Activation of the nuclear receptor FXR induces fibrinogen expression: a new role for bile acid signaling

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Abstract Three genes, fibrinogen-α (FBGα), -β, and -γ, encode proteins that make up the mature FBG protein complex. This complex is secreted from the liver and plays a key role in coagulation in response to vascular disruption. We identified all three FBG genes in a screen designed to isolate genes that are regulated by the farnesoid X receptor (FXR; NR1H4). Treatment of human hepatoma cells with either naturally occurring or synthetic [3-(2,6-dichlorophenyl)-4-(3′-carboxy-2-chloro-stilben-4-yl)-oxymethyl-5-isopropyl-isoxazole] FXR ligands resulted in the induction of transcripts for all three genes. The induction of FBGβ mRNA in response to activated FXR appears to be a primary transcriptional response, as it is blocked by actinomycin D but not by cycloheximide. Four FXR isoforms were recently identified that differ either at their N termini and/or by the presence of four amino acids in the hinge region. Interestingly, the activities of the human FBGβ promoter-reporter constructs were highly induced by FXR isoforms that lack the four amino acid insert. The observation that all three FBG subunits are induced by specific FXR isoforms, in response to FXR ligands, suggests that bile acids and FXR modulate fibrinolytic activity.—Anisfeld, A. M., H. R. Kast-Woelbern, H. Lee, Y. Zhang, F. Y. Lee, and P. A. Edwards. **Activation of the nuclear receptor FXR induces fibrinogen expression: a new role for bile acid signaling. *J. Lipid Res.* 2005.** 46: **458–468.**

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Secretion of fibrinogen (FBG) from hepatocytes into the blood is a key component of the coagulation pathway that ultimately leads to the formation of a fibrous clot in response to vascular disruption (1). The mature FBG protein is a hexamer that is formed from disulfide-linked equimolar ratios of three peptides encoded by the FBGα, -β, and -γ genes (2). FBG expression is restricted almost entirely to the hepatocyte (3). In response to disruption of the vasculature, a signaling cascade originating from either the intrinsic or the extrinsic coagulation pathway leads to the activation of thrombin, which then cleaves small peptides, termed A and B, from the FBG hexamer, allowing fibrin to form (3). Fibrin can self-associate to form filaments that aggregate to form a meshwork of interconnected thick fibers that are a critical component of the clot (3). The activation of thrombin, and thus the production of fibrin, is regulated by a series of enzymes that respond to disruption of the vasculature to initiate the blood coagulation process (3). The production of fibrin is also regulated at the transcriptional level by expression of the FBGα, -β, and -γ genes in the hepatocyte (4).

The three FBG genes are clustered in a 65 kb region on human chromosome 4 (5, 6). Hepatic-specific expression is achieved by the requirement for the liver-enriched transcription factor HNF-1 (7, 8). Expression of the three FBG genes is tightly regulated in a coordinated manner so that the expression of all three genes is induced in response to the same signal (9). Induction of rat and human FBG mRNAs occurs as part of the acute phase response that is activated by interleukin-6 (IL-6) and glucocorticoid signaling pathways (9–11). The coordinated regulation of all three FBG genes in response to IL-6 and glucocorticoids is achieved by the presence of distinct transcription factor binding sites flanking each of the three FBG genes rather than through a single common regulatory element (4, 10, 11).

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Abbreviations: apoC-II, apolipoprotein C-II; CDCA, chenodeoxycholic acid; DR-1, direct repeat with a 1 bp spacer; ER-8, everted repeat with an 8 bp spacer; FBG, fibrinogen; FXR, farnesoid X receptor; FXRE, farnesoid X receptor response element; GW4064, 3-(2,6-dichlorophenyl)-4-(3′-carboxy-2-chloro-stilben-4-yl)-oxymethyl-5-isopropyl-isoxazole; hFXR, human farnesoid X receptor; hRXRα, human retinoid X receptor α; I-BABP, ileal bile acid binding protein; IL-6, interleukin-6; IR-1, inverted repeat with a 1 bp spacer; LG100153, a synthetic RXR agonist; mFXR, murine farnesoid X receptor; MRP2, multidrug resistance-associated protein 2; PLTP, phospholipid transfer protein; PXR, pregnane X receptor; rFXR, rat farnesoid X receptor; RXRα, retinoid X receptor α; SDC1, syndecan-1; SHP, small heterodimer partner.

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The farnesoid X receptor (FXR; NRIH4) is a member of a subclass of the nuclear hormone receptor superfamily of transcription factors that form heterodimers with a common partner, the 9-*cis* retinoid X receptor (RXR). Such transcription factors function by binding to *cis*-acting response elements located within the promoters, introns or enhancers of their target genes, and regulate gene expression, usually in response to the binding of small lipophilic ligands (12). Studies with a limited number of RXR heterodimers suggest that ligands induce a conformational change of the nuclear receptor that promotes the release of corepressor proteins and the subsequent recruitment of coactivator proteins; the net result is increased transcription of the target gene (13, 14).

Differential use of two distinct promoters of the single FXR gene, coupled with alternative mRNA splicing, results in the formation of four FXR isoforms (FXRα1, -α2, -α3, and -α4; originally called FXRα1, -α2, -β1, and -β2) (15, 16). The α1 and α2 isoforms are distinguished from the α3 and α4 isoforms by a truncated N terminus. The FXRα1 and -α3 isoforms contain a four amino acid insert in the hinge region that is absent from both FXRα2 and -α4 (15, 16). The four isoforms differ in their tissue distribution and induce the expression of partially overlapping sets of targets (15, 17).

In 1999, three groups independently reported that specific bile acids are ligands for human and rat FXR (hFXR and rFXR, respectively) and as such bind to and activate FXR at physiologically relevant concentrations (18–20). The most potent of these natural ligands is the primary bile acid chenodeoxycholic acid (CDCA) (18–20). These observations have helped to define a new physiological function for bile acids as metabolically derived regulators of gene expression.

