 and 62 kb upstream of the structural gene (11). Within this region, 315-, 485-, and 680-bp enhancer elements have been identified. These elements increase the expression of the basal promoter activity in intestinal cells (12–14). Thus, the tissue-specific expression of the apoB gene depends on the far upstream, proximal, and distal sequences.

The apoB gene transcription is believed to be constitutive, and apoB levels are thought to change primarily by co- and post-translational mechanisms. First, it was demonstrated that various perturbations that increase apoB secretion do not affect apoB mRNA levels (15). Second, it was demonstrated that oleic acid supplementation increases apoB secretion in HepG2 cells by inhibiting the intracellular degradation (16). Subsequent studies led to the understanding that co- and post-translational mechanisms involving degradation of nascent apoB are primarily involved in the modulation of apoB secretion (17–21). However, apoB expression studies have challenged this concept and indicated that transcriptional mechanisms may also play a role in the control of apoB secretion. For example, the amounts of apoB secreted by rat hepatoma McA-RH7777 cells stably transfected with human apoB cDNAs were correlated with increases in apoB mRNA levels (22). Similarly, increased plasma apoB levels were correlated with the human transgene copy number in mice (3, 23–25). It is conceivable that human apoB might have escaped the co- and post-translational mechanisms in rats and mice, leading to increased secretion. It is also possible that overexpression might have burdened the post-translational control mechanisms and enhanced apoB secretion. It remains to be determined whether modest changes in the transcription of the endogenous apoB gene would affect apoB secretion in human cells.

Transforming growth factor β (TGF-β) is a family of cytokines that play a widespread role in various biological processes such as growth, development, differentiation, apoptosis, embryogenesis and anti-inflammation (26, 27). These cytokines are produced by most cell types and exert paracrine, autocrine, and endocrine effects by interacting with their cell surface serine/threonine kinase TGF-β receptors type I and type II. TGF-β binds to type II receptors and induces phosphorylation of the type I receptors. The phosphorylated receptor I in turn phosphorylates the receptor SMADs. The phosphorylated receptor SMADs bind to SMAD4, and the complex translocates to the nucleus. The receptor SMADs and SMAD4 complex affects the transcription of various genes by directly interacting with the DNA sequences present in the promoter, enhancer, or repressor elements or through physical interactions with other transcriptional co-activators or co-repressors (26, 27). Ubiquitlation and proteosome-dependent degradation of receptor SMADs in the nucleus provide a way to terminate the TGF-β
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**EXPERIMENTAL PROCEDURES**

**Materials—**Recombinant TGF-β2 was from R & D Systems, Inc. (Minneapolis, MN). Antibodies used for the determination of apoB have been described (43, 44). Bovine serum albumin (BSA), MAP kinase inhibitor PD98059 (Promega), and other chemicals were from Sigma. Monoclonal anti-apoA-1 antibody, 4H1, was from the University of Ottawa Heart Institute. Polyclonal anti-apoA-1 antibodies were from Roche Molecular Biochemicals. The β-galactosidase assay kit was from Invitrogen. [14C]Chloramphenicol and Trans-35S label were from ICN Biomedicals.

**Treatments—**HepG2 cells received 2.0 ml of methionine/cysteine-free DMEM containing 0.1% BSA and 10 ng/ml of TGF-β2. In the case of Caco-2 cells, the apical sides received 2.0 ml of methionine/cysteine-free DMEM plus 0.1% BSA, and the basalateral side received 2.0 ml of methionine/cysteine-free DMEM plus 0.1% BSA containing 10 ng/ml of TGF-β2. After 1 h, 100 µCi of Trans-[35S]label were added to each well (to the apical side in the case of Caco-2 cells), and the cells were then incubated for the indicated time periods. Cells were washed with cold DMEM containing methionine and cysteine and were lysed with 0.5 ml of immunoprecipitation lysis buffer (phosphate-buffered saline containing 0.5% deoxycholate, 1% SDS, 1% Triton X-100, 200 mM methionine, 1 mM cysteine, and protease inhibitor mixture). The cell lysates were cleared by centrifugation at 10,000 rpm for 4 °C for 10 min. The supernatants were precleared with 10 µl of protein A + G-Sepharose and used for immunoprecipitation. Precleared cell lysates and the media (basolateral media in the case of Caco-2 cells) were incubated overnight at 4 °C in a rocker with 5 µl of sheep anti-human apoB antibodies. The antigen-antibody complexes were precipitated by adding 20 µl of protein A + G-Sepharose and rocked at 4 °C for 2 h. The complexes were spun at 4 °C for 2 min, and the supernatants were discarded. The pellets were washed three times with immunoprecipitation lysis buffer and once with PBS and were finally suspended in 1× Laemmli sample buffer. The suspensions were heated at 95 °C for 5 min and then centrifuged at 10,000 rpm for 2 min. The clear supernatants were applied to SDS-polyacrylamide gels, and proteins were separated by electrophoresis. The gels were fixed, dried, and exposed to the PhosphorImager screen. The intensity of each band was quantified with ImageQuant software (Amersham Biosciences).

