LIGAND SPECIFICITY AND CONFORMATIONAL DEPENDENCE OF THE
HEPATIC NUCLEAR FACTOR-4α (HNF-4α)

by

Anca D. Petrescu¹, Rachel Hertz², Jacob Bar-Tana²,
Friedhelm Schroeder¹, and Ann B. Kier³,*

from the

¹ Dept. of Physiology and Pharmacology
Texas A&M University, TVMC
College Station, TX 77843-4466

the

²Dept. of Human Nutrition and Metabolism
Hebrew University Medical School
Jerusalem 91120, Israel

and the

³Dept. of Pathobiology
Texas A&M University, TVMC
College Station, TX 77843-4467

*To whom correspondence should be addressed.
TEL: 979-862-1509
FAX: 979-862-4029
E-MAIL: akier@cvm.tamu.edu

RUNNING TITLE: HNF-4αLBD binds acyl-CoAs with nanomolar affinities

KEY WORDS: HNF-4α, fluorescence, circular dichroism, secondary structure, ligand binding, fatty acyl CoA.
This work was supported by the USPHS National Institutes of Health grants DK41402 (FS) and NIEHS ES09106 (AK); United States-Israel Binational Science Foundation #1998219 (JB).

**Abbreviations**: apo, apolipoprotein; C16:0, palmitic acid; C18:0, stearic acid; C18:2, linoleic acid; C20:4, arachidonic acid; CoA, coenzyme A; cPNA, cis parinaric acid; cPNA-CoA, cis parinaroyl-CoA; COUP-TF, chicken ovalbumin upstream-promoter transcription factor; CYP, cytochrome P450; FFA, free fatty acid; FRET, fluorescence resonance energy transfer; glut2, glucose transporter 2; HNF-4αLBD, hepatic nuclear factor-4α ligand binding domain; LCFA, long-chain fatty acids; Lp(a), lipoprotein (a); Medica 16, β,β’-tetramethyl-hexadecane dioic acid; MTP, microsomal triglyceride transfer protein; NBD-stearic acid, (12-(N-methyl)-N-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-octadecanoic acid); PEPCK, phosphoenolpyruvate carboxykinase; PPs, peroxisome proliferators; PPAR, peroxisome proliferator activated receptors; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; Tyr, tyrosine; Trp, tryptophan.
ABSTRACT

Hepatic nuclear factor-4α (HNF-4α) controls the expression of genes encoding proteins involved in lipid and carbohydrate metabolism. Fatty acyl-CoA thioesters have recently been proposed to be naturally occurring ligands of HNF-4α and to regulate its transcriptional activity as function of their chain length and degree of unsaturation (Hertz et al., 1998, Nature 392, 512–516). However, the apparent low affinities (μM Kd) obtained with a radiolabeled fatty acyl-CoA ligand binding assay raised questions regarding the physiological significance of this finding. Furthermore, it is not known whether interaction with fatty acyl-CoA alters the structure of HNF-4α. These issues were examined using rat recombinant HNF-4α ligand binding domain (HNF-4αLBD) in conjunction with photon counting fluorescence and circular dichroism. First, fluorescence resonance energy transfer (FRET) between HNF-4αLBD tryptophan (Trp) and cis-parinaroyl-CoA (cPNA-CoA) yielded an intermolecular distance of ≤ 42Å, thus pointing to direct molecular interaction rather than nonspecific coaggregation. Second, quenching of HNF-4αLBD intrinsic Trp fluorescence by fatty acyl CoAs (e.g., palmitoyl-, stearoyl-, linoleoyl-, arachidonoyl-CoAs) yielded a single binding site with Kd's of 1.6–4.0 nM. These affinities were 2–3 orders of magnitude higher than those previously derived by radiolabeled fatty acyl-CoA ligand binding assay. Third, binding of fatty acyl-CoAs was specific as the binding affinities of the respective free fatty acids or free CoA (Kd's of 421–742 nM) were significantly lower. Fourth, circular dichroism demonstrated that the HNF-4αLBD secondary structure was significantly and differentially altered by fatty acyl-CoA binding. The opposite effects of saturated vs polyunsaturated fatty acyl-CoAs on HNF-4αLBD secondary structure correlated with their opposite regulatory effects on HNF-4α function. Fifth, the CoA thioesters of some hypolipidemic peroxisome proliferators bind with high affinity (Kd's as low as 2.6 nM) to HNF-4αLBD thus indicating that HNF-4α may serve as target for these drugs. In summary, these data demonstrate for the first time high affinity binding to HNF-4α of fatty and xenobiotic acyl-CoAs in the physiological range, resulting in significantly altered HNF-4α conformation.
INTRODUCTION

Hepatic nuclear factor 4 (HNF-4) is a member of the superfamily of nuclear receptors which includes steroid hormone receptors and nonsteroid ligand dependent transcription factors, e.g. thyroid hormone receptor, retinoid receptors (RXR, RAR) peroxisome proliferator activated receptors (PPARs) (rev. in (1)). HNF-4α isoforms (α1–α3) have been cloned and characterized and are expressed in mammals in liver, kidney, intestine and pancreas (rev. in (2)). Unlike RXRα, with which it has 40% amino acid sequence homology, HNF-4 does not form heterodimers with any other nuclear receptor, but binds to direct repeat-1 (DR-1) DNA sequences as homodimer (3). DR-1 motifs are promiscuous binding sites for HNF-4, PPAR, RAR, RXR, COUP-TFI and COUP-TFII homo or heterodimers (4).