High levels of FXR expression are limited to the liver, intestine, kidney, and adrenals, with low levels reported in the stomach, fat, and heart (15, 16, 21). However, with the exception of the intestinally expressed ileal bile acid binding protein (I-BABP) gene, all other FXR target genes reported to date were identified from analysis of hepatic tissue or cells (22). Hepatic FXR target genes fall into a limited number of groups. One group, which includes the ABC transporters BSEP (21, 23), multidrug resistance-associated protein 2 (MRP2) (24), multidrug resistance protein, MDR3 (human) (25), and rodent Mdr2 (26), together with the fibroblast growth factor-19 (27) and the small heterodimeric partner (SHP) (28–31), functions to decrease hepatic bile acid concentrations by increasing export and decreasing bile acid synthesis. A second group of FXR target genes encode proteins that influence lipoprotein levels in the serum and decrease plasma triglycerides (17, 22, 28, 32–35). The identification of this latter group of FXR target genes may help explain the molecular mechanism underlying the observations that administration of chenodeoxycholate to humans resulted in decreased plasma triglyceride levels (36). Activated FXR also has a hepatoprotective role (26, 37) and regulates genes involved in gluconeogenesis (38). In a recent report, FXR was also shown to induce the human kininogen gene, suggesting a role in anticoagulation (39). In addition, the microarray data reported by Downes et al. (40) suggest that there may be numerous other hepatic FXR target genes that modulate diverse biochemical pathways still to be elucidated. Surprisingly, the latter microarray data also suggest that three structurally unrelated FXR ligands, CDCA, 3-(2,6-dichlorophenyl)-4-(3′-carboxy-2-chloro-stilben-4-yl)-oxymethyl-5-isopropyl-isoxazole (GW4064), and fexaramine, regulate distinct subsets of hepatic genes (40).

In an effort to identify new FXR target genes and define new signaling pathways by which bile acids influence gene expression in the hepatocyte, we performed a microarray-based screen of RNA populations from HepG2 hepatoma cells that overexpress FXR and compared expression profiles from cells that were treated with FXR agonists or vehicle. Here, we report the identification of three novel FXR targets (FBGα, -β, and -γ). These data suggest an unexpected link between bile acid signaling and fibrinolytic activity, and this represents a new paradigm for bile acid function.

MATERIALS AND METHODS

Materials

GW4064 and LG100153, a synthetic RXR agonist, were gifts from Dr. Patrick Maloney (GlaxoSmithKline) (41) and Dr. Richard Heyman (Ligand Pharmaceuticals) (42), respectively. The retroviral vector MSCV-IRES-Neo was a gift from Dr. Owen Witte (UCLA). Mammalian expression vectors for rFXR (pCMX-rFXR) and human RXRα (pCMX-hRXRα) were gifts from Dr. Ron Evans (Salk Institute, La Jolla, CA). Mammalian expression vectors for murine FXR (pCMX-mFXRα1, -α2, -α3, and -α4) have been described (in earlier publications, FXRα3 and -α4 were termed FXRβ1 and -β2, respectively) (15). Cycloheximide and actinomycin D were purchased from Sigma. The sources of other reagents have been noted elsewhere (24).

Cell culture and stable cell lines

The generation and maintenance of HepG2 and stably infected HepG2-rFXR or HepG2-Neo cells have been described (42). HuH7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. CV-1 cells were maintained as described (15).

RNA isolation and Northern blot hybridization

Unless otherwise indicated, HepG2 and HuH7 cells were cultured in medium containing superstripped FBS for 24 h before the addition of ligands or Me_2SO (vehicle) for an additional 8–24 h. Total RNA was isolated using TRIzol reagent and was resolved (5–10 μg/lane) on a 1% agarose, 2.2 M formaldehyde gel, transferred to a nylon membrane (Hybond N^+; Amersham Biosciences, Inc.), and cross-linked to the membrane with ultraviolet light. cDNA probes were radiolabeled with [^{32}P]dCTP using the Rediprime™ II labeling kit (Amersham Biosciences, Inc.). Membranes were hybridized using the QuikHyb hybridization solution (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Blots were normalized for variations of RNA loading by hybridization to a control probe, either 18S ribosomal cDNA or the ribosomal protein 36B4. The RNA levels were quantitated using a PhosphorImager (ImageQuant software; Molecular Dynamics, Inc., Sunnyvale, CA).

