Activation of the nuclear receptor FXR induces Fibrinogen Expression;
A New Role for Bile Acid Signaling

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Running Title: FXR Regulates Fibrinogen Expression

Key Words- FXR, fibrinogen, chenodeoxycholic acid, GW4064, bile acid, nuclear hormone receptor.

Abbreviations
The abbreviations used are: FXR, farnesoid-X receptor; hFXR, human farnesoid-X receptor; rFXR, rat farnesoid-X receptor; mFXR, murine farnesoid-X receptor; FBG, Fibrinogen; apoC-II, apolipoprotein C-II; CDCA, chenodeoxycholic acid; DR-n, direct
repeat with n-bp spacer; IR-n, inverted repeat with n-bp spacer, ER-8, everted repeat with 8-bp spacer; GW4064, 3-(2,6-dichlorophenyl)-4-(3’carboxy-2-chloro-stilben-4-yl)-oxymethyl-5-isopropyl-isoxazole; I-BABP, ileal bile acid-binding protein; MRP2, multidrug resistance-associated protein 2; RXRa, retinoid X receptor α; SHP, small heterodimer partner; FXRE, Farnesoid-X receptor response element; SDC1, Syndecan-1.
Abstract

Three genes, FBGα, β, and γ, encode proteins that make up the mature fibrinogen protein complex. The 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 (CDCA) or synthetic (GW4064) FXR ligands resulted in 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 have recently been identified that differ either at their amino terminus 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 acids 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.
Introduction

Secretion of fibrinogen (FBG) from hepatocytes into the blood is a key component of the coagulation pathway which ultimately leads to the formation of a fibrous clot in response to vascular disruption (1). The mature FBG protein is a hexamer which 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 extrinsic coagulation pathway leads to the activation of thrombin which then cleaves small peptides, termed A and B, from the fibrinogen hexamer, thus allowing fibrin to form (3). Fibrin can self associate to form filaments which 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 which respond to disruption of the vasculature in order 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 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).

The farnesoid X receptor (FXR, NRIH4) is a member of a subclass of the nuclear hormone receptor (NHR) superfamily of transcription factors that form heterodimers with a common partner, the 9-cis retinoic acid 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 which 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 FXR α1, α2, β1, β2) (15, 16). The α1 and α2 isoforms are distinguished from the α3 and α4 isoforms by a truncated N-terminus. The FXRα1 and FXRα3 isoforms contain a four amino acid insert in the hinge region that is absent from both FXRα2 and FXRα4 (15, 16). The four isoforms differ in their tissue distribution, and induce 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, 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 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), MRP2 (24), MDR3 (25), Mdr2 (26) together with the fibroblast growth factor-19 (FGF-19) (27) and the small heterodimeric partner SHP (28-31) function to decrease hepatic bile acid concentrations by increasing export and decreasing bile acid synthesis. A second group of FXR target genes encode proteins which influence lipoprotein levels in the serum and lower 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 paper, FXR was also shown to induce the human kininogen gene,
suggesting a role in anti-coagulation (39). In addition, the microarray data reported by Downes et al suggest that there may be numerous other hepatic FXR target genes that modulate diverse biochemical pathways that remain to be elucidated (40).

Surprisingly, the latter microarray data also suggest that three structurally unrelated FXR ligands, CDCA, 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 on RNA populations from HepG2 hepatoma cells which over-express FXR, and compared expression profiles from cells which were treated with FXR agonists or vehicle. Here, we report the identification of three novel FXR targets (FBGα, β, γ). The 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 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 rat FXR (pCMX-rFXR), and human RXRa (pCMX-hRXRa), were gifts from Dr. Ron Evans (Salk Institute, La Jolla, CA). Mammalian expression vectors for murine FXR (pCMX FXRα1, 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 has 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 Me2SO (vehicle) for an additional 8-24 hours. 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 UV light. cDNA probes were radiolabeled with [32P]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 Phosphoimager (ImageQuant software; Molecular Dynamics, Inc., Sunnyvale, CA).