HNF-4α responsive genes encode transcription factors (HNF-1α, PXR), proteins involved in fatty acid, lipoprotein and lipid metabolism (apo A-I, A-II, B, C-II, C-III, Lp(a), microsomal triglyceride transfer protein, mitochondrial fatty acyl-CoA dehydrogenases, fatty acid binding protein), carbohydrate metabolism (insulin, glut2, glucose-6-phosphatase, PEPCK, pyruvate kinase, aldolase B, glyceraldehyde-3-phosphate dehydrogenase), amino acid and protein metabolism (ornithine transcarbamylase, tyrosine amino transferase, phenylalanine hydroxylase, antitrypsin α1), P450 enzymes (steroid 15α-hydroxylase, fatty acyl ω-hydroxylase, cholesterol-7α-hydroxylase, drug metabolizing P450 enzymes (cyp3α4–6)), hematopoiesis (erythropoietin, transferrin), blood coagulation (factors VII, IX and X, fibrinogen) and others (e.g., cellular retinol binding protein, transthyretin) (rev. in (2,5-14)). Since HNF-4α activates the transcription of some nuclear receptors (HNF-1α) and may further directly interact with other transcription factors (HNF-1α), the above list of HNF-4α responsive genes may include some which are transcriptionally affected by HNF-4α indirectly.
The first demonstration of putative HNF-4 ligands showed that long chain fatty acyl-CoA (LCFA-CoAs) thioesters (15) as well as CoA-thioesters of hypolipidemic peroxisome proliferators (e.g., fibrate drugs, Medica homologues) (12) bind to HNF-4α and, depending on their chain length, degree of saturation, and respective substitutions are able to activate or inhibit transcription of a reporter gene enhanced by the C3P element of the apo CIII promoter (12,15). However, the radioligand competition assay used to demonstrate acyl-CoA binding in these previous studies yielded $K_d$s in the $\mu M$ range (12,15). Although total tissue levels of LCFA-CoAs (free + bound) are in the range 0.4–164 $\mu M$, depending on tissue, cell, nutrition, and pathology (rev. in (16,17)), free (unbound) LCFA-CoA are estimated to be 5–200 nM in cytosol (17) and only 1–10 nM in the nucleus (18). Based on these considerations as well as computer modeling of HNF-4α structure, it has been suggested that HNF-4α does not significantly bind LCFA-CoAs (19). However, it must be considered that radioligand competition assays for lipidic ligand binding proteins typically yield $K_d$s that are 2–3 orders of magnitude higher (i.e. lower affinity) than $K_d$s determined by direct fluorescence or microcalorimetry binding assays (rev. in (20)). Thus, it seems possible that the radioligand acyl-CoA competition binding results significantly underestimated the affinity of HNF-4α for its endogenous ligands.

The objective of the present investigation was to resolve these issues through (i) direct fluorescent ligand binding assays based on quenching of tryptophan emission by putative HNF-4α ligands using a recombinant HNF-4α ligand binding domain (HNF-4αLBD), (ii) fluorescence resonance energy transfer (FRET) between HNF-4αLBD aromatic amino acids and bound cis-parinaroyl CoA (naturally occurring fluorescent LCFA-CoA) to calculate the intermolecular distance between ligand and its binding site, and (iii) circular dichroism to characterize the secondary structure of HNF-4αLBD as well as potential changes induced therein by LCFA-CoA binding.
EXPERIMENTAL PROCEDURES

Chemicals: Cis-parinaric acid and NBD-stearic acid were purchased from Molecular Probes (Eugene, OR). Coenzyme A, palmitoyl-CoA, stearoyl-CoA, linoleoyl-CoA, arachidonoyl-CoA, arachidonic acid, and bezafibrate were from Sigma Chemical Co. (St. Louis, MO). Medica 16 as well as the coenzyme A thioesters of Medica 16 and bezafibrate were chemically synthesized as described (21,22).

Expression of recombinant rat HNF-4αLBD: Rat HNF-4α1 cDNA encoding the sequence from amino acid 132 to 455 was synthesized by PCR with the following primers: 5’-CCGGCTCGAGGATGGCTTCCTGCTT-3’ and 5’-GCGCCATATGAGGTCAAGCTACGAG-3’. This DNA fragment was cloned into the NdeI/XhoI site of pET21b plasmid (Novagen, Milwaukee, WI) and sequenced. The recombinant protein was expressed in BL21(DE3)plyS strain of E. coli.

