

# Identification of PLTP as an LXR target gene and apoE as an FXR target gene reveals overlapping targets for the two nuclear receptors

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**Abstract** Affymetrix microarray data and Northern blot assays demonstrated that phospholipid transfer protein (PLTP) was induced 6-fold when either murine or human macrophages were incubated in the presence of ligands for the liver X receptor (LXR) and the retinoid X receptor. Two functional LXR response elements (LXREs) were identified and characterized in the proximal promoter of the human PLTP gene. One LXRE corresponds to a traditional direct repeat separated by 4 bp. However, the second LXRE is novel in that it corresponds to an inverted repeat separated by 1 bp, and is identical to the farnesoid X receptor response element. These studies demonstrate that PLTP is a direct target for activated LXR and farnesoid X receptor (FXR). In addition, apolipoprotein E (apoE), a known LXR target gene in macrophages, was shown to be activated in liver cells by FXR ligands. Taken together, the current data suggest that a small number of genes that currently include PLTP, apoE, and apoC-II, are induced in macrophages by activated LXR and in liver by activated FXR.—Mak, P. A., H. R. Kast-Woelbern, A. M. Anisfeld, and P. A. Edwards. Identification of PLTP as an LXR target gene and apoE as an FXR target gene reveals overlapping targets for the two nuclear receptors. *J. Lipid Res.* 2002. 43: 2037–2041.

**Supplementary key words** macrophages • foam cells • hepatocytes • phospholipid transfer protein • liver X receptor • retinoid X receptor

Phospholipid transfer protein (PLTP) is a member of the lipid transfer/lipopolysaccharide binding protein gene family that includes CETP, the lipopolysaccharide binding protein, and the bactericidal/permeability increasing protein (1). PLTP has broad substrate specificity and functions to transfer phospholipids from triglyceride rich particles (VLDL and chylomicrons) to HDL during lipoprotein lipolysis (2). PLTP mRNA is expressed in a number of tissues, including liver, ovary, thymus, and placenta (3). Recent studies have demonstrated that PLTP is a tar-

get gene for activated farnesoid X receptor (FXR) (4, 5). This conclusion was based on the findings that *i*) FXR ligands induced PLTP mRNA levels in cultured human hepatocytes (4) and in the livers of wild type but not FXR null mice (6), and *ii*) the proximal promoter of the human gene contains a functional FXR response element (FXRE) (4, 5).

In the current report, we utilized murine and human macrophages to demonstrate that PLTP is also an liver X receptor (LXR) target gene. Since macrophages do not express FXR, the results demonstrate that PLTP can be regulated independently by either receptor. Apolipoprotein E (apoE) is a known LXR target gene in macrophages (7, 8). We now demonstrate that the hepatic expression of apoE is induced by FXR ligands. Taken together, these studies provide evidence that a subset of genes, which currently includes PLTP, apoE, and apoC-II (4–8), are induced following activation of LXR or FXR and control lipid metabolism.

## EXPERIMENTAL PROCEDURES

### Reagents, RNA isolation, Northern blot analysis, transient transfections, and reporter gene assays

Expression plasmids, sources of all reagents, and the indicated procedures have been described (6, 7, 9, 10). T0901317, chenodeoxycholate (CDCA), LG100153 (LG), and GW4064 (GW) were obtained from Cayman Chemical (Ann Arbor, MI), Sigma (St. Louis, MO), Dr. R. Heyman (Ligand Pharmaceuticals, La Jolla, CA), and Dr. T. Willson (GlaxoSmithKline), respectively.

### Cell culture

Murine peritoneal macrophages, human macrophages, and HepG2 cells were isolated and/or cultured as described (6, 7).

Abbreviations: apo, apolipoprotein; CDCA, chenodeoxycholic acid; EMSA, electrophoretic mobility shift assay; FXR, farnesoid X receptor; FXRE, FXR response element; GW, GW4064, a synthetic FXR agonist; LG, LG100153, a synthetic RXR agonist; LXR, liver X receptor; LXRE, LXR response element; RXR, retinoid X receptor.