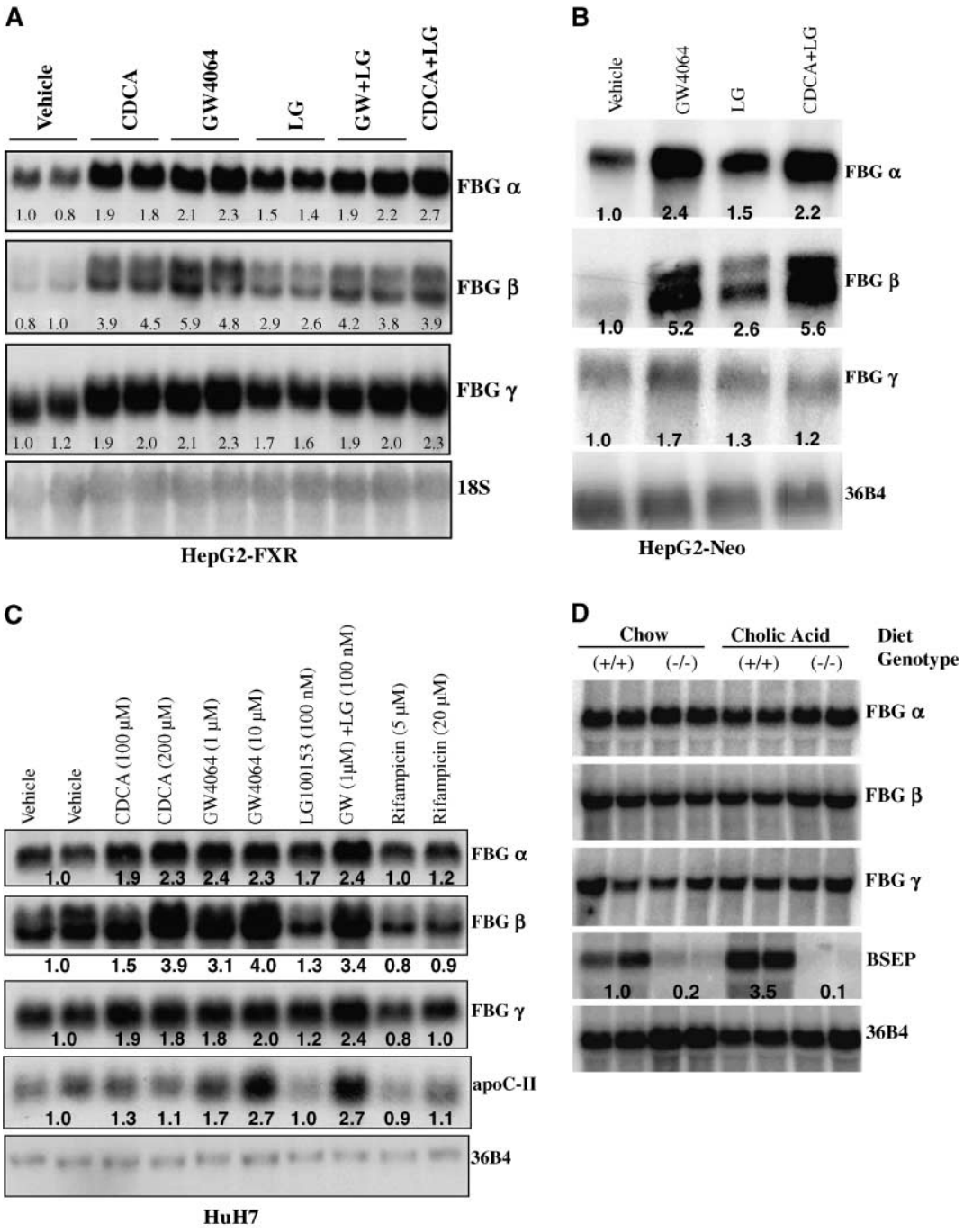

Fig. 1. Induction of fibrinogen α (FBGα), -β, and -γ mRNAs by farnesoid X receptor (FXR) ligands in human hepatoma cells. A: FBGα, -β, and -γ mRNAs are induced by FXR ligands in HepG2-FXR cells. HepG2-FXR cells were treated with vehicle (Me_2SO), chenodeoxycholic acid (CDCA; 100 μM), 3-(2,6-dichlorophenyl)-4-(3′-carboxy-2-chloro-stilben-4-yl)-oxymethyl-5-isopropyl-isoxazole (GW4064; 1 μM), and/or LG100153 (100 nM) for 24 h. Total RNA was isolated, separated on a 1% agarose/formaldehyde gel, transferred to a nylon membrane, and sequentially hybridized to radiolabeled cDNA probes for FBGα, -β, -γ, and 18S rRNA as described in Materials and Methods. The relative FBG mRNA levels are indicated. B: FBGα, -β, and -γ mRNAs are induced by FXR ligands in HepG2-Neo cells. HepG2-Neo cells were treated for 24 with vehicle (Me_2SO), GW4064 (1 μM), LG100153 (100 nM), or CDCA (100 μM) as indicated. RNA was isolated and analyzed as described for A. C: FBGα, -β, and -γ mRNAs are induced in HuH7 cells by ligands for FXR but not pregnane X receptor (PXR). HuH7 cells were treated for 24 h with vehicle (Me_2SO) or the indicated concentrations of ligands for FXR (CDCA and GW4064), retinoid X receptor (RXR; LG100153), or PXR (rifampicin). RNA was isolated and analyzed as described for A. Induction of apolipoprotein C-II (apoC-II) served as a positive control (42). D: Murine FBG hepatic mRNA levels are unaffected by deletion of FXR or after administration of cholic acid to mice. Wild-type (+/+) and FXR null (−/−) mice were fed normal chow or chow supplemented with 1% cholic acid for 5 days before RNA isolation and Northern blot analysis. The relative levels of BSEP mRNA are shown. Values for FBGα, -β, and -γ mRNAs did not differ significantly from the chow fed wild-type mice.

Reporter genes

The promoters for the human FBGβ gene were amplified from the human bacterial artificial chromosome clone RPCI11-21G22 and cloned into the *KpnI/NheI* sites of the pGL3 vector (Promega). The FBGβ −2500/+1 construct was amplified using the 5′ primer 5′-ACACGGTACCACATGATAATATTCTTTG and the 3′ primer 5′-ACACGCTAGCCCATCCTTTTCATGTAGACT. Additional constructs used the same 3′ primer and internal 5′ primers. All constructs were sequenced before transfection and shown to contain wild-type sequence.

Transient transfections and reporter gene assays

HepG2 cells were transiently transfected using the modified bovine serum (MBS) mammalian transfection kit (Stratagene), with minor modifications. HepG2 cells, on 48-well plates, were transiently transfected with a reporter plasmid (100 ng) and 50 ng of pCMX-rFXR, pCMX-mFXRα1, pCMX-mFXRα2, pCMX-mFXRα3, pCMX-mFXRα4, or VP16-hFXR together with 5 ng of pCMX-hRXRα and 50 ng of pCMV-β-galactosidase, as indicated in the figure legends. After 3.5 h, the cells were treated with 10% superstripped FBS and one of the following ligands: CDCA, LG100153 (a synthetic RXR agonist), or GW4064. The cells were lysed, and the luciferase activities were normalized to β-galactosidase activity (24). All transfections were performed in triplicate, and similar results were obtained in at least three independent experiments.

RESULTS

Induction of FBGα, -β, and -γ by natural and synthetic FXR ligands

As detailed in previous reports (24, 42), we screened for target genes that are regulated by the bile acid receptor using HepG2 that were infected with retroviral vectors expressing either rFXRα2 and the neomycin-resistant gene (HepG2-FXR) or the neomycin-resistant gene alone (HepG2-Neo). Total RNA was isolated from HepG2-Neo or HepG2-FXR cells that had been treated for 24 h with vehicle (Me_2SO), the FXR ligand CDCA (100 μM), or the synthetic FXR ligand GW4064 (1 μM). These RNA samples were then used to prepare biotinylated cRNAs that were hybridized to high-density microarrays containing ~6,000 cDNAs/expressed sequence tags (Affymetrix HuFL Gene Chip). This approach identified several genes, including those encoding FBGα, -β, and -γ, whose mRNAs appeared to be induced by treatment of the cells with either natural or synthetic FXR ligands. Other genes identified by this approach, including apolipoprotein C-II (apoC-II), syndecan-1 (SDC1), and MRP2, have been reported elsewhere and are involved in either bile acid or lipoprotein transport and metabolism (17, 24, 42). We chose to explore the regulation of the FBG genes by FXR because their function in clotting represents an entirely new signaling paradigm that potentially links coagulation and clotting to bile acids.

Initially, we used Northern blot analyses to confirm that FBG mRNAs were induced in response to FXR ligands; HepG2-FXR cells were treated with the FXR ligands CDCA (100 μM) or GW4064 (1 μM) or the RXR synthetic ligand LG100153 (100 nM) for 24 h. Treatment with FXR ligands resulted in a 2- to 5.9-fold induction of transcripts from all three FBG genes (**Fig. 1A**). Because GW4064 has been shown to be highly specific for FXR (41), these data suggest that induction of FBGα, -β, and -γ is dependent upon activation of FXR. Addition of the RXR ligand LG100153 also resulted in the induction of all three FBG mRNAs (Fig. 1A).

The studies described above used HepG2 cells that stably overexpress rFXR. To determine whether FBG transcripts are induced in HepG2 cells that do not stably overexpress high levels of FXR, we treated HepG2-Neo cells with GW4064 or the RXR ligand LG268 (RXR agonist) in the presence or absence of CDCA (Fig. 1B). Treatment with these FXR and RXR ligands led to a marked induction of FBGα and -β mRNAs, although only minor changes were noted for FBGγ (Fig. 1B). Thus, the induction of FBG mRNAs in HepG2 cells in response to FXR/RXR ligands is not dependent on stable overexpression of rFXR.