**RNA Quantifications—**HepG2 cells and differentiated Caco-2 cells were incubated for 17 h either with or without 10 ng/ml TGF-β2. The total RNA from cells using Trizol reagent (Invitrogen) by following the manufacturer’s instructions. The total RNA (15 µg) was then run on a denaturing agarose gel and transferred to a nitrocellulose membrane in 20× SSC (20× 0.15 M NaCl and 0.015 M sodium citrate). The RNA was cross-linked to the membrane by exposing it to the UV light. Prehybridization was carried out for 4 h in Quikhybrid hybridization solution (Stratagene), and hybridization was carried out in the same solution in the presence of radiolabeled probes and 100 µg/ml denatured and sheared salmon sperm DNA. To prepare different radiolabeled probes, apoB and GAPDH fragments were amplified and labeled with [α-32P]dCTP by using the Random Primers DNA Labeling System (Invitrogen). Membranes were then washed with 2× SSC, 0.1% SDS at 50 °C and 3× SSC at 65 °C. The blots were then exposed to a phosphorimager screen, and RNA levels were quantified with ImageQuant software (Amersham Biosciences).

**For RT-PCR, 1 µg of total RNA isolated from nontreated and TGF-β-treated Caco-2 and HepG2 cells was used. A blend of Omniscript and Sensiscript reverse transcriptase provided in the Quantitect RT Mix (Qiagen) was used according to the manufacturer’s instructions to reverse transcribe and amplify apoB and GAPDH sequences. Primers used for apoB and GAPDH were gcactgctcaggcggtccagtcgagcagcctgctatg (apoB) and cagcagcactactcctggtttgcagctacacg (GAPDH). The reverse transcription was performed at 50 °C for 15 min followed by incubating at 95 °C for 15 min. The PCR also denatures the newly synthesized template cDNA and activates TaqDNA polymerase. PCR conditions were 94 °C for 15 s, 55 °C for 20 s, and 72 °C for 20 s. The products were electrophoresed on 2% agarose gel, and the bands were quantified by scanning.**
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FIG. 1. Effect of different concentrations of TGF-β on the secretion of apoB by differentiated Caco-2 and HepG2 cells. Caco-2 cells were plated on six-well Transwells and used after 16 days. For experiments, the cells were preincubated with DMEM for 24 h and subsequently treated with TGF-β. For this treatment, Caco-2 cells received DMEM on the apical side and DMEM containing different concentrations of TGF-β (0–20 ng/ml) on the basolateral side. After 17 h, the amounts of apoB present in the basolateral media were quantified by ELISA performed in triplicate as described under “Experimental Procedures.” The data are representative of three independent experiments. HepG2 cells were plated in 24-well plates and used after 2 days. For experiments, the cells were preincubated with DMEM for 24 h and subsequently treated with TGF-β. HepG2 cells received different concentrations of TGF-β in DMEM. After 17 h, the amounts of apoB present in the media were quantified by ELISA performed in triplicate.

Transient Expression of Transgenes—Varying amounts (5–10 μg) of plasmid DNAs along with 1 μg of an internal reference plasmid (pCMV-β-gal) were incubated with Fugene-6 (Roche Diagnostic) and introduced to subconfluent (~70%) monolayers of Caco-2 and HepG2 cells in 75-mm² flasks and then incubated for 24 h. An equal number of cells were then transferred to six-well plates or Transwells. After 24 h, the cells were washed with DMEM and incubated for 8 h with DMEM containing 0.1% BSA, washed, and treated with or without TGF-β. Three wells received DMEM plus 0.1% BSA containing 10 ng/ml TGF-β, whereas the remaining three wells served as controls (in the case of Transwells, TGF-β was added to the basolateral side). The cells were incubated for 17 h at 37 °C, 5% CO₂ in humidified atmosphere, and the medium (from the basolateral side in the case of Transwells) was collected and assayed for apoB mass. Cells were washed and collected in lysis buffer (Promega Corp., Madison, WI). The cell lysates were cleared by centrifugation at 10,000 rpm for 10 min at 4 °C, and supernatants were used for the determination of cellular protein levels and different enzyme activities.