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11). HUH7 cells were maintained in DMEM containing 10% calf serum, and were incubated for 24 h in DMEM containing 10% super stripped FBS prior to addition of ligands or vehicle.

### Reporter genes

BLAST searches using the human PLTP promoter sequence (U38950) (3) identified three overlapping 5' EST clones (BM908408, BM019368 and BM050369) that extended the 5' end of the PLTP transcript (3, 5) by 90 bp (+1 in the current study).

The human PLTP proximal promoter fragments were amplified from human genomic DNA and cloned into *SacI/HindIII* sites of the pGL3 basic vector (Promega). The -2776/+86 and -539/+86 constructs were amplified using the common 3' primer 5'-acacaagcttggcgggcagggtgtcc-3' and 5' primers 5'-acacga gctctgacagagcgagactccacc-3' or 5'-acacgagctctggcagacctctctg-3', respectively. Four-base pair mutations of LXR response element (LXRE1) at -2669 and LXRE2 at -249 were introduced by QuikChange Mutagenesis Kit (Stratagene) using sense primers 5'-gcttgagccaggctctaggaagAAtactagagAcaaaaggaac-3' and 5'-ggaag aaactgaggAAcagtgTTcaagtgaagtgtgcc-3', respectively. Mutations are capitalized.

### Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed as described (6). The sense strand sequences were: LXRE1 5'-ctctaggaaggttactagagttcaaaaggaaca-3', mut LXRE1 5'-ctctaggaagAAtactagagAcaaaaggaaca-3', Cyp7a 5'-gtttgcttggcactcaagttca agttattg-3', LXRE2 5'-aagaaactgagggtcagtgacccaagtgaagtg-3', mut LXRE2 5'-aagaaactgaggAAcagtgTTcaagtgaagtg-3', and I-BABP 5'-ttcctaaggtgaataaccttggggctcct. Mutations are capitalized.

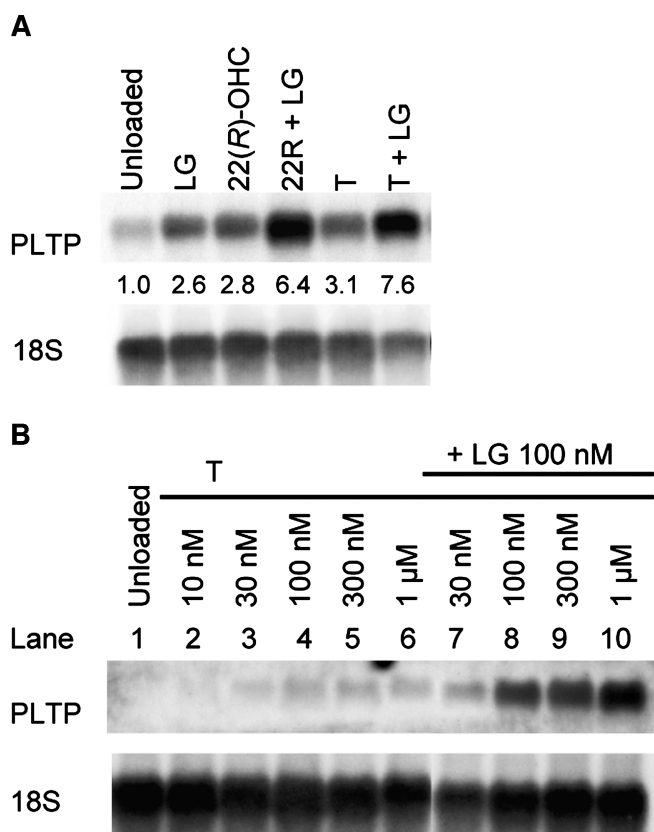
## RESULTS AND DISCUSSION

### Identification of PLTP as an LXR target gene in primary macrophages

Murine peritoneal macrophages were isolated from C57BL/6 mice 4 days after thioglycollate injection, allowed to adhere to tissue culture dishes for 15 h, and then incubated for an additional 36 h either in a cholesterol-poor medium or in medium supplemented with 1  $\mu$ M T0901317 and 100 nM LG, ligands that are specific for LXR and retinoid X receptor (RXR), respectively. RNA was isolated and processed for hybridization to Affymetrix microarrays (MG-U74Av2) as described (7).