**Reporter Genes**- The promoters for the human FBG β gene were amplified from the human BAC 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’-aacctggtaccacatgataatattctttg and the 3’ primer 5’-aacaagctagccacatccttttcatgtagact. Additional constructs utilized the same 3’ primer and internal 5’ primers. All constructs were sequenced prior to transfection and shown to contain wild-type sequence.

**Transient Transfections and Reporter Gene Assays**- HepG2 cells were transiently transfected using the MBS mammalian transfection kit (Stratagene), with minor modifications. HepG2 cells, in 48 well plates, were transiently transfected with a reporter plasmid (100 ng) and either 50 ng of pCMX-rFXR, pCMX-mFXRα1, pCMX-mFXRα2, pCMX-mFXRα3, pCMX-mFXRα4, or VP16-hFXR together with 5 ng pCMX-hRXRα, and 50 ng of pCMV-β-galactosidase, as indicated in the specific figure legends. After 3.5 h, the cells were treated with 10% superstripped FBS (SS-FBS) and one of the following ligands: CDCA, LG100153 (synthetic RXR agonist), 3-[(2,6-dichlorophenyl)-4-(3’-carboxy-2-chloro-stilben-4-yl)-oxymethyl-5-isopropyl-isoxazole (GW 4064). 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 that express either rat FXRα2 (rFXR) and the neomycin-resistant gene (HepG2-FXR), or the neomycin resistance gene alone (HepG2-Neo). Total RNA was isolated from HepG2-Neo or HepG2-FXR cells that had been treated for 24 h with either vehicle (Me2SO), the FXR ligand CDCA (100 µM), or the synthetic FXR ligand GW4064 (1 µM). These RNA samples were then used to prepare biotinylated cRNAs which were hybridized to high-density micro-arrays containing ~6,000 cDNA/ESTs (Affymetrix HuFL Gene Chip). This approach identified several genes, including those encoding FBGα, FBGβ, and FBGγ, whose mRNA levels appeared to be induced by treatment of the cells with either natural or synthetic FXR ligands. Other genes identified by this approach, including apoC-II, 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 as their function in clotting represents an entirely new signaling paradigm which potentially links coagulation and clotting to bile acids.
Initially we used northern blot analyses to confirm that FBG mRNA levels 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). Since 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 induction of all three FBG mRNAs (Fig. 1A).

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

To ensure that 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 following 24 h treatment with the FXR (CDCA or GW4064) and/or RXR ligands. The data of Fig. 1C show that the all three FBG transcripts were induced following 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 serves 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 the treatment of HepG2-FXR cells was due to activation of PXR, rather than FXR, we treated HuH7 cells with the PXR specific ligand rifampicin. Figure 1C shows that addition of rifampicin at two different concentrations failed to induce mRNA levels for FBG transcripts. Taken together, the data presented in Fig. 1 indicate that the induction of mRNAs for 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/− mice a standard chow, or chow supplemented with 1% cholic acid for five 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 of Fig. 1D show that hepatic FBG transcript levels were similar in both wild-type and FXR null mice and were unchanged following administration of the diet supplemented with cholic acid (Fig. 1D). These data indicate that the murine fibrinogen gene is not responsive to FXR. The observation that FBG mRNA levels 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 fibrinogen assembly (4, 43), was the most highly induced of the three FBG genes in response to FXR ligands (Fig 1). Consequently, subsequent more detailed studies focused on FBGβ. In order 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 of Fig. 2 demonstrate that induction of FBGβ mRNA by FXR ligands is independent of protein synthesis. The super-induction of FBGβ mRNA levels 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 mRNA levels 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 polymerase II. The data of Figure 3A show that induction of both FBGβ and SHP mRNA levels, 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 mRNA levels in response to FXR ligands is a transcriptional response.