Other Methods—Cell protein was quantified by the Bradford method (49) using Coomassie Blue reagent (Fierce). ApoB and apoA-I were quantified by sandwich ELISA (43, 44). The β-galactosidase and CAT activities were assayed as described previously (50, 51). The CAT activity levels were quantified with PhosphorImager analysis and the ImageQuant program and were corrected for transfection efficiencies between the flasks by dividing with the β-galactosidase activity values. Luciferase activity was measured as per the manufacturer’s instructions (Promega Corp.).

RESULTS

Differential Effects of TGF-β on ApoB Secretion by Caco-2 and HepG2 Cells—To investigate the effect of TGF-β on apoB secretion by HepG2 and differentiated (17–20 days postplating), Caco-2 cells were incubated with increasing concentrations of TGF-β (Fig. 1). The amount of apoB secreted by the control Caco-2 and HepG2 cells was 944 ± 59 and 1266 ± 82 ng/well, respectively, in agreement with our earlier studies (44, 48, 52–54). In Caco-2 cells, TGF-β2 showed a concentration-dependent increase in the amount of apoB secreted into the basolateral medium. At 10 ng/ml, TGF-β2 increased apoB secretion by 56 ± 10%. The increases ranged from 20 to 80% in different experiments. At higher concentrations, no further increase in apoB secretion was observed, indicating that the maximum effect was achieved at 10 ng/ml TGF-β2. In contrast, TGF-β2 showed a concentration-dependent decrease in the amount of apoB secreted by HepG2 cells (Fig. 1). The decrease in apoB secretion was 20% at 5 ng/ml and reached a maximum of 30% inhibition at 15 ng/ml.

Next, we compared the effect of TGF-β1 and TGF-β2 on apoB secretion in Caco-2 and HepG2 cells (Fig. 2). Both TGF-β1 and TGF-β2 augmented (16–25%) apoB secretion in Caco-2 cells and attenuated (23–30%) apoB secretion in HepG2 cells, indicating that both of these molecules have similar biologic effects. In subsequent experiments, we only used TGF-β2 because of more consistent results and relatively better responses. To determine the earliest time point required for TGF-β2 to exert its effects on apoB secretion, we performed time course experiments. These experiments revealed that statistically significant differential effects on apoB secretion in both cell lines were first apparent after 8 h of treatment (data not shown). These studies showed that TGF-β2 differentially regulates apoB secretion in intestine and liver-derived cell lines.

Effect of TGF-β Is Independent of the State of Differentiation of Caco-2 Cells—In the studies described above, a major difference between Caco-2 and HepG2 cells was their state of differentiation; the Caco-2 cells were plated in Transwells and allowed to differentiate for about 2 weeks, whereas HepG2 cells were used 2–3 days after plating. Thus, diverse effects of TGF-β might be related to the differentiation of Caco-2 cells. To test this hypothesis, we studied the effect of TGF-β on Caco-2 cells after 2 days of plating along with HepG2 cells (Fig. 3). As seen before, treatment of HepG2 cells with TGF-β decreased apoB secretion by ~25%. As expected, nondifferentiated Caco-2 cells secreted (54 ± 7 ng/well) significantly smaller amounts of apoB than the differentiated cells (see above), in agreement with other studies (48, 52). Nonetheless, treatment of these cells increased apoB secretion by 26% (Fig. 3). These experiments were extended to study the effect of TGF-β during the entire course of differentiation of Caco-2 cells. Determination of the differentiation of Caco-2 cells by measuring sucrase activity has been described before (48). Cells were plated in Transwells, and the effect of TGF-β was studied at different times (5, 10, 11, 12, 14, 16, 18, 20, 22, 24, 30, and 36 days after plating) as described in the legend to Fig. 1. TGF-β increased (20–90%) the secretion of apoB by Caco-2 cells at all of the time periods tested. Such long-term cell culture experiments could not be performed with HepG2 cells because these cells do not differentiate with time; instead, they come off the plates. These
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Studies point out that the increased secretion of apoB by TGF-β was independent of the differentiation of Caco-2 cells.