The Affymetrix data suggested that PLTP mRNA levels were induced 7-fold in response to T0901317 and LG. Consequently, we isolated RNA from murine peritoneal macrophages that had been incubated in the presence of ligands for LXR (22(*R*)-hydroxycholesterol or T0901317), RXR (LG), or FXR. As shown in **Fig. 1A**, PLTP mRNA levels were induced  $\leq$ 7.6-fold in response to ligands for LXR and/or RXR. In contrast to the liver (4–6), macrophages do not express FXR (data not shown) and thus FXR ligands alone do not induce PLTP mRNA levels (data not shown).

Figure 1B shows that the human PLTP gene was also induced when primary human macrophages were incubated with specific LXR/RXR ligands. PLTP mRNA levels were undetectable in sterol-deprived human macrophages (Fig. 1B, lane 1), were induced significantly in response to T0901317 (lanes 2–6), and were further increased in the



**Fig. 1.** Induction of PLTP mRNA following addition of ligands for liver X receptor (LXR) and/or retinoid X receptor (RXR) to primary macrophages. Murine peritoneal macrophages (A) or human monocytes macrophages (B) were incubated for 36 h with either vehicle, 5  $\mu$ M compactin and 100  $\mu$ M mevalonic acid (unloaded) or the indicated ligands. A: The ligand concentrations were: T0901317, 5  $\mu$ M; 22(*R*)-OHC, 5  $\mu$ M (LXR ligands); and/or LG100153 (LG), 100 nM (RXR ligand). The PLTP mRNA level in unloaded cells was arbitrarily given a value of 1.0 in A and was undetectable in B. The results are representative of two experiments.

presence of both LG and T0901317 (lanes 7–10). PLTP mRNA levels were induced within 7 h and remained elevated ( $>17$ -fold) after 60 h in the presence of both ligands (data not shown).

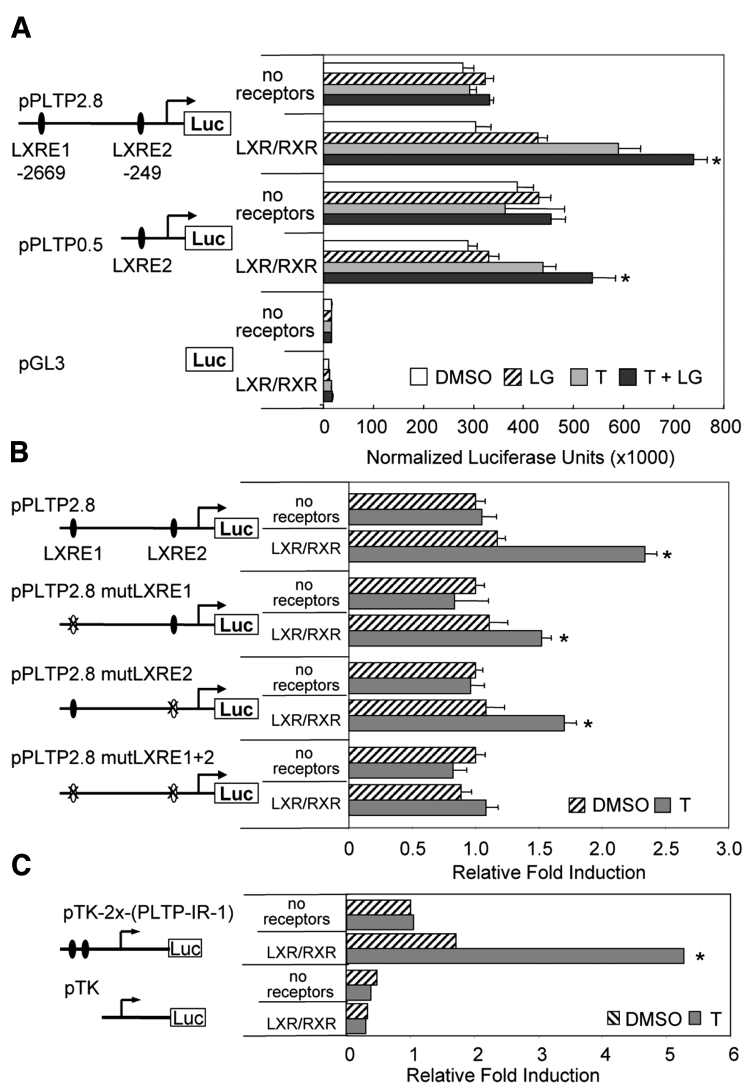
### The proximal promoter of the human PLTP gene contains two functional LXREs