Purification, SDS-PAGE and western blotting of recombinant HNF4αLBD: His-tagged HNF-4αLBD was purified by affinity chromatography on a nickel-NTA resin (Quiagen, Chatsworth, CA), desalted, lyophilized and stored at −70°C. Prior to use, the protein was solubilized in 20 mM Tris-HCl buffer, pH 8.0, containing 0.3 M NaCl, 10% glycerol and 1 mM 2-mercaptoethanol or dithiothreitol (binding buffer). Protein was determined by BCA Protein Assay (Pierce, Rockford, IL). Purity of recombinant rat His-HNF-4αLBD (amino acids 132–455 of the wild type rat HNF-4α1 with 6 His residues attached at the C-terminus) was assessed by SDS-PAGE and western blotting (Fig. 1). SDS-PAGE separated a single protein band of 36 kDa as detected by staining with Coomassie Blue and indicated 97% purity (Fig. 1, panel A). Western blotting with rabbit anti-rat HNF-4αLBD polyclonal antibody followed by goat anti-rabbit-IgG-alkaline phosphatase conjugate was performed as described (23). A single reacting band at the same molecular weight was detected by western blot analysis (Fig. 1, panel B).

Synthesis and purification of fluorescent cis-parinaroyl-CoA (cPNA-CoA). Fluorescent cPNA-CoA was chemically synthesized (22) utilizing a naturally occurring
fluorescent fatty acid, cis-parinaric acid (Molecular Probes, Eugene, OR). Unreacted, free cis-parinaric acid was retained into the organic phase of a chloroform:methanol:water mix (24). Further removal of free CoA from cPNA-CoA in the aqueous phase was performed by HPLC, as described (25). The purity level of the HPLC fraction containing cPNA-CoA was checked by assessing the absorption spectrum with a UV/VIS spectrometer, model Lambda 2 (Perkin Elmer, Norwalk, CT). HPLC of the aqueous extract showed two major peaks, 1 and 4 separated by over 32 min, as well as two minor peaks 2 and 3 with retention times between 3-6 min (Fig. 2A). The absorbance spectrum of the partially purified cPNA-CoA (Fig. 2B) exhibited a 260 nm maximum characteristic for CoA as well as three maxima within 280-324 nm, characteristic for the conjugated double bonds of cPNA-CoA. The absorbance spectrum of HPLC-peak 1 (Fig. 2C) did not reveal the presence of a conjugated tetraene (peaks between 280-324 nm) of cPNA-CoA, but instead showed absorbance maximum near 260 nm, typical of free CoA (not shown). In contrast, the absorbance spectrum of peak 4 taken from the HPLC chromatogram in Fig. 2A exhibited absorbance characteristics of both the conjugated tetraene and thioester linkage present in cPNA-CoA (Fig. 2D). Absorbance spectra of oleoyl CoA demonstrated only the presence of the thioester linkage but not the conjugated tetraene present only in cPNA-CoA (not shown).

**Direct ligand binding assay: fluorescent ligands.** Direct binding assays not requiring separation of bound from free ligand were performed using cis-parinaroyl CoA as described (25-30). To establish specificity for LCFA-CoA, this assay was repeated using fluorescent fatty acids: cis-parinaric acid and NBD-stearic acid. Briefly, HNF-4αLBD was added to phosphate buffer saline, pH 7.4 or binding buffer described above to yield final concentration of 170nM HNF-4αLBD. Increasing fluorescent ligand was then added to yield final concentrations of 10-4000 nM cis-parinaroyl CoA. Fluorescence emission spectra were obtained using a PC1 photon counting fluorimeter (ISS Inc., Urbana, IL) and maximal intensities measured. The $K_d$ and number of binding sites (n) were calculated as previously described (26,27,30). Where it was possible (e.g. NBD-stearic acid) the number of binding sites was determined with a higher degree of accuracy from the reverse titration binding curve in which a constant amount of ligand was titrated with increasing concentration of HNF-4αLBD.
Direct ligand binding assay: quenching of intrinsic fluorescence of HNF-4\textsubscript{\textalpha} LBD aromatic amino acids Tyr/Trp or Trp. To investigate direct binding of nonfluorescent LCFA-CoAs to HNF-4\textsubscript{\textalpha}LBD, the effect of LCFA-CoA binding on intrinsic fluorescence of HNF-4\textsubscript{\textalpha}LBD aromatic amino acids was examined. Briefly, 170 nM HNF4\textsubscript{\textalpha}LBD were titrated with increasing ligand (5-4000 nM). Two types of fluorescence measurements were obtained. First, both Tyr and Trp residues were excited at 280 nm. Second, the contribution of Trp from Tyr residues was resolved by selective excitation of Trp at 295 nm. Since fluorescence emission was maximal at 333 nm in both cases, Trp was the main contributor to HNF-4\textsubscript{\textalpha}LBD fluorescence emission. All steady state fluorescence measurements were performed using a PC1 photon counting fluorimeter (ISS, Champaign, IL) in the L-format with 300 watt xenon arc lamp light source and 4 nm band-passes in both excitation and emission monochromators. In ligand-induced HNF-4\textsubscript{\textalpha}LBD fluorescence quenching experiments the number of binding sites (n) was estimated by fitting the binding curve to a Hill plot according to the equation 
\[ y = ax^b/(c^b + x^b), \]
where \( a \) is Bmax =nE\textsubscript{0}, \( b \) is the number of binding sites (n) and \( c \) is K\textsubscript{d}.