To determine the specificity of TGF-β action, we studied the effects of TGF-β on cellular protein levels and apoA-I secretion (Table I). TGF-β treatment had no significant effect on cellular protein levels in both cell lines. In accordance with the studies described above, TGF-β treatment significantly increased (43%) apoB secretion in Caco-2 cells. In contrast, TGF-β had no significant effect on apoA-I secretion in these cells. In HepG2 cells, TGF-β significantly decreased (35%) apoB secretion and is in concert with the studies described above. The secretion of apoA-I appeared to increase by ~11%, but this effect was no longer statistically significant when corrected for cell protein. We also studied the effect of TGF-β on the secretion of apoA-I in differentiated Caco-2 cells and observed no significant effect (data not shown). Thus, TGF-β specifically and differentially modulates apoB secretion and exerts no significant effect on the cellular protein levels and apoA-I secretion in these cells.

TGF-β Alters Steady State ApoB mRNA Levels—Next, we attempted to understand the mechanisms involved in the differential regulation of apoB secretion in these cells. It is known that apoB synthesis is constitutive (15), and the amounts of apoB secreted are generally modulated by intracellular degradation (17, 19, 20). To evaluate the role of intracellular degradation as a possible mechanism for TGF-β effects, we performed pulse-chase experiments. These studies revealed that the major effect of TGF-β was at the end of the pulse period. After a 30-min pulse, the amounts of apoB100 in HepG2 cells treated and untreated with TGF-β were 33291 and 22065 PhosforImager units, respectively. Thus, TGF-β-treated HepG2 cells synthesized 33% less apoB100 than the control cells. To study this effect further, cells were either treated or not with TGF-β for 17 h and pulsed with Trans-35S-label for various times, and the amounts of apoB in cells were quantified after immunoprecipitation (Fig. 4). The amounts of apoB100 present in TGF-β-treated HepG2 cells were lower (30–60%) at all times than in the control cells (Fig. 4A). Except for one time point, the amounts of apoB100 present in TGF-β-treated Caco-2 cells were higher (40–110%) than in the control cells (Fig. 4B). Similarly, TGF-β-treated cells contained more apoB48 than the control Caco-2 cells (Fig. 4C). These studies indicate that TGF-β differentially affects apoB synthesis in these cells.

Consideration was then given to the possibility that the modulation of apoB mRNA synthesis may be related to changes in the steady state apoB mRNA levels. Total RNA was isolated from the control cells and cells treated with 10 ng/ml TGF-β for 17 h. Northern blot analysis revealed that TGF-β increased steady state levels of apoB mRNA in Caco-2 cells by 54% and decreased its levels by 39% in HepG2 cells (Fig. 5A). Changes in mRNA levels were also studied by RT-PCR. In these experiments, conditions were optimized for the maximum apoB amplification, and the size of the apoB fragment amplified was smaller than that of GAPDH. Probably for these reasons, the amounts of apoB amplified were qualitatively higher than GAPDH. Nonetheless, comparative studies showed that TGF-β treatment increased (50%) apoB mRNA levels in Caco-2 cells and decreased (38%) in HepG2 cells (Fig. 5B). Thus, TGF-β differentially affects the steady state levels of apoB mRNA in these cells.

TGF-β Signaling via SMADs Dissimilarly Affects ApoB Secretion in Caco-2 and HepG2 Cells—The effect of TGF-β on apoB mRNA levels indicated that TGF-β might affect apoB gene transcription. To our knowledge, TGF-β has not been shown to affect mRNA stability. However, TGF-β is known to alter gene expression by modulating gene transcription (26, 27, 55, 56). It is also known that mitogen-activated protein kinases (MAP kinases) and SMAD proteins are involved in the downstream mediation of the TGF-β response in many cell types (27, 55, 55–59). In order to investigate the involvement of MAP kinases, we used a specific inhibitor, PD98059 (27, 32–34). We reasoned that, if MAP kinases were involved, inhibition of MAP kinases would abolish the modulation of apoB secretion by TGF-β. Caco-2 and HepG2 cells were treated with TGF-β in the presence or absence of PD98059 (Table II). TGF-β increased apoB secretion by 39% in Caco-2 cells. Surprisingly, PD98059 alone increased apoB secretion by 80% in these cells. Treatment of these cells with TGF-β and PD98059 augmented apoB secretion greater than that observed for the individual treatments. In fact, the 111% increase was close to the calculated additive increase of 119%. Again, in HepG2 cells, TGF-β decreased apoB secretion by 25%. Surprisingly, PD98059 alone attenuated apoB secretion. In this case, the decrease was 27%. Both TGF-β and PD98059 decreased apoB secretion by 41%, an inhibition that was more than the individual responses and was comparable with the expected additive change of 52%. Most likely, TGF-β and PD98059 independently and additively modulate apoB secretion in these cells. Thus, TGF-β does not appear to modulate apoB secretion via the MAP kinase pathway.