To date, all functional LXREs identified in LXR target genes correspond to a DR-4 (12, 13). Analysis of the proximal promoter of the human PLTP gene, using both visual scanning and FINDPATTERNS algorithm from the Genetic Computing Group package, identified a DR-4 element (-2669 AGGTTActagAGTTCA -2654). In order to determine whether the LXRE motif is important in the transcriptional activation of the human PLTP gene, we generated a number of human PLTP promoter-luciferase constructs (**Fig. 2A**). HepG2 cells were transiently transfected with the indicated reporter plasmids in the presence or absence of the plasmids encoding LXR and RXR, and the cells were then incubated for 24 h in the presence of specific ligands. Figure 2A shows that pPLTP2.8 (-2776/+86) was activated by ligands for LXR and/or

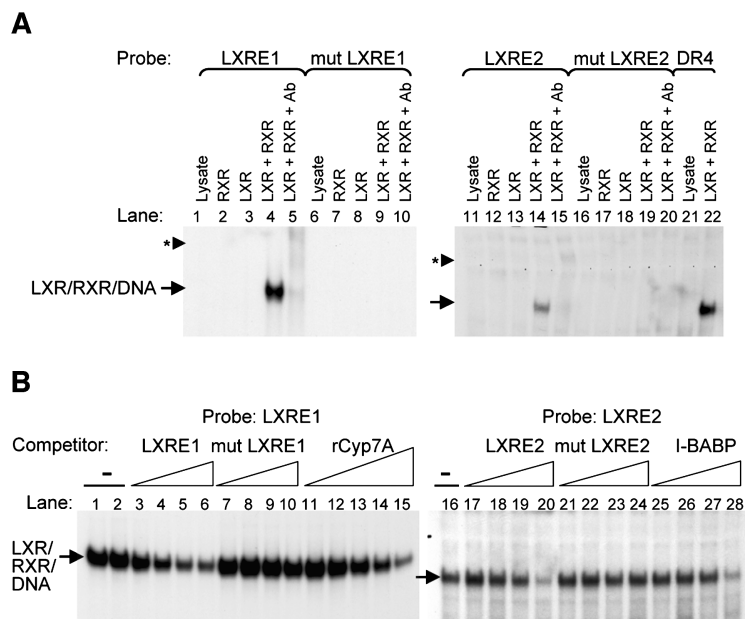
RXR in the presence of both nuclear receptors. However, we consistently noted that a shorter construct (pPLTP0.5), which does not contain the DR-4, was modestly activated (Fig. 2A). These data suggest that there may be an additional LXRE within the  $-539/+86$  region. The only hexanucleotide consensus sequence present within this region corresponds to an IR-1 ( $-249$  GGGTCAGTGACCC  $-236$ ) that is known to function as an FXRE (4, 5). In order to investigate the importance of the DR-4 and IR-1 motifs, named LXRE1 and LXRE2 respectively, we introduced a 4-bp mutation into each of the response elements. The data of Fig. 2B show that the wild type promoter was induced 2.3-fold by T0901317 in the presence of LXR/RXR, and that the induction was attenuated, but not abolished,

when mutations were incorporated into either LXRE1 or LXRE2. In contrast, mutation of both response elements (pPLTP2.8-mutLXRE1+2) completely abolished the induction by T0901317 (Fig. 2B).