Fluorescence resonance energy transfer (FRET): determination of intermolecular distance between the HNF-4\textsubscript{\textalpha}LBD Trp and bound cis-parinaroyl-CoA. Examination of the excitation and emission spectra of HNF4\textsubscript{\textalpha}LBD revealed that the emission of Trp overlapped significantly with excitation of cis-parinaroyl CoA, a condition ideal for Forster FRET (31). Since FRET varies as (intermolecular distance)\( ^{1/6} \), the donor (HNF-4\textsubscript{\textalpha}LBD Trp) and acceptor (cis-parinaroyl CoA) residues must be in very close proximity, generally a few Å, for efficient FRET to occur. To determine the average intermolecular distance between HNF4\textsubscript{\textalpha}LBD Trp residues and bound cis-parinaroyl CoA, HNF-4\textsubscript{\textalpha}LBD (170 nM) was titrated with increasing cis-parinaroyl-CoA (5-4000 nM final concentration). The HNF-4\textsubscript{\textalpha}LBD Tyr/Trp or Trp only, were excited at 280 and 295 nm, respectively. Maximal fluorescence emission intensities of HNF-4\textsubscript{\textalpha}LBD Tyr/Trp or Trp were obtained at 333 nm while maximal emission of cis-parinaroyl-CoA was measured at 430 nm. If bound cis-parinaroyl CoA acceptor was located within the optimal FRET distance from the HNF-4\textsubscript{\textalpha}LBD Trp, then efficient energy transfer occurred such that HNF-4\textsubscript{\textalpha}LBD Tyr/Trp or Trp fluorescence emission was quenched while, concomitantly, sensitized fluorescence emission of cis-parinaroy-
CoA was at 430 nm. The intermolecular distance between HNF-4LBD Trp and bound cis-parinaroyl-CoA was calculated from 
\[ E = \frac{R_0^6}{(R_0^6 + R_{2/3}^6)} \], where \( E \) is the FRET efficiency, \( R_0 \) is the critical distance for 50% efficiency, and \( R_{2/3} \) is the actual distance between donor and acceptor (31-33). The energy transfer efficiency was calculated from quenching of HNF-4LBD Trp according to 
\[ E = 1 - \frac{F_{DA}}{F_D} \], where \( F_{DA} \) and \( F_D \) are the fluorescence intensities of HNF-4LBD Trp (energy donor) at 333 nm in the presence and absence of cis-parinaroyl-CoA (energy acceptor) upon excitation of HNF-4LBD Trp at 295 nm. The transfer efficiency (\( E \)) was also calculated from the excitation spectrum of the energy acceptor (i.e. cis-parinaroyl-CoA) as previously described by Stryer (34) according to 
\[ E = \frac{G(\lambda_2)G(\lambda_1)}{G(\lambda_1)G(\lambda_2)} \times \frac{\varepsilon_D(\lambda_1)}{\varepsilon_A(\lambda_2)} \], where \( G(\lambda) \) is the magnitude of the corrected excitation spectrum of the energy acceptor excited at wavelength \( \lambda_1 \); \( \varepsilon_D(\lambda) \) and \( \varepsilon_A(\lambda) \) are the extinction coefficients of the donor and acceptor at wavelength \( \lambda \). \( G \) is measured at two wavelengths: at \( \lambda_1 \), where the donor has low absorption (320 nm), and at \( \lambda_2 \), where the extinction coefficient of the donor is large compared to that of the acceptor (280 nm). This second method is especially useful when the local environment of the donor is different in the presence of the acceptor, provided that the absorption spectra of the donor and acceptor moieties are known (34). The critical distance for 50% efficiency (\( R_0 \) in Å) was calculated as described (35,36) using 
\[ R_0 = 0.211[k_2n^{-4}QDJ(\lambda)]^{1/6} \] with wavelength expressed in nm and \( J(\lambda) \), the overlap integral, expressed as M\(^{-1}\)cm\(^{-1}\)nm\(^4\). \( Q_D \), the quantum yield for HNF-4LBD Trp and \( J(\lambda) \) were calculated as described earlier (35,36). The orientation factor \( k_2 \) and the refractive index \( n \) were assumed to be 2/3 and 1.4, respectively, as for proteins in solution.