To investigate the potential role of SMAD proteins in the TGF-β-mediated signal transduction, we overexpressed these proteins in HepG2 and Caco-2 cells (Fig. 6). First, the effect of overexpression of SMADs on the expression of luciferase in the 3TP-Lux reporter plasmid was studied in HepG2 cells untreated and treated with TGF-β (Fig. 6A). In 3TP-Lux, luciferase is under the control of SMAD binding enhancer elements derived from the PAI-1 gene (46). HepG2 cells transfected with 3TP-Lux showed basal levels of luciferase activity, and this activity was increased 3-fold after TGF-β treatment (Fig. 6A). Co-expression of 3TP-Lux with SMAD3 and SMAD4 resulted in a 43-fold increase in the basal expression of luciferase. TGF-β treatment increased this activity to 69-fold of the basal activity in untreated cells. These control studies indicate that HepG2 cells respond to TGF-β and SMAD expression and are in agreement with other studies (31, 46). Next, we studied the effect of the overexpression of SMADs on apoB (Fig. 6B) and apoA-I (Fig. 6C) secretion in HepG2 cells. The treatment of control cells (not overexpressing SMADs) with TGF-β resulted in 29% decreased secretion of apoB by HepG2 cells (Fig. 6B). More importantly, overexpression of SMAD3 and -4 decreased the secretion of apoB by ~40% when compared with untreated cells.
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Caco-2 cells from a 75-mm² flask were plated in six-well Transwells and cultured in DMEM containing 20% FBS. After 48 h, the cells were switched to DMEM plus 0.1% BSA for 24 h. Subsequently, they were treated either with or without 10 ng/ml TGF-β in triplicate for 17 h in DMEM containing 0.1% BSA. HepG2 cells from a 75-mm² flask were plated in six-well plates and cultured in DMEM plus 10% FBS. After 24 h, the cells were changed to DMEM plus 0.1% BSA for 24 h. Subsequently, they were treated either with or without 10 ng/ml TGF-β in triplicate for 17 h in DMEM plus 0.1% BSA. ApoB and apoA-I levels were quantified in triplicate by ELISA. Cell monolayers were extracted in lysis buffer and used for protein determinations using the Bradford method (49). The data are representative of three independent experiments.

|                  | Caco-2 cells | HepG2 cells |
|------------------|--------------|-------------|
| **Control**      |              |             |
| Cell protein (mg/well) | 0.38 ± 0.02 | 0.46 ± 0.02 |
| ApoB (ng/well)   | 109 ± 8      | 1258 ± 116  |
| ApoB (ng/mg cell protein) | 288 ± 21 | 2722 ± 352 |
| ApoA-I (μg/well) | 26.3 ± 2.0   | 42.7 ± 1.2  |
| ApoA-I (μg/mg cell protein) | 74.0 ± 3.5 | 92.3 ± 6.8  |
| **TGF-β**        |              |             |
| Cell protein (mg/well) | 0.36 ± 0.04 | 0.48 ± 0.02 |
| ApoB (ng/well)   | 147 ± 11     | 850 ± 87    |
| ApoB (ng/mg cell protein) | 412 ± 45 | 1766 ± 107 |
| ApoA-I (μg/well) | 28.8 ± 0.9   | 47.4 ± 1.8  |
| ApoA-I (μg/mg cell protein) | 80.8 ± 7.7 | 98.8 ± 3.7  |

| Percentage of control | 95 | 104 | 68 | 111 | 107 |
| p values             | 0.482 | 0.288 | 0.005 | 0.020 | 0.220 |

* Statistically significant differences between control and TGF-β-treated cells.

| A. HepG2, ApoB100 | B. Caco-2, ApoB100 | C. Caco-2, ApoBm8 |
|-------------------|--------------------|-------------------|

**Table I.** Effect of TGF-β on apoB and apoA-I secretion

Regulation of ApoB Secretion by TGF-β—It is known that SMADs modulate gene transcription by interacting with either cis-elements or transcription factors (27, 55–57, 59). To determine whether SMADs were modulating apoB gene expression by directly interacting with cis-elements, we studied the effects of TGF-β on the expression of CAT under the control of a minimal apoB promoter. The minimal promoter sequences required for apoB expression in the liver and intestinal cells are −85 to +121 bp (4). For this reason, the −85CAT construct (Fig. 7A) was transiently transfected in HepG2 and Caco-2 cells, and the effect of TGF-β was studied on the expression of the CAT gene (Fig. 7B). CAT expression was minimal under the control of a minimal promoter (−85CAT) in both cell lines. More importantly, the expression of CAT was not affected by TGF-β treatment in these cells. Furthermore, overexpression of SMADs had no effect on −85CAT expression in Caco-2 cells. These studies indicated that the minimal apoB promoter does not respond to TGF-β treatment and SMAD overexpression.