In order to demonstrate that the IR-1 element functions as an LXRE, we transfected cells with a TK-reporter gene under the control of two copies of the IR-1 from the PLTP promoter. This reporter gene was activated 5.3-fold by T0901317 in the presence of LXR/RXR, while the empty TK-luciferase construct was unresponsive (Fig. 2C). Taken together, these data identify human PLTP as a direct target gene of LXR and suggest that both the LXRE1 and LXRE2 are necessary for maximal induction in response to activated LXR/RXR. Since LXR ligands also ac-



**Fig. 2.** LXR/RXR activates the PLTP promoter via LXRE response element (LXRE)1 and LXRE2. A and B: HepG2 cells were transiently transfected in triplicate with 100 ng of the indicated human PLTP promoter-reporter gene construct containing wild-type LXREs (solid ovals) or mutated LXREs (crossed open ovals) and, where indicated, pLXR $\alpha$  (50 ng) and pRXR $\alpha$  (5 ng). Cells were incubated for 24 h in the presence of DMSO, LG (100 nM) and/or T0901317 (1  $\mu$ M). Luciferase activities were normalized for variations in transfection efficiency. Error bars represent standard deviations. B: Luciferase activities are given relative to the untreated cells. C: Triplicate dishes of HepG2 cells were transiently transfected with pTK-2x-(PLTP-IR-1) or the empty vector (pTK-Luc) as described above. Results are representative of three experiments. Asterisks indicate statistical significant differences between the ligand treated and untreated control, where  $P < 0.01$ .



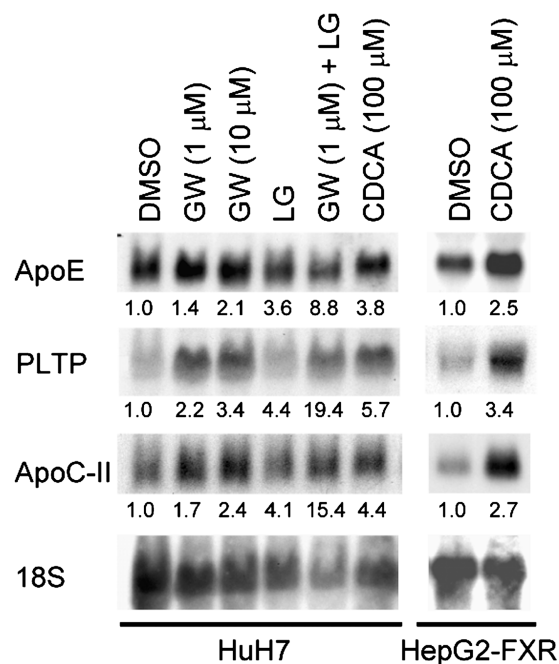
**Fig. 3.** LXR/RXR binds to DR-4 and IR-1 sequences in the human PLTP promoter. A: Electrophoretic mobility shift assays (EMSAs) were performed using the indicated radiolabeled probes. The arrows indicate the positions of the complex containing the DNA probe, LXR and RXR, and the asterisks with arrowheads denote the positions of the supershifted bands. B: Sequence-specific competition defines the binding of LXR $\alpha$ /RXR $\alpha$  to LXRE-1 and LXRE-2. The EMSA was performed in the absence of competitor (-) or presence of increasing concentrations (10-, 50-, 250-, and 1,000-fold excess) of unlabeled LXRE-1, mutant LXRE-1 (mut LXRE1), (rat) rCyp7a DR-4 (up to 5000-fold excess), LXRE-2, mutant LXRE-2 (mut LXRE-2), and I-BABP IR-1, as indicated.

tivate murine PLTP (Fig. 1) (14), it is likely that the murine gene is also a direct LXR target, although to date a functional LXRE has not been identified.