**Circular dichroism (CD) of HNF-4LBD:** Far UV CD spectra of HNF-4LBD (2 mM) were taken in the absence and presence of ligands as described (37) except that 2 mM Tris-HCl, pH 8 containing 30 mM NaCl, 1% glycerol and 0.1 mM DTT was used as buffer. The CD measurements were performed with a J-710 Spectropolarimeter (Jasco, Baltimore, MD) using a 1 mm cuvette. Spectra were recorded from 250 to 195 nm at 50 nm/min with a time constant of 1 s and a bandwidth of 2 nm. For each CD profile an average of ten scans was obtained. Percentages of various secondary structures in HNF-4LBD were calculated from the CD spectra by using the CDssstr program (38,39).
RESULTS

Determination of the binding parameters of HNF-4αLBD for a naturally occurring fluorescent fatty acyl CoA: cis-parinaroyl-CoA quenching of HNF-4αLBD Trp. Cis-parinaroyl CoA was chosen as a fluorescent ligand to examine the fatty acyl CoA binding properties (K_d and B_max) of HNF4(LBD) because: (i) cis-parinaroyl CoA is a naturally occurring fluorescent fatty acyl CoA with structure similar to other fatty acyl CoAs (Table 1); (ii) cis-parinaroyl CoA absorbance overlaps with aromatic amino acid fluorescence emission, thereby allowing fluorescence resonance energy transfer; (iii) cis-parinaroyl CoA has been extensively used in fatty acyl CoA fluorescence binding assays (25-30). Furthermore, cis-parinaroyl CoA fluorescence binding assay does not underestimate fatty acyl CoA binding as do radioligand binding assays (rev. in (20,40)). Thus, quenching of HNF-4LBD Trp emission at 333 nm was examined upon titration with increasing concentration of cis-parinaroyl-CoA. HNF-4LBD was excited either at 280 nm to excite both Trp/Tyr (Fig. 3A) or at 295 nm to selectively excite HNF-4LBD Trp (Fig. 3C). In both cases, the fluorescence emission of HNF-4LBD was quenched by increasing concentrations of cis-parinaroyl-CoA. Upon excitation of both Trp/Tyr (at 280 nM), the binding curve of cis-parinaroyl-CoA to HNF-4LBD demonstrated saturation binding (Fig. 3B). A reciprocal plot of the binding curve (Fig. 3B, inset) was linear, suggesting either a single binding site or two binding sites with the same K_d. The number of binding sites was resolved by fitting the binding curve to a Hill plot, as described in Methods. The Hill plot yielded n=1.08, i.e. one binding site with a K_d of 248.5 nM (Table 2). The number of binding sites calculated from direct titration, considering the molecular weight of HNF-4LBD monomer (36 kD), was 0.73. On selective excitation of Trp at 295 nm, a saturation binding curve was again obtained (Fig. 3D) with linear reciprocal plot of the binding curve (Fig. 3D, inset). Fitting to Hill plot resulted in n=1.42 and K_d of 238.2 nM (Table 2). Taken together, measurement of the direct quenching of HNF-4LBD aromatic amino acid fluorescence by cis-parinaroyl-CoA indicated that HNF-4LBD bound cis-parinaroyl-CoA with nanomolar affinity at one binding site per monomer.

Molecular interaction of cis-parinaroyl-CoA with HNF4α-LBD: fluorescence resonance energy transfer (FRET). To investigate in more detail the close molecular
interaction of HNF4α with cis-parinaroyl CoA, advantage was taken of the spectral properties of cis-parinaroyl CoA absorbance and HNF-4ζBD Trp emission. FRET provided a sensitive technique for determining if interaction between cis-parinaroyl CoA and HNF-4ζBD represented close molecular binding rather than coaggregation. Comparison of a portion of the absorbance characteristics of cis-parinaroyl-CoA (Fig. 4, dashed line) with the emission of HNF-4ζBD Trp, selectively excited at 295 nm (Fig. 4, solid line), demonstrated partial but significant overlap. This overlap of the energy acceptor absorbance with the donor fluorescence emission is a required condition for efficient FRET. To determine if FRET occurred between HNF-4αLBD and bound cis-parinaroyl-CoA, HNF-4ζBD was titrated with increasing concentrations of cis-parinaroyl-CoA followed by determination of fluorescence emission spectra from 300 to 450 nm upon excitation of Tyr/Trp at 280 nm or of Trp only at 295 nm (Figs. 3 and 5). When HNF-4ζBD Tyr/Trp were excited at 280 nm, Trp emission at 333 nm was highly quenched with increasing cis-parinaroyl-CoA (Fig. 3A). While this suggested that efficient FRET occurred between HNF-4ζBD and cis-parinaroyl-CoA, a conformational change in HNF-4ζBD upon ligand binding may also cause Trp fluorescence quenching. This possibility was resolved by the appearance of cis-parinaroyl-CoA sensitized emission near 430 nm upon increasing cis-parinaroyl-CoA concentration (Fig. 5A). The increase in the acceptor cis-parinaroyl-CoA sensitized fluorescence emission was even more apparent when HNF-4ζBD Trp was selectively excited at 295 nm (Fig. 5C). Since a conformational change in HNF-4ζBD cannot elicit sensitized emission of cis-parinaroyl-CoA, these data indicated the close molecular interaction between HNF4-ζBD and bound cis-parinaroyl-CoA.