In some instances, it has been shown that SMADs bind to CAGAC sequences and modulate gene transcription (56, 60). To identify the presence of the TGF-β response element, we searched for SMAD binding sequences in the apoB gene. We found a SMAD-binding site in a 690-bp sequence 55 kb upstream of the mouse apoB gene that confers intestinal expression (12). To determine whether these sequences were responsible for the increased apoB secretion after TGF-β treatment in Caco-2 cells, we expressed CAT under the control of a minimal
apoB promoter in the presence and absence of the mouse intestinal enhancer element (Fig. 7B, 690CAT). The 690-bp enhancer element increased the expression of CAT by 17-fold in Caco-2 cells. More importantly, the CAT expression increased (40%) after TGF-β treatment. Furthermore, co-expression of 690CAT with SMAD3 and -4 (690CAT + S3 + 4) resulted in a 21-fold increase in the enzyme activity. However, TGF-β treatment did not potentiate this response in the presence of SMADs. Next, we determined whether 690CAT would respond to TGF-β in HepG2 cells. Expression of 690CAT in HepG2 cells was higher than ~85 CAT, and this expression was increased after TGF-β treatment. These studies indicated that the TGF-β-responsive element is indeed present in the 690-bp enhancer element present in the mouse apob gene. If functionally active, this sequence would enhance apoB secretion in both liver and intestine-derived cell lines.

Subsequently, we turned our attention to the regulatory elements in the human apob gene. Antes et al. (12) identified a 315-bp human intestinal enhancer element that was homologous to the 690-bp mouse intestinal enhancer element. Subsequently, they also identified a 485-bp sequence upstream of the 315 bp required for the intestinal expression of apoB (14). The

**TABLE II**

| ApoB               | Percentage of Control (%) | Percentage change (%) |
|--------------------|---------------------------|-----------------------|
| Caco-2 cells       |                           |                       |
| Control            | 415 ± 11                  | 100 ± 3               |
| TGF-β (10 ng/ml)   | 576 ± 40                  | 139 ± 10              |
| PD98059 (50 μM)    | 747 ± 83                  | 180 ± 20              |
| TGF-β + PD98058    | 876 ± 49                  | 211 ± 12              |
| HepG2 cells        |                           |                       |
| Control            | 2314 ± 71                 | 100 ± 3               |
| TGF-β (10 ng/ml)   | 1740 ± 210                | 75 ± 9                |
| PD98059 (50 μM)    | 1691 ± 76                 | 73 ± 3                |
| TGF-β + PD98058    | 1361 ± 141                | 59 ± 6                |

**Fig. 5.** TGF-β treatment affects steady state apoB mRNA levels in Caco-2 and HepG2 cells. A, Northern analysis. HepG2 and differentiated Caco-2 cells were treated either with or without TGF-β (10 ng/ml) for 17 h as described in the legend to Fig. 1 and under “Experimental Procedures.” The bands corresponding to apoB and GAPDH were quantified, and the ratios were calculated. The ratios in the control cells were normalized to 100%. C and T, RNA obtained from control and TGF-β-treated cells, respectively. The data are representative of four independent experiments. B, RT-PCR. 1 μg of total RNA from nontreated (C) and TGF-β-treated (T) cells were used for RT-PCR using a QuantiTect RT-PCR kit as described under “Experimental Procedures.” The products were separated on agarose gels and quantified.
485-bp sequence contains a SMAD binding site, whereas the 315-bp sequence does not. We examined whether these sequences respond to TGF-β (Fig. 7). In accordance with the studies of Antes et al. (12, 13), both 315-bp (315CAT) and 485-bp (485(F)CAT) enhancer elements increased the level of CAT expression in Caco-2 cells. TGF-β treatment had no significant effect on the CAT activity when expressed under the control of the 315-bp enhancer element. In contrast, TGF-β increased the CAT expression under the control of the 485-bp enhancer element. Furthermore, expression of the CAT activity under the 485-bp enhancer (485(R)CAT) element in a reverse orientation failed to respond to TGF-β. Note that the 485-bp element in reverse orientation does not act as an enhancer and is in concert with earlier reports (14). These studies establish that the 485-bp enhancer element that contains a SMAD binding site in the forward orientation responds to TGF-β in Caco-2 cells.