To ensure that the induction of FBG by FXR ligands is not limited to a single hepatoma HepG2 cell line, we also isolated RNA from human hepatoma HuH7 cells after 24 h of treatment with the FXR (CDCA or GW4064) and/or RXR ligands. Figure 1C shows that the all three FBG transcripts were induced after treatment with FXR ligands. The RXR ligand LG100153 led to a relatively low level of induction of FBG transcripts. ApoC-II is a known FXR target and served as a positive control (Fig. 1C).

At pathological concentrations, bile acids can act as ligands for a second nuclear receptor, the pregnane X receptor (PXR). To rule out the possibility that the induction of FBG mRNAs observed upon treatment of HepG2-FXR cells was attributable to the activation of PXR rather than FXR, we treated HuH7 cells with the PXR-specific ligand rifampicin. Figure 1C shows that the addition of rifampicin at two different concentrations failed to induce mRNAs for FBG transcripts. Taken together, the data presented in Fig. 1 indicate that the induction of mRNAs for

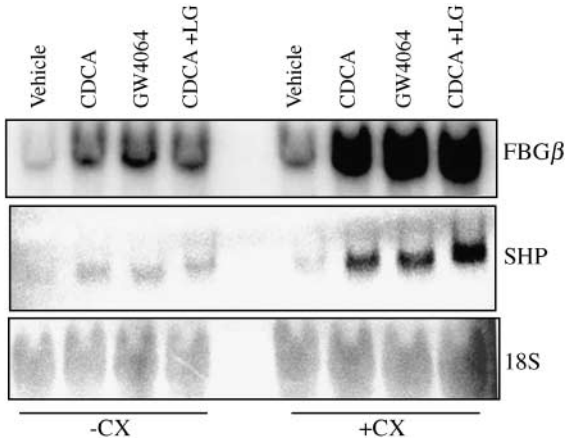

Fig. 2. The induction of FBGβ mRNA by FXR ligands does not require protein synthesis. HepG2-FXR cells were treated for 8 h with 100 μM CDCA with or without 100 nM LG100153 or 1 μM GW4064 in the presence (+) or absence (−) of cycloheximide (CX; 10 μg/ml), as indicated. RNA isolation and Northern blot analysis were performed as described in the legend to Fig. 1. SHP, small heterodimer partner.

all three chains of human FBG is specifically induced in response to FXR ligands.

To examine whether FBG transcripts are also induced in murine liver in response to FXR activation, we fed wild-type and FXR null mice a standard chow or chow supplemented with 1% cholic acid for 5 days. Total liver RNA was isolated and analyzed by Northern blot. As expected, expression of BSEP, a known FXR target gene (21), was induced when the wild-type mice were fed the diet supplemented with cholic acid, whereas BSEP mRNA levels were low in the FXR null mice and were not induced by cholic acid (Fig. 1D). The data in Fig. 1D show that hepatic FBG transcript levels were similar in both wild-type and FXR null mice and were unchanged after administration of the diet supplemented with cholic acid (Fig. 1D). These data indicate that the murine FBG gene is not responsive to FXR. The observation that FBG mRNAs are induced by FXR ligands in two human hepatoma cell types, but not in the livers of mice fed an FXR ligand, suggests that the induction of the three FBG genes is species specific.

Induction of FBGβ by FXR ligands occurs in the presence of cycloheximide

FBGβ, considered to be the nucleating chain for FBG assembly (4, 43), was the most highly induced of the three FBG genes in response to FXR ligands (Fig. 1). Consequently, subsequent studies focused on FBGβ. To determine whether mRNA induction is a primary response that occurs in the absence of protein synthesis, we treated HepG2-FXR cells with vehicle or FXR ligands for 8 h in the presence or absence of cycloheximide before harvesting RNA. The Northern blot data shown in **Fig. 2** demonstrate that induction of FBGβ mRNA by FXR ligands is independent of protein synthesis. The superinduction of FBGβ mRNAs noted in the presence of cycloheximide was also observed for SHP, a well-characterized primary target gene of FXR/RXR (Fig. 2).

Induction of FBG mRNAs by FXR ligands is attenuated by actinomycin D

We next treated HepG2-FXR cells for 8 h in the presence or absence of actinomycin D, an inhibitor of RNA