**Calculation of the intermolecular distance between HNF-4ζBD and bound cis-parinaroyl-CoA by FRET.** To determine the intermolecular distance between HNF-4ζBD and bound cis-parinaroyl-CoA it was essential to remove complicating HNF-4ζBD internal energy transfer from Tyr to Trp residues. This process occurred upon excitation of HNF-4ζBD at 280 nm (excites both Trp and Tyr residues) since fluorescence emission was detected only from Trp near 333 nm (Fig. 3A). In contrast, preferential excitation of HNF-4ζBD Trp at 295 nM resulted in maximal emission near 333 nm as expected (Fig. 3C). Since separate emission of Tyr (typically near 308 nm)
was not observed upon excitation at 280 nm (Fig. 3A), this was consistent with efficient internal nonradiative transfer of energy from Tyr to Trp within HNF-4αLBD.

Based on the significant overlap between the absorbance of cis-parinaroyl-CoA (Fig. 4, dashed line) with the emission of HNF-4αLBD Trp (Fig. 4, solid line), it was possible to calculate \( R_0 \), the critical distance for 50% energy transfer, as described in Experimental procedures. The calculated \( R_0 \) for the HNF-4αLBD Trp/cis-parinaroyl-CoA donor/acceptor pair was 30 Å (Table 3). Since FRET decreases with (intermolecular distance)\(^{1/6}\), efficient FRET will only occur between HNF-4αLBD Trp and cis-parinaroyl-CoA if these donors/acceptors are close to 30 Å apart.

To determine \( R_{2/3} \), the actual intermolecular distance between HNF-4αLBD and cis-parinaroyl-CoA, HNF-4αLBD was titrated with increasing concentrations of cis-parinaroyl-CoA followed by selective excitation of HNF-4αLBD Trp at 295 and determination of fluorescence emission between 300 to 450 nm, so that changes in both HNF-4αLBD Trp fluorescence at 333 nm and of cis-parinaroyl-CoA emission at 430 nm were observed (Figs 3C, 5C). The energy transfer efficiency was calculated from these data as described in Experimental Procedures. Based on the equation that determines energy transfer efficiency with the assumption that no protein conformational change is induced upon ligand binding, a value of 85% energy transfer efficiency between HNF-4αLBD Trp and cis-parinaroyl-CoA was obtained (Table 3). Thus, in the absence of a ligand induced conformational change in HNF-4αLBD, an intermolecular distance between HNF-4αLBD and cis-parinaroyl-CoA, i.e. \( R_{2/3} = 22.6 \) Å was determined. However, if fatty acyl-CoA binding elicits a conformational change in HNF-4αLBD then this value may overestimate how close the cis-parinaroyl-CoA approaches HNF-4αLBD. Stryer’s equation in Experimental Procedures allows calculation of energy transfer efficiency when both FRET and protein conformational changes upon ligand binding contribute to the sensitized emission of the bound ligand (34). For this case a much lower energy transfer efficiency, i.e. 12% was found and the resultant intermolecular distance between HNF-4αLBD and cis-parinaroyl-CoA, \( R_{2/3} \), was 42 Å (Table 3). Thus, depending on whether fatty acyl-CoA binding elicits a conformational change in HNF-4αLBD structure, the intermolecular distance between HNF-4αLBD Trp and bound cis-parinaroyl-CoA was in the range of 23-42 Å. In either case, these data were consistent
with fatty acyl-CoA binding to HNF-4αLBD being due to close molecular interaction between these molecules. Whether 23 Å or 42 Å is the correct intermolecular distance was resolved by circular dichroism (see below).

**Determination of the binding parameters of HNF-4αLBD for cis parinaroyl-CoA from FRET data.** The appearance of sensitized cis-parinaroyl-CoA fluorescence emission upon FRET between HNF-4αLBD and bound cis-parinaroyl CoA was used to obtain binding parameters. Plotting the increase in sensitized emission of HNF-4αLBD-bound ligand (excited at 280 nm) as a function of increasing cis-parinaroyl-CoA yielded a saturation binding curve (Fig. 5B). A double reciprocal plot of the binding curve was linear (Fig. 5B, inset), consistent with a binding site or two binding sites with the same affinity. A Hill plot of the binding data resolved this issue by demonstrating n= 0.71, i.e. consistent with a single binding site, with a K_d of 250.4 nM (Table 2). Upon selective excitation of HNF-4αLBD Trp at 295 nm (Fig. 5C), the binding curve based on cis-parinaroyl-CoA sensitized emission again demonstrated binding to saturation (Fig.5D). A double reciprocal plot of the binding data was linear again, consistent with one binding site or two binding sites of similar affinity (Fig.5D, inset). A Hill plot yielded n=1.02, consistent with a single binding site, and a K_d of 758.9 nM (Table 2).