**DISCUSSION**

**Differential Effects of TGF-β on ApoB Secretion in Liver and Intestine-derived Cells**—The present studies demonstrate that TGF-β has dissimilar effects on apoB secretion in Caco-2 and HepG2 cells. These two cell lines have been used extensively as models for intestinal and hepatic lipoprotein assembly and secretion and to study tissue-specific cytokine responses (39, 40). TGF-β increased apoB secretion in Caco-2 cells and decreased secretion in HepG2 cells (Figs. 1–3). The differential effect was specific to apoB, since apoA-I secretion was unaffected by TGF-β. The increased secretion of apoB by Caco-2 cells after TGF-β treatment is in agreement with the studies of Murthy et al. (42). They showed that TGF-β increased apoB synthesis, had no effect on intracellular apoB degradation, and increased the secretion of apoB and triacylglycerols. The effect of TGF-β on apoB secretion in HepG2 cells has not been de-
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A.

-85CAT

690CAT

315CAT

485(F)CAT

485(R)CAT

Fig. 7. Identification of cis-elements in the apob gene required for increased apoB secretion in Caco-2 cells. A, schematic diagram of different expression vectors. In the -85CAT expression vector, CAT is under the control of a minimal apoB promoter composed of -85 to +121 bp. The numbers represent the location of the base pairs in relation to the transcription start site of the apob gene. In the 690CAT expression plasmid, a mouse 690-bp intestinal enhancer element is placed upstream of the minimal apoB promoter in a forward direction as found in the apob gene. In the 315CAT construct, CAT expression is under the control of the 315-bp human intestinal enhancer element in the forward direction. CAT expression in 488(F)CAT and 488(R)CAT vectors is under the control of the 485-bp human intestinal enhancer element in the forward and reverse orientations, respectively. B, HepG2 and Caco-2 cells were transfected with -85CAT or 690CAT expression vectors with or without SMAD3 and -4 (S 3+4) expression vectors as described in the legend to Fig. 6. Cells were subcultured in six-well plates and treated or not with TGF-β as described in the legend to Fig. 6 and under “Experimental Procedures.” C, Caco-2 cells (75-mm² flasks, ~70% confluent) were transfected with various indicated expression vectors. After 24 h, each flask was subcultured into six-well plates. After 32 h, the cells were treated or not with TGF-β for 17 h as described in the legend to Fig. 6 and under “Experimental Procedures.” Cell lysates were used to measure protein concentration, β-galactosidase, and CAT activities.
sion of apoB in mice results in increased plasma apoB levels (23, 24, 67). A mutation in the human apoB gene promoter that increases transcription has been correlated with increased apoB plasma levels in humans (68). Thus, it is likely that tweaking the transcriptional regulatory mechanisms might modulate apoB levels. Alterations in the transcriptional control are expected to result in modest changes due to the interplay between various positive and negative control mechanisms that coordinate apoB gene transcription. Modest changes in apoB levels are desirable, because both overexpression and deficiency of apoB lead to metabolic and pathologic disorders.

One Command, Same Messengers, Different Outcomes—TGF-β is known to transduce signals by MAP kinases (27, 32–34) or by SMADs (27, 55–57, 59). We had anticipated that the two different mechanisms might lead to different apoB secretion in two different cell lines. Inhibition of MAP kinases resulted in an additive response with TGF-β/34) or by SMADs (27, 55–57, 59). We had anticipated that the two different mechanisms might lead to different apoB secretion in two different cell lines. Inhibition of MAP kinases resulted in an additive response with TGF-β (Table II) excluding the involvement of MAP kinases as downstream mediators/ regulators of the TGF-β response in both of the cell lines. In contrast, overexpression of SMAD3 and -4 in these cells mimicked the TGF-β response, and cells overexpressing SMADs did not alter apoB secretion in response to TGF-β. Thus, we conclude that the differential response to TGF-β in both cells is mediated by SMADs.