### LXR/RXR binds to LXRE1 and LXRE2

EMSAs utilized radiolabeled probes corresponding to wild-type LXREs or mutated LXREs (containing the 4-bp mutation utilized in the promoter-luciferase constructs) and nuclear receptors derived from in vitro transcription/translation reactions. The data indicate that LXR and RXR bind to both the DR-4 (LXRE1) and IR-1 (LXRE2) (Fig. 3A, lanes 4 and 14). Antibody to RXR had two effects: *i*) the total amount of radiolabeled probe that was shifted decreased significantly, consistent with the antibody interfering with the complex formation and *ii*) a minor supershifted band was observed (Fig. 3A, lanes 5 and 15). In contrast, no protein/DNA complex was observed when the probe was incubated with either LXR or RXR alone (lanes 2, 3, 12, and 13) or when the probe contained a mutated LXRE (lanes 7–10 and 17–20). The positive control contained an idealized DR-4 LXRE (lane 22).

Competition studies demonstrate that the formation of the LXR/RXR complex with either LXRE1 or LXRE2 is reduced in the presence of excess unlabelled DNA containing sequences corresponding to the LXRE1 and the DR-4 of rat Cyp7A1 (Fig. 3B, left panel), or LXRE2 and the IR-1 of I-BABP (Fig. 3B, right panel), respectively. In contrast, the formation of the LXR/RXR/DNA complexes was unaffected when the competitor DNA contained four mutations in the LXRE (Fig. 3B, lanes 7–10 and 21–24).



**Fig. 4.** Induction of apolipoprotein E mRNA in response to farnesoid X receptor ligands. HuH7 or HepG2-FXR (6) liver cells were maintained in DMEM + 10% super stripped FBS and treated for 24 h with either vehicle or ligands for FXR (GW4064 or chenodeoxycholic acid) and/or RXR (LG). Northern blots were performed as described (6).



Together with the transfection data, these studies demonstrate that PLTP is an LXR-target gene that contains two functional LXREs (a DR-4 and an IR-1) in the proximal promoter. To our knowledge, an IR-1 has not previously been shown to function as an LXRE and transactivate a target gene. Since the IR-1 can also function as an FXRE (4, 5), we conclude that either FXR/RXR or LXR/RXR can bind to this sequence. Additional studies will be required to determine what parameters regulate the preferential binding of distinct heterodimers to the same element in the liver.

### Identification of apoE as a new FXR target gene

The findings that PLTP is transcriptionally activated in macrophages by LXR (Fig. 1) and in liver by FXR (4, 5, 15) and that the apoC-II gene is induced in macrophages by LXR (7) and in liver by FXR (6) suggest that there may be other genes involved in lipid metabolism that are also activated by both of these nuclear receptors.

The data of Fig. 4 demonstrate that apoE, a known LXR target gene in macrophages (8), is induced in liver cells by FXR ligands (CDCA and GW). ApoE is a member of a gene cluster (apoE/C-I/C-IV/C-II) that is known to be regulated in the liver by two distal enhancers, termed hepatic control regions (16–19). We recently demonstrated that FXREs, contained within these enhancers, regulate the expression of hepatic apoC-II in response to activated FXR (6). Consequently, we hypothesize that the same FXREs are involved in the induction of hepatic apoE mRNAs in response to FXR ligands (Fig. 4). Since apoE is induced by ligands for FXR (Fig. 4) and LXR (8), these data suggest that a subset of genes, currently limited to apoE, PLTP, and apoC-II, can each be independently regulated by either FXR (Fig. 4) or LXR, thus providing alternative mechanisms that control lipid homeostasis in different tissues.

The function of macrophage derived PLTP remains to be established. Our studies suggest that PLTP expression and secretion would be highly induced as macrophages convert to lipid-loaded foam cells in the fatty streak of the artery wall. Since treatment of macrophages with LXR ligands also activates LPL (20), its obligate cofactor apoC-II, as well as apo E, C-I, and C-IV (7), we hypothesize that this milieu of proteins will alter lipoprotein metabolism in the artery wall and possibly affect the development of the atherosclerotic plaque. ■

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