In summary, the binding curves constructed from the FRET induced sensitized emission of HNF-4αLBD bound cis-parinaroyl-CoA indicated that HNF-4αLBD bound cis-parinaroyl-CoA to one site which, in general, showed a similar affinity for cis-parinaroyl-CoA as based on HNF-4αLBD Trp emission quenching at 333 nm (Table 2).

**Binding specificity of HNF-4αLBD for cis-parinaroyl-CoA: interaction with cis-parinaric acid and CoA.** The ligand binding specificity of HNF-4αLBD was determined by titration with increasing amounts CoA or cis-parinaric acid. Upon HNF-4αLBD Trp/Tyr excitation at 280 nm, CoA slightly quenched HNF-4αLBD fluorescence emission at 330 nm (Fig. 6A). The CoA concentration plot was hyperbolic, indicating saturation binding (Fig. 6B). The double reciprocal plot of this curve was linear (Fig. 6B, inset) and a Hill plot resolved one binding site (n=1.53) with K_d of 429.1 nM (Table 2). Similarly, excitation of HNF-4αLBD Trp and determination of fluorescence resulted in quenching of fluorescence upon the presence of increasing concentrations of CoA (Fig. 6C). The binding curve constructed from these data was hyperbolic (Fig.6D) indicating
saturation binding. Both the double reciprocal plot (Fig. 6D, inset) and Hill plot (n=0.98) indicated a single binding site for CoA. The binding affinity of HNF-4LBD to CoA was low, i.e. \( K_d \) of 680.4 nM (Table 2). Thus, quenching of HNF-4LBD Trp (regardless of whether both Trp/Tyr or only Trp were excited) fluorescence suggested that (i) HNF-4LBD binds CoA at one binding site, and (ii) HNF-4LBD has 2-fold lower affinity for CoA at that site as compared to cis-parinaroyl-CoA.

Binding of free cis-parinaric acid to HNF-4LBD was assayed similarly as for CoA binding above, except that increasing concentrations of cis-parinaric acid were used. When HNF-4LBD Trp/Tyr were excited at 280 nm, increasing cis-parinaric acid also quenched HNF-4LBD Trp emission (Fig. 7A). However, this binding of cis-parinaric acid did not result in FRET as indicated by the absence of sensitized emission of at 430 nm (Fig. 7A, inset). The latter was in contrast to titration with cis-parinaroyl-CoA binding which induced sensitized emission at 430 nm (Fig. 5A). The plot of cis-parinaric acid concentration versus HNF-4LBD Trp fluorescence quenching indicated weak binding (Fig. 7B). The double reciprocal plot of the binding curve showed a single binding site (Fig. 7B, inset); this was confirmed by Hill plot which yielded \( n=1.09 \) and a \( K_d \) of 421.3 nM (Table 2). When only HNF-4LBD Trp was selectively excited at 295 nm, quenching of HNF-4LBD fluorescence was observed (Fig. 7C) but, again, there was no appearance of cis-parinaric acid sensitized emission at 430 (Fig. 7C, inset). Construction of the cis-parinaric acid binding curve showed a hyperbolic shape (Fig. 7D). The double reciprocal plot again was linear, consistent with a single binding site (Fig. 7D, inset). This was confirmed by Hill plot which yielded \( n=0.82 \) and \( K_d \) of 588.9 nM (Table 2). Thus, quenching of HNF-4LBD Trp (regardless of whether both Trp/Tyr or only Trp were excited) fluorescence suggested that (i) HNF-4LBD binds cis-parinaric acid at one binding site, and (ii) HNF-4LBD has about 2-fold lower affinity for cis-parinaric acid at that site then for cis-parinaroyl-CoA.

In summary, these data based on quenching of HNF-4LBD aromatic amino acid fluorescence emission indicated that HNF-4LBD has a single binding site with \( K_d \) near 250 nM for cis-parinaroyl-CoA. The individual residues, i.e. CoA and cis-parinaric acid, constituting cis-parinaroyl-CoA, interact with HNF-4LBD at lower-affinities (i.e. \( K_d \)s
in the range of 420-680 nM), thus indicating that both portions of the cis-parinaroyl CoA molecule apparently contributed to binding.