Several studies have established that the TGF-β signaling pathway is mediated by SMADs. In HepG2 cells, transcriptional regulation of SMAD7 by TGF-β requires the participation of SMAD2, SMAD3, and SMAD4 (69). In these cells, TGF-β induces furin transcription involving SMAD2 and SMAD4 (70). Liu et al. have demonstrated that TGF-β-induced phosphorylation of SMAD3 is required for the inhibition of epithelial cell proliferation (71). SMADs have been shown to participate in TGF-β-induced regulation of p21 and apoCIII in HepG2 cells (30, 31). Furthermore, constitutive phosphorylation and nuclear localization of SMAD3 have been correlated with increased collagen gene transcription in activated hepatic stellate cells (72). In the intestinal epithelial cells, overexpression of oncogenic ras has been shown to decrease SMAD4 expression, inhibit interaction of SMAD4 with SMAD2/SMAD3, and repress TGF-β-mediated growth inhibition (73).

How do SMADs bring about differential regulation in two different cell lines? We have identified the SMAD binding site in the intestinal enhancer element of the opab gene and showed that this enhancer element responds to TGF-β and increases the expression of a reporter gene. Thus, the increased apoB secretion in Caco-2 cells is due to the binding of SMADs to the intestinal enhancer 55 kb upstream of the apoB gene. We speculate that SMADs may interact with other transcription factors required for liver expression, inhibit their binding to cis-elements, and decrease apoB secretion in HepG2 cells. Thus, the cell-specific response is most likely determined by the tissue-specific regulatory elements of the gene. TGF-β uses SMADs in both cell lines as messengers to convey its signal. The outcome of the signal in the two cell lines is different, because the apoB gene uses different tissue-specific enhancer elements for its expression in the intestine and liver.

Genetic Context Defines TGF-β Responsiveness of the apoB Gene—TGF-β is known to cause different responses in different types of cells, and its effect is generally explained in terms of the “cellular” context (27, 60). For example, TGF-β stimulates cellular proliferation in fibroblasts and inhibits it in keratinocytes (27, 60). It is generally believed that the binding of SMADs to the CAGAC sequence and their association with adapters, partners, co-activators, or co-repressors define the cellular context. Based on the following discussion, we propose that the gene itself may define the TGF-β response and that SMADs are the transducers and not the determining factors in regulating apolipoprotein gene expression. Furthermore, a combination of different responses by individual genes may define the cellular context of TGF-β action.

First, the presence of the CAGAC sequence does not always confer TGF-β responsiveness. It is known that apoCIII, a member of the apoAI-apoCII-apoAIV gene complex, responds to TGF-β (31). However, our studies indicate that apoAI does not respond to TGF-β. Thus, the presence of a SMAD binding site in the control region of a gene does not always confer TGF-β responsiveness. Second, different control elements respond differently to the same signal, and the extent of the response varies in the same cell. For example, TGF-β treatment increases 3TP-Lux expression and decreases apoB expression in HepG2 cells (Fig. 6). SMAD overexpression increases 3TP-Lux expression by severalfold and is further augmented after TGF-β treatment (Fig. 6A). In contrast, SMAD overexpression decreases apoB secretion by ~30%, and this response is resilient to further TGF-β treatment. Most likely, the effect of SMADs is counterbalanced by other factors that bind apoB enhancer elements. Third, evidence for the genetic context comes from the studies with 690CAT (Fig. 7). The 690-bp enhancer elements respond to TGF-β by increasing the CAT in both HepG2 and Caco-2 cells. Thus, if an enhancer element is transcriptionally active, then it will respond similarly in both the cell lines. Thus, we propose that the genetic context determines the TGF-β responsiveness.

In summary, we have shown that TGF-β dissimilarly affects apoB secretion in Caco-2 and HepG2 cells. TGF-β binds to its cell surface receptors and activates SMADs that in turn move to the nucleus and dissimilarly regulate apoB gene transcription in these cells. In Caco-2 cells, SMADs most likely bind to the 485-bp intestinal enhancer in the apoB gene and enhance its transcription, leading to an increase in apoB secretion. The differential regulation of apoB secretion by TGF-β appears to represent a novel mode of regulation by cytokines involving signaling mechanisms that target tissue-specific enhancer elements. In this type of regulation, two organs express the same gene using two different tissue-specific regulatory elements. The presence of different cytokine-responsive elements in these tissue-specific regulatory regions allows for dissimilar regulation by cytokines. These studies raise the possibility that signaling mechanisms modulate apoB secretion, and a tweaking of these mechanisms can be of therapeutic interest in changing apoB levels.

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