To rule out the possibility that FXR ligands induced FBGβ mRNA levels by a process that involved mRNA stabilization, we determined the half life of the mRNA;
HepG2-FXR cells were pre-treated for 24 h with either vehicle or the FXR agonist GW4064 to induce FBGβ mRNA levels. 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 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 less than 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 of Fig. 3B, the rapid induction of FBGβ mRNA in response to FXR ligands (Figs. 1 and 2) cannot result from stabilization of the FBGβ mRNA. The data of Fig. 3B also shows that the SHP mRNA half life approximates 4 h, and is also unaffected by GW4064 treatment. Based on the results of Figs. 1-3, we conclude that induction of FBGβ mRNA levels 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, ER-8, and DR-1 arrangements of the traditional nuclear hormone receptor binding hexad can function as 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. In order 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 co-transfected into HepG2 cells in the presence or absence of plasmids encoding RXR and either rat FXRα2 (which lacks the four amino insert), or the constitutively active VP16-hFXRα2 fusion protein. The data of Fig. 4A show that co-transfection 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 to cells either 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 co-transfected with pGL3-FBGβ-2500 and a plasmid encoding the constitutively active VP16-hFXR (Fig. 4A).

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). Addition of GW4064 activated reporter genes containing from 2172 to 2500 bp of the FBGβ promoter but failed to activate genes containing ≤1700 bps (Fig. 4B). Extensive additional studies failed to identify a bona fide FXRE between -2281 and -1700 bps 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 the FBGβ by FXR is isoform specific*- Alternate splicing and promoter usage produces four FXR isoforms from the single murine and human FXR genes (15, 16). Some FXR target genes, which include SHP, BSEP and PLTP, are
transcriptionally activated to similar levels by all four isoforms (15). Other genes, including I-BABP (15) and syndecan-1 (17), are activated in an FXR isoform-specific manner (15, 17). To explore the possibility that FBGβ levels are induced by FXR in an isoform specific manner, we co-transfected cells with the pGL3-FBGβ-2500 luciferase reporter, RXR and individual murine FXR isoforms, and then treated the cells with the FXR-specific ligand GW4064. As shown in Fig. 5, panel A, luciferase activity was highly induced in cells co-transfected with plasmids encoding FXRα2 or FXRα4 and treated with GW4064. In contrast, GW4064 treatment did not increase luciferase activity in the presence of either FXRα1 or FXRα3 (Fig. 5). In the presence of GW4064, all four FXR isoforms are capable of activating appropriate target genes, that 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. 5, panels C, D). Taken together, the transfection data indicate that the FBGβ proximal promoter contains elements which control transcriptional induction of the gene in response to either FXRα2 or α4 isoforms.

The mechanistic basis for the isoform specific induction of some FXR targets is not fully understood. The difference may have to do with 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 4 amino acid insert MYTG) to induce expression of I-BABP is due, at least in part, to impaired binding to the FXRE
in the promoter of the I-BABP gene (15). It is attractive 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 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 carboxy terminus, and differ only by the inclusion/exclusion of the four amino acid insert (Fig. 6). We hypothesized that the recruitment of co-activators to both isoforms would be similar since 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 co-transfected with FXRα2, and then treated with GW4064 (Fig. 6). Co-transfection of this reporter with a plasmid encoding the VP16-hFXRα2 (-MYTG) fusion protein, led to an even higher induction of reporter activity, that occurred in the absence of ligand (Fig. 6). In contrast, co-transfection 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 due to differences in co-activator recruitment. Rather, the data suggest that the different activation profile of pGL3-FBGβ-2500 in response to the two VP16-hFXR fusion proteins is likely due to 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 and 6 utilized murine FXR isoforms. The data in Fig. 7 show that the human fibrinogen promoter-reporter gene was also induced by ligand-activated human FXR isoforms that lack the MYTG motif, but was unresponsive to the MYTG-containing isoforms. These data suggest that the human and murine FXR 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 induction of FBGα, β, and γ mRNA levels. The proteins encoded by the three FBG genes form an equimolar hexamer that comprise 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 mRNA levels are also induced following activation of FXR. Since 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 be human/primate specific, since murine hepatic FBG mRNA levels did not increase following activation of FXR (Fig 1D). In other studies, Zhao et al demonstrated that FXR activated the human kininogen gene, and it was proposed that FXR and bile acids have a role in vasodilation and anti-coagulation (39). Additional studies will be necessary in order to determine the effect of FXR activation on coagulation in humans.