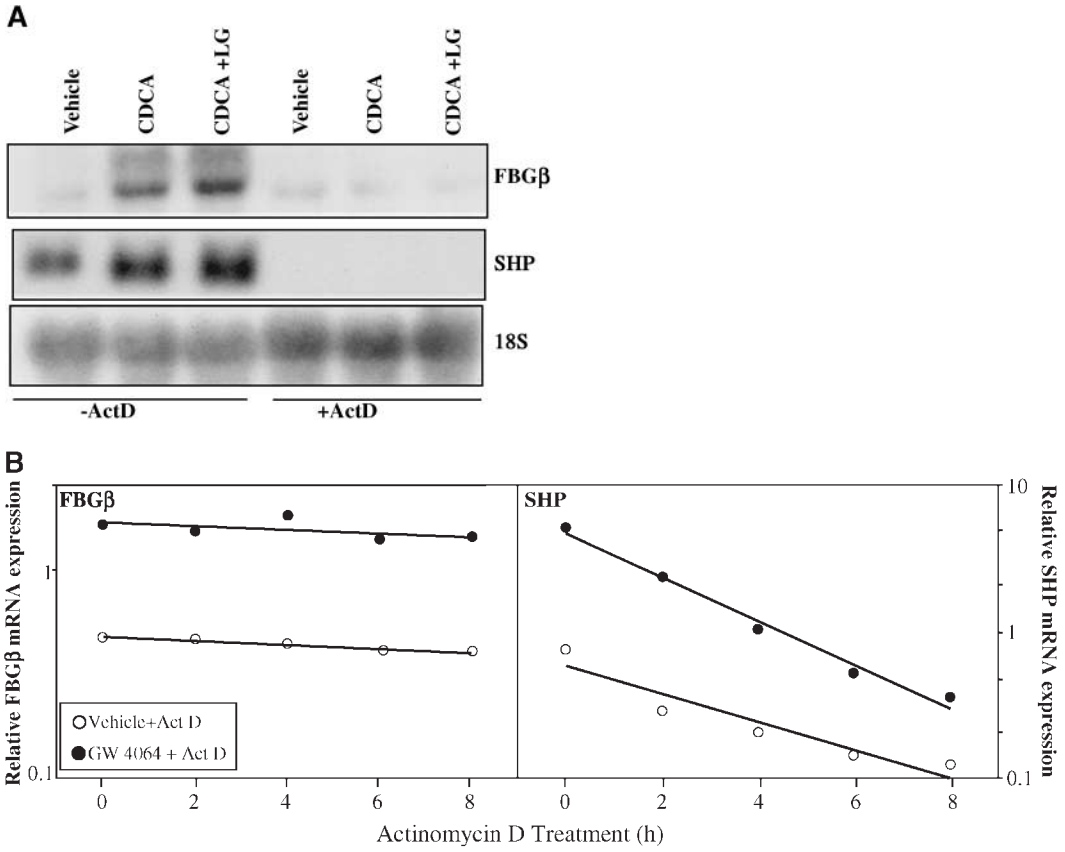

Fig. 3. Induction of FBGβ mRNA by FXR ligands is attenuated by an inhibitor of transcription. A: Actinomycin D (ActD) prevents the FXR ligand-dependent induction of FBGβ. HepG2-FXR cells were cultured in the presence of actinomycin D (5 μg/ml) for 20 min before the addition of vehicle (Me_2SO) or 100 μM CDCA with or without 100 nM LG100153 (a synthetic RXR agonist), as indicated. After 8 h, total RNA was isolated, and Northern blot analysis performed as described in the legend to Fig. 1. B: The half-life of FBGβ mRNA is unaffected by FXR ligands. Cells were cultured for 24 h in the presence of vehicle (Me_2SO) or GW4064 (1 μM). Actinomycin D (5 μg/ml) was then added (0 h) to all dishes, and RNA was isolated after the indicated times. The relative mRNA levels for FBGβ and SHP were determined from Northern blot assays and the data plotted for vehicle-treated (open circles) or GW4064-treated (closed circles) cells.

polymerase II. **Figure 3A** shows that induction of both FBGβ and SHP mRNAs, in response to FXR ligands, was attenuated when cells were simultaneously treated with actinomycin D (Fig. 3A). These data are consistent with the proposal that the induction of FBGβ mRNAs in response to FXR ligands is a transcriptional response.

To rule out the possibility that FXR ligands induced FBGβ mRNA by a process that involved mRNA stabilization, we determined the half-life of the mRNA. HepG2-FXR cells were pretreated for 24 h with either vehicle or the FXR agonist GW4064 to induce FBGβ mRNA. Actinomycin D was then added to all cells to inhibit transcription, and FBGβ mRNA levels were determined during the subsequent 8 h (Fig. 3B). The results demonstrate that the

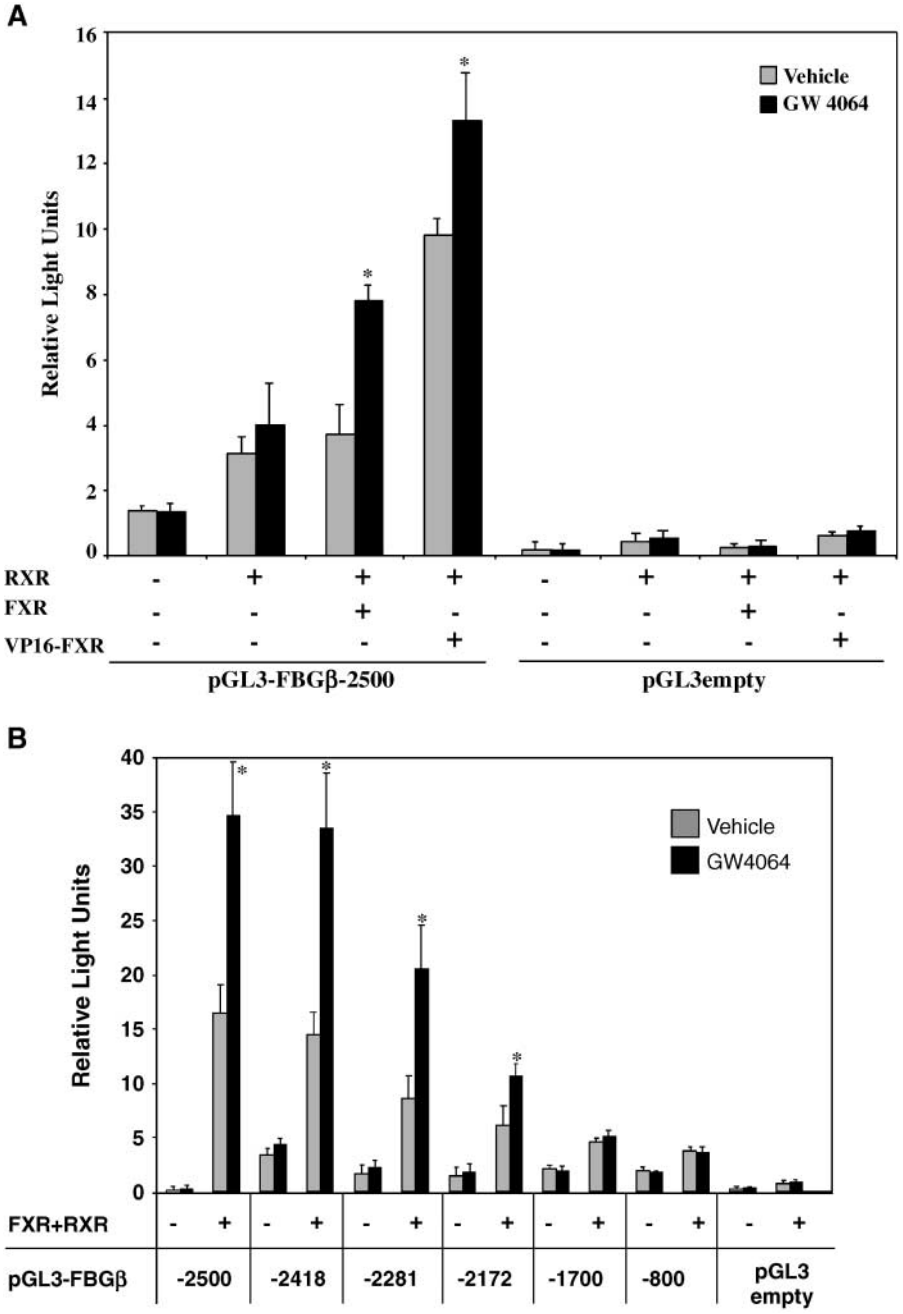

Fig. 4. Transactivation of the human FBGβ promoter by FXR. A: Triplicate dishes of HepG2 cells were cotransfected with plasmids encoding RXRα and either rat FXR (rFXR) or an FXR-VP16 fusion protein and a luciferase reporter gene under the control of the human FBGβ proximal promoter. After transfection, cells were treated with vehicle (Me_2SO) or GW4064 (1 μM) for 24 h. Relative light units are shown after normalization with β-galactosidase. The results are representative of three independent experiments. B: Triplicate dishes of HepG2 cells were cotransfected with plasmids encoding rFXR, RXR, and the indicated reporter gene. Cells were treated for 24 h as indicated, and relative luciferase values were determined as described for A. * $P < 0.01$ vs. controls using Student's *t*-test. Values are shown as mean ± SD.

half-life of FBGβ is greater than 8 h and is not significantly affected by GW4064 treatment (Fig. 3B). The finding that FBGβ mRNA levels decline by <20% after 8 h precludes an accurate determination of the half-life, as such studies would necessarily involve incubation of the cells in the presence of actinomycin D for >24 h. Such conditions are toxic to cells. Nonetheless, based on the data in Fig. 3B, the rapid induction of FBGβ mRNA in response to FXR ligands (Figs. 1, 2) cannot result from stabilization of the FBGβ mRNA. Figure 3B also shows that the SHP mRNA half-life is ~4 h and is also unaffected by GW4064 treatment. Based on the results shown in Figs. 1–3, we conclude that the induction of FBGβ mRNAs is dependent on increased transcription of the gene in response to activated FXR.