**Binding of nonfluorescent, long chain fatty acyl-CoA to HNF-4\(\alpha\)LBD.** The interaction of nonfluorescent, saturated and unsaturated naturally-occurring fatty acyl-CoAs with HNF-4\(\alpha\)LBD was examined. Palmitoyl-CoA (C-16 acyl chain) and stearoyl-CoA (C-18 acyl chain) were representative, highly-prevalent, straight-chain saturated fatty acyl CoAs while linoleoyl-CoA (C-18 acyl chain with 2 double bonds) and arachidonoyl-CoA (C-20 acyl chain with 4 unconjugated double bonds) were representative polyunsaturated, kinked-chain, fatty acyl-CoAs typically found in tissues.

When excited at 280 nm, HNF-4\(\alpha\)LBD Trp emission at 333 nm was significantly quenched in the presence of palmitoyl-CoA (Fig 8A), stearoyl-CoA (Fig.8B), linoleoyl-CoA (Fig.8C), and arachidonoyl-CoA (Fig.8D). HNF-4\(\alpha\)LBD exhibited hyperbolic, saturation binding curves with each of these fatty acyl-CoAs. Furthermore, linearization of each binding curve (shown in the insets of Fig.8A, B, C, D, respectively) indicated the presence of a single binding site for each of these fatty acyl-CoAs. The binding affinities calculated from the latter linear plots showed that HNF-4\(\alpha\)LBD exhibited very high affinity for palmitoyl-CoA, stearoyl-CoA, linoleoyl-CoA, and arachidonoyl-CoA as evidenced by \(K_d\)’s of 1.7 nM, 3.8 nM, 4.4 nM and 4.0 nM, respectively (Table 4).

In summary, the very high affinity (\(K_d\)s of 1.7-4.4 nM) of HNF-4\(\alpha\)LBD for non-fluorescent, naturally-occurring fatty acyl CoAs showed that fatty acyl CoA binding to HNF-4\(\alpha\)LBD was not just a unique property of cis-parinaroyl-CoA. Furthermore, the two-order of magnitude higher \(K_d\)s of HNF-4\(\alpha\)LBD for cis-parinaroyl CoA (\(K_d\)s near 250 nM, depending on the type of assay, Table 2) suggested that presence of the conjugated tetraene double bonds in cis-parinaroyl CoA reduced HNF-4\(\alpha\)LBD’s affinity for 18-carbon, naturally-occurring fatty acyl-CoA.

**Binding specificity of HNF-4\(\alpha\)LBD: free fatty acids.** In order to further assess the binding specificity of HNF-4\(\alpha\)LBD, the binding of two long-chain free fatty acids was examined: NBD-stearic acid (a fluorescently labeled, synthetic, 18-carbon saturated fatty acid) and arachidonic acid (a nonfluorescent, naturally-occurring, 20-carbon polyunsaturated fatty acid). In aqueous buffer, NBD-stearic acid fluoresced weakly with maximal emission near 562 nm (Fig. 9A, spectrum 1). However, when a constant
amount of NBD-stearic acid was titrated with increasing concentrations of HNF-4\(_{\alpha}\)LBD (Fig.9A, spectra 2-8), three types of changes in the NBD-stearic acid fluorescence emission occurred. First, NBD-stearic acid fluorescence emission maximum exhibited a 32 nm blue shift from 562 nm in the absence of HNF-4\(_{\alpha}\)LBD, to 530 nm at saturation with HNF-4\(_{\alpha}\)LBD (Fig. 9A). When compared to a calibration curve of NBD-stearic acid in solvents with increasing dielectric constants (41), the maximal emission wavelength of NBD-stearic acid bound to HNF-4\(_{\alpha}\)LBD corresponded to a dielectric constant near 2, indicating that the HNF-4\(_{\alpha}\)LBD-bound NBD-stearic acid was localized in a highly hydrophobic microenvironment. Second, excitation of HNF-4\(_{\alpha}\)LBD protein at 280 nm resulted in a small, but visible increase in the NBD-stearic acid fluorescence emission at 530 nm, due to FRET between HNF-4\(_{\alpha}\)LBD (donor) and NBD-stearate (acceptor) (Fig. 9B). Third, NBD-stearic acid exhibited a saturable increase in fluorescence emission intensity with increasing HNF-4\(_{\alpha}\)LBD (Fig. 9A). Titration of a constant amount of HNF-4\(_{\alpha}\)LBD with increasing concentrations of NBD-stearic acid followed by measurement of the increase in the NBD-stearic acid fluorescence emission intensity at 530 nm (upon excitation at 480 nm), resulted in a hyperbolic, saturable, binding curve (Fig. 9C). The linear plot of this curve demonstrated a single binding site (Fig. 9C, inset) and allowed calculation of a \(K_d\) of 93 nM for NBD-stearic acid.

Since the affinity of HNF-4\(_{\alpha}\)LBD for NBD-stearic acid (\(K_d\) of 93 nM) was 5-7 fold higher than for the other fluorescent fatty acid examined, i.e. cis-parinaric acid (\(K_d\) of 538-642 nM, Table 2), it was important to determine if this binding was dependent on the presence of the fluorophores (NBD, conjugated tetraene) present in these free fatty acids. Therefore, the affinity