The findings that hepatic FBGα, β and γ mRNA levels 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 due to a toxic compound. This conclusion is supported by the observation that the PXR ligand rifampicin did not illicit an induction of FBG mRNA levels.
Analysis of the FBGβ promoter failed to identify any motifs corresponding to traditional FXREs. Co-transfection 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 which may involve several cis elements that act in an additive or synergistic fashion to induce expression of FBGβ mRNA. Further, detailed analysis of the promoter will be necessary to conclusively define the molecular mechanism by which FXR activates transcription of the fibrinogen genes.

Four FXR isoforms are transcribed from the murine and human FXR genes. The four isoforms differ in both their tissue distribution, and their ability to activate expression of FXR targets (15, 16). Thus, while some FXR target genes (PLTP, SHP), respond similarly to all four isoforms, others (I-BABP, SDC1), are highly induced in response to FXR isoforms that do not contain the 4 amino acids insert, and are either less responsive, or entirely unresponsive to the isoforms that do contain this insert (15). The current data indicates that FBGβ belongs to the second class since FBGβ promoter-reporter construct activity was highly induced in response to co-transfection with constructs encoding murine or human FXRα2 and α4 isoforms (which lack the 4 amino acid insert), but were unresponsive to constructs which contain this insert (FXRα1 and α3). These results expand the subset of genes which respond to ligand
bound FXR in an isoform specific manner. Furthermore, co-transfection 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 isoform specific response of the FBGβ promoter to FXR is due, 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 mRNA levels 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), the current studies suggest unexpected consequences of activating FXR as a means to reduce plasma triglyceride levels.
Acknowledgments

We thank Drs R. Evans, R. Heyman, P. Tontonoz, O. Witte, and P. Maloney for providing plasmids and reagents. We thank members of the Edwards laboratory for critical comments during these studies. This work was supported by National Institutes of Health Grants HL30568 and HL68445 (to P.A.E.), a grant from the Laubisch Fund (to P.A.E.), from the United States Department of Education Grant P200A80224 (to A.M.A. and H.R.K) and from the American Heart Association, Western Affiliate (Y.Z.).
References


**Figure Legends**

Fig. 1. **Induction of FBGα, β, and γ mRNAs by FXR ligands in human hepatoma cells.**

A, FBGα, β, and γ mRNA levels are induced by FXR ligands in HepG2-FXR cells. HepG2-FXR cells were treated with vehicle (Me2SO), CDCA (100 µM), 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, FBGβ, FBGγ, and 18S ribosomal RNA, as described under “Experimental Procedures.” The relative FBG mRNA levels are indicated.

B, FBGα, β, and γ mRNA levels are induced by FXR ligands in HepG2-Neo cells. HepG2-Neo cells were treated for 24 with vehicle (Me2SO), GW4064 (1 µM), LG100153 (100 nM), or CDCA (100 µM), as indicated. RNA was isolated and analyzed as described in “A.”

C, FBGα, β and γ mRNA levels are induced in HuH7 cells by ligands for FXR, but not PXR. HuH7 cells were treated for 24 h with vehicle (Me2SO), or the indicated concentrations of ligands for FXR (CDCA, GW4064), RXR (LG100153), or PXR (Rifampicin). RNA was isolated and analyzed as described in “A.” Induction of apoC-II serves as a positive control (42).

D, murine FBG hepatic mRNA levels are unaffected by deletion of FXR or following administration of cholic acid to mice. Wild-type (+/+) and FXR null (-/-) mice were fed normal chow or chow supplemented with 1% cholic acid for five days prior to RNA isolation and northern blot analysis. The relative levels of BSEP mRNA are shown. Values for fibrinogen α, β, and γ mRNAs did not differ significantly from the chow fed wild-type mice.
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 analysis were performed as described in the legend to Fig. 1.