The FBGβ promoter is responsive to FXR

Previous studies have shown that IR-1 (an inverted repeat with a 1 bp spacer), ER-8 (an everted repeat with an 8 bp spacer), and DR-1 (a direct repeat with a 1 bp spacer) arrangements of the traditional nuclear hormone receptor binding hexad can function as farnesoid X receptor response elements (FXREs) (24, 44–46). Computer-assisted analysis of 10 kb of the published nucleotide sequence upstream of the transcriptional start site of all three human FBG genes failed to identify any sequences that correspond to a putative IR-1, ER-8, or DR-1 response element. To determine if the proximal FBGβ promoter is responsive to FXR-mediated transcription, ~2.5 kb of the FBGβ proximal promoter was cloned into a luciferase reporter to produce pGL3-FBGβ-2500. This reporter was cotransfected into HepG2 cells in the presence or absence of plasmids encoding RXR and either rFXRα2 (which lacks the four amino insert) or the constitutively active VP16-hFXRα2 fusion protein. The data shown in **Fig. 4A** show that cotransfection of the reporter pGL3-FBGβ-2500 with FXR, followed by treatment with the FXR agonist GW4064, led to a 2- to 5.5-fold induction of luciferase activity compared with cells not treated with ligand and/or not transfected with plasmids encoding FXR and RXR. Luciferase activity was induced to even greater levels when cells were cotransfected with pGL3-FBGβ-2500 and a plasmid encoding the constitutively active VP16-hFXR (Fig. 4A).

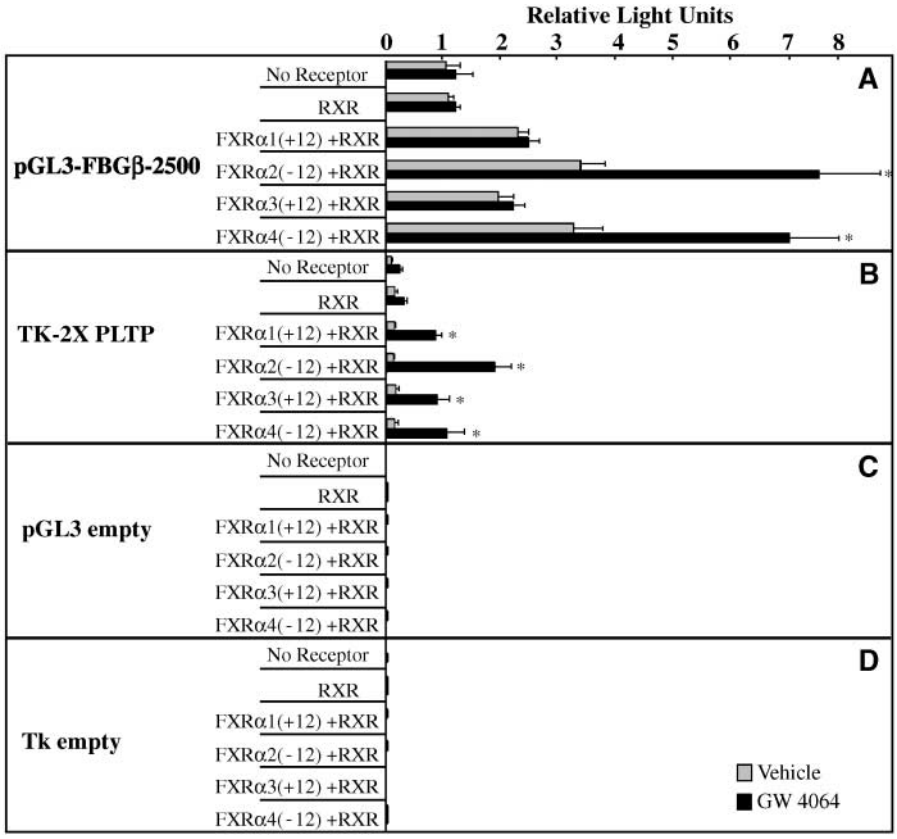

Fig. 5. Transactivation of the human FBGβ promoter by FXR is isoform specific. Triplicate dishes of HepG2 cells were cotransfected with plasmids encoding one of the four murine FXR isoforms (FXRα1, -α2, -α3, or -α4), RXRα, and reporter genes under the control of either the human FBGβ proximal promoter (pGL3-FBGβ-2500) or two copies of the IR-1 (inverted repeat with a 1 bp spacer) farnesoid X receptor response element (FXRE) from the human phospholipid transfer protein (PLTP) gene upstream of a minimal promoter (pTK-2x-PLTP). The presence (+12) or absence (−12) of the 12 bp/4 amino acid insert in the hinge region is indicated for clarification. After transfection, cells were treated with vehicle (Me_2SO) or GW4064 (1 μM) for 24 h. Relative light units are shown after normalization with β-galactosidase activity. The results are representative of three independent experiments. * $P < 0.01$ vs. controls with no GW4064. Values are shown as mean ± SD.

In an attempt to localize the critical *cis* element in the FBGβ promoter, we constructed a series of reporter genes containing 5′ deletions and transiently transfected these into HepG2 cells (Fig. 4B). The data show that successive 5′ deletions resulted in a stepwise decrease in reporter activity (Fig. 4B). The addition of GW4064 activated reporter genes containing from 2,172 to 2,500 bp of the FBGβ promoter but failed to activate genes containing ⩽1,700 bp (Fig. 4B). Extensive additional studies failed to identify a bona fide FXRE between −2,281 and −1,700 bp of the FBGβ promoter. Consequently, we conclude that transcriptional activation of the FBGβ gene by ligand-activated FXR likely depends upon multiple *cis* elements in the proximal promoter, thus making it difficult to identify the FXRE.

Induction of FBGβ by FXR is isoform specific

Alternate splicing and promoter use produces four FXR isoforms from the single mFXR and hFXR genes (15, 16). Some FXR target genes, including SHP, BSEP, and phospholipid transfer protein (PLTP), are transcriptionally activated to similar levels by all four isoforms (15). Other genes, including I-BABP (15) and SDC1 (17), are activated in an FXR isoform-specific manner (15, 17). To explore the possibility that FBGβ is induced by FXR in an isoform-specific manner, we cotransfected cells with the pGL3-FBGβ-2500 luciferase reporter RXR and individual mFXR isoforms and then treated the cells with the FXR-specific ligand GW4064. As shown in **Fig. 5A**, luciferase activity was highly induced in cells cotransfected with plasmids encoding FXRα2 or -α4 and treated with GW4064. In contrast, GW4064 treatment did not increase luciferase activity in the presence of either FXRα1 or -α3 (Fig. 5). In the presence of GW4064, all four FXR isoforms are capable of activating appropriate target genes, which include a luciferase reporter under the control of IR-1 elements from the PLTP gene (Fig. 5B) (15). Luciferase activities from the empty pGL3 and Tk vectors were low and unaffected by these treatments (Fig. 5C, D). Taken together, the transfection data indicate that the FBGβ proximal promoter contains elements that control transcriptional induction of the gene in response to either the FXRα2 or -α4 isoform.

The mechanistic basis for the isoform-specific induction of some FXR targets is not fully understood. The difference may involve different DNA binding properties of the four isoforms, differential association of the isoforms with coactivator proteins, or other factors. Previous reports have suggested that the inability of FXR isoforms α1 and α3 (which contain the four amino acid insert MYTG) to induce the expression of I-BABP is attributable, at least in part, to impaired binding to the FXRE in the promoter of the I-BABP gene (15). It is reasonable to consider that the location of the four amino acid insert, directly adjacent to the DNA binding domain, may account for this observation. Despite extensive studies, we have been unable to identify the response elements responsible for FXR-mediated induction of the FBGβ gene. This precludes

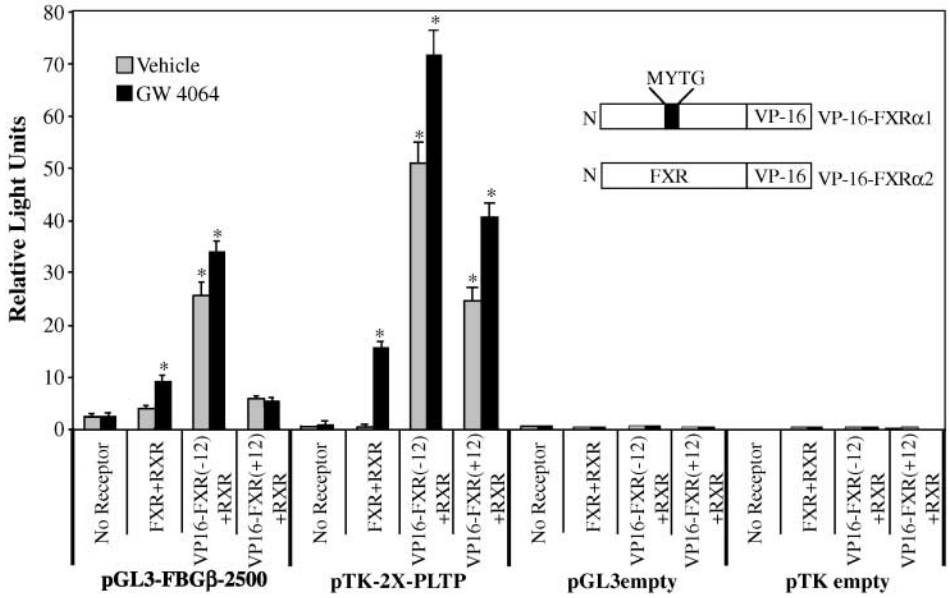

Fig. 6. Isoform-specific activation of the FBGβ promoter is maintained with VP16-FXR fusion proteins. Triplicate dishes of HepG2 cells were cotransfected with plasmids encoding RXRα, rFXRα2, or VP16-human FXR (hFXR) fusion proteins and luciferase reporter genes under the control of either the human FBGβ proximal promoter (pGL3-FBGβ-2500) or two copies of the IR-1 FXRE from the human PLTP gene upstream of a minimal promoter (pTK-2x-PLTP). The presence (+12) or absence (−12) of the 12 bp/4 amino acid insert in the hinge region of the FXR fusion proteins is indicated for clarification. After transfection, cells were treated with vehicle (Me_2SO) or GW4064 (1 μM) for 24 h. Relative light units are shown after normalization with β-galactosidase activity to account for small variations in transfection efficiency. The results are representative of three independent experiments. * $P < 0.01$ vs. controls transfected with FXR/RXR but with no GW4064 treatment. Values are shown as mean ± SD.

studies that would directly assay the ability of various FXR isoforms to bind a critical FXRE necessary for FBGβ induction. Consequently, we generated plasmids that encode either VP16-hFXRα1 or VP16-hFXRα2. The two fusion proteins contain the same VP16 transactivation domain at the carboxyl terminus and differ only by the inclusion/exclusion of the four amino acid insert (**Fig. 6**). We hypothesized that the recruitment of coactivators to both isoforms would be similar because they both contain the potent VP-16 transactivation domain.

HepG2 cells were transiently transfected with the pGL3-FBGβ-2500 luciferase reporter plasmids and, where indicated, plasmids encoding RXRα, FXRα2 (−MYTG), VP16-hFXRα1 (+MYTG), or VP16-hFXRα2 (−MYTG). As expected from earlier results, the pGL3-FBGβ-2500 reporter was induced when cells were cotransfected with FXRα2 and then treated with GW4064 (Fig. 6). Cotransfection of this reporter with a plasmid encoding the VP16-hFXRα2 (−MYTG) fusion protein led to an even higher induction of reporter activity, which occurred in the absence of ligand (Fig. 6). In contrast, cotransfection of the same reporter with a plasmid encoding VP16-hFXRα1 (+MYTG) failed to induce reporter activity in the presence or absence of an FXR ligand (Fig. 6). pTK-2x-PLTP served as a positive control and, as expected (15), was induced by ligand-activated FXR and by both VP16-hFXR constructs even in the absence of added ligand (Fig. 6). As both VP16-hFXRα1 and VP16-hFXRβ1 fusion proteins contain the same highly active VP16 activation domain, it is unlikely that the differences in reporter activation potential on the pGL3-FBGβ-2500 reporter are attributable to differences in coactivator recruitment. Rather, the data suggest that the different activation profiles of pGL3-FBGβ-2500 in response to the two VP16-hFXR fusion proteins is likely the result of differences in binding of FXR/RXR to a response element in the FBGβ promoter. Identification of the this element will be necessary to confirm this hypothesis.

Studies described in Figs. 5, 6 used mFXR isoforms. The data in **Fig. 7** show that the human FBG promoter-reporter gene was also induced by ligand-activated hFXR isoforms that lack the MYTG motif but was unresponsive to the MYTG-containing isoforms. These data suggest that the hFXR and mFXR isoforms are functionally interchangeable in such transient transfection assays.

DISCUSSION

The current study demonstrates that activation of FXR in human-derived liver cells results in the induction of FBGα, -β, and -γ mRNAs. The proteins encoded by the three FBG genes form an equimolar hexamer that constitutes the mature FBG complex (3). FBG is synthesized almost exclusively in the hepatocyte and is secreted into the blood as part of the acute phase inflammatory response (4, 11, 16). FBG expression is known to be activated by IL-6 and glucocorticoids (7, 9). The current data demonstrate that hepatic FBG mRNAs are also induced after activation of FXR. Because FBG is involved in the formation of fibrin, our data suggest that bile acids may also activate the fibrinolytic system. Interestingly, this response appears to

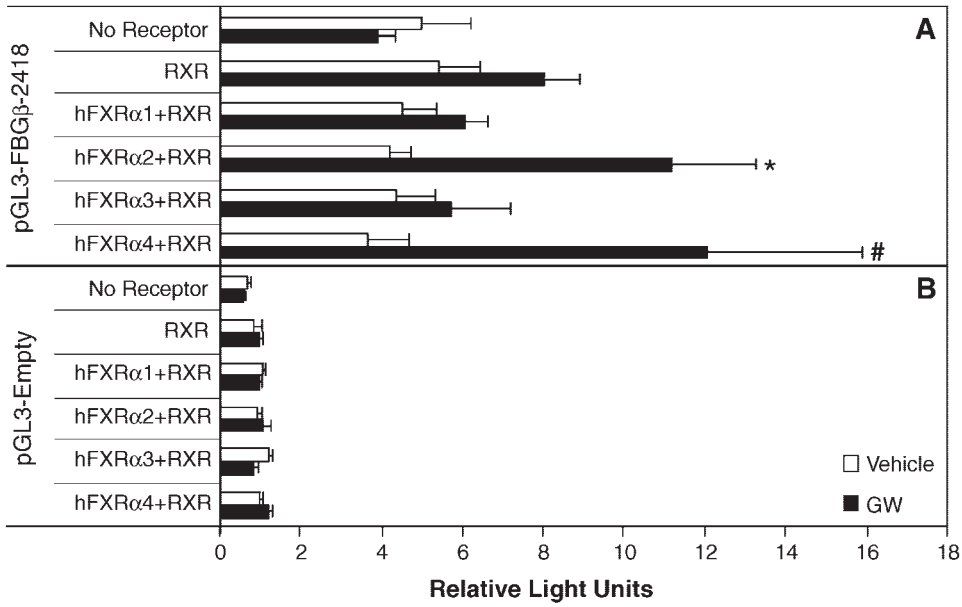

Fig. 7. Isoform-specific transactivation of the human FBGβ promoter is preserved when using different hFXR isoforms. Triplicate dishes of CV-1 cells were cotransfected with plasmids encoding one of the hFXR isoforms (FXRα1, -α2, -α3, or -α4), human RXRα, and reporter gene under the control of the human FBGβ proximal promoter (pGL3-FBGβ-2418) (A) or the empty pGL3 plasmid (B). After transfection, cells were treated with vehicle (Me_2SO) or GW4064 (1 μM) for 48 h. Relative light units are shown after normalization with β-galactosidase activity. * $P < 0.01$, # $P < 0.05$ compared with controls incubated in the absence of GW4064. Values are shown as mean ± SD.

be human/primate specific, because murine hepatic FBG mRNA levels did not increase after activation of FXR (Fig. 1D). In other studies, Zhao et al. (39) demonstrated that FXR activated the human kininogen gene, and it was proposed that FXR and bile acids have a role in vasodilation and anticoagulation. Additional studies will be necessary to determine the effect of FXR activation on coagulation in humans.

The findings that hepatic FBGα, -β and -γ mRNAs were induced in response to multiple FXR ligands, including the natural ligand CDCA or low levels of the synthetic FXR agonist GW4064, suggest that this response is not simply an inflammatory response caused by a toxic compound. This conclusion is supported by the observation that the PXR ligand rifampicin did not elicit the induction of FBG mRNAs.

Analysis of the FBGβ promoter failed to identify any motifs corresponding to traditional FXREs. Cotransfection of a promoter-reporter construct containing 2.5 kb of the FBGβ promoter with FXR and RXR, followed by treatment with FXR ligands, indicated that the FBGβ promoter is responsive to ligand-bound FXR. In addition, activated transcription of FBGβ is a primary response and thus independent of protein synthesis. Our attempts to use deletion analysis to further localize the response element necessary for this induction have been complicated by an apparent complex system of regulation that may involve several *cis* elements that act in an additive or synergistic manner to induce the expression of FBGβ mRNA. Further analysis of the promoter will be necessary to conclusively define the molecular mechanism by which FXR activates transcription of the FBG genes.

Four FXR isoforms are transcribed from the mFXR and hFXR genes. The four isoforms differ in both their tissue distribution and their ability to activate the expression of FXR targets (15, 16). Thus, although some FXR target genes (PLTP and SHP) respond similarly to all four isoforms, others (I-BABP and SDC1) are highly induced in response to FXR isoforms that do not contain the four amino acid insert and are either less responsive or entirely unresponsive to the isoforms that do contain this insert (15). The current data indicate that FBGβ belongs to the second class, because FBGβ promoter-reporter construct activity was highly induced in response to cotransfection with constructs encoding mFXR or hFXR α2 and α4 isoforms (which lack the four amino acid insert) but were unresponsive to constructs that contain this insert (FXRα1 and -α3). These results expand the subset of genes that respond to ligand-bound FXR in an isoform-specific manner. Furthermore, cotransfection assays using plasmids encoding constitutively active VP16-FXR fusion proteins demonstrate that isoform specificity is maintained in the face of a potent heterologous transactivation domain. These results, coupled with previous reports, suggest that the mechanistic basis for the isoform-specific response of the FBGβ promoter to FXR is attributable, at least in part, to differential DNA binding between isoforms that either contain or lack the four amino acid insert in the hinge region.

Our observations that bile acids and FXR induce mRNAs for all three genes necessary for the formation of mature FBG define a new functional paradigm for FXR signaling. Studies that explore the consequences of increased hepatic expression of FBG would help to shed light on an unexpected link between cholesterol metabolism and fibrogenesis. To our knowledge, there are no reports that link serum bile acid levels in humans with FBG levels. As high serum FBG levels are known to be a risk factor for the development of atherosclerosis (1, 47), this study suggests unexpected consequences of activating FXR as a means of reducing plasma triglyceride levels.

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