Fig. 3. **Induction of FBGβ mRNA by FXR ligands is attenuated by an inhibitor of transcription.** A, Actinomycin D prevents the FXR ligand-dependent induction of FBGβ. HepG2-FXR cells were cultured in the presence of actinomycin D (ActD)(5 µg/ml) for 20 minutes before the addition of vehicle (Me2SO) or 100 µM CDCA with or without 100 nM LG100153, as indicated. After 8 h, total RNA was isolated, and Northern 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 (Me2SO), or GW4064 (1 µM). Actinomycin D (5 µg/ml) was then added (0 h) to all dishes and RNA isolated after the indicated time period. The relative mRNA levels for FBGβ and SHP were determined from northern blot assays and the data plotted for vehicle (○) or GW4064 (●) treated cells.

Fig. 4. **Transactivation of the human FBGβ promoter by FXR.** A, Triplicate dishes of HepG2 cells were co-transfected with plasmids encoding RXRα and either rat FXR, or an FXR-VP16 fusion protein, and a luciferase reporter gene under the control of the human FBGβ proximal promoter. Following transfection, cells were
treated with vehicle (Me₂SO) or GW4064 (1µM) for 24 h. Relative light units are shown following normalization with β-galactosidase. The results are representative of three independent experiments. B, Triplicate dishes of HepG2 cells were co-transfected with plasmids encoding rFXR, RXR, and the indicated reporter gene. Cells were treated for 24 h as indicated and relative luciferase values determined as described in 4A. *, P<0.01 vs controls, using Student t-test.

Fig. 5. Transactivation of the human FBGβ promoter by FXR is isoform specific. A, Triplicate dishes of HepG2 cells were co-transfected 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 FXRE from the human PLTP gene upstream of a minimal promoter (pTK-2X PLTP). The presence (+12), or absence (-12) of the 12 base pair/4 amino acid insert in the hinge region is indicated for clarification. Following transfection, cells were treated with vehicle (Me₂SO) or GW4064 (1 µM) for 24 h. Relative light units are shown following normalization with β-galactosidase activity. The results are representative of three independent experiments. *, P<0.01 vs controls with no GW4064.

Fig. 6. Isoform specific activation of the FBGβ promoter is maintained with VP16-FXR fusion proteins. A, Triplicate dishes of HepG2 cells were co-transfected with plasmids encoding RXRα, rat FXR α2, or Vp16-human FXR 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 base pair/4 amino acid insert in the hinge region of the FXR fusion proteins is indicated for clarification. Following transfection, cells were treated with vehicle (Me2SO) or GW4064 (1 µM) for 24 h. Relative light units are shown following 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.

Fig. 7. Isoform-specific transactivation of the human FBGβ promoter is preserved when using different human FXR isoforms. Triplicate dishes of CV-1 cells were co-transfected with plasmids encoding one of the human FXR 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). Following transfection, cells were treated with vehicle (Me2SO) or GW4064 (1 µM) for 48 h. Relative light units are shown following normalization with β-galactosidase activity. *p<0.01; #p<0.05, compared to controls incubated in the absence of GW4064.
### Fig. 1A

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Fig. 3B

![Graph showing relative FBGβ and SHP mRNA expression](image)

- FBGβ
  - Vehicle + Act D (empty circle)
  - GW4064 + Act D (filled circle)
- SHP
  - Vehicle + Act D (empty circle)
  - GW4064 + Act D (filled circle)

Relative mRNA expression on the y-axis and Actinomycin D treatment (h) on the x-axis.
Fig. 4A

A

Relative Light Units

RXR

FXR

V216-FXR

pGL3-FBΔp-2500

pGL3-empty

Vehicle

GW 4064

*
Fig. 4B

![Bar graph showing relative light units for different conditions](image)

- **Vehicle**
- **GW4064**

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Fig. 5

Relative Light Units

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**pGL3-FBGP-2500**

**TK-2X PLTP**

**pGL3 empty**

**Tk empty**

[Legend: Vehicle, GW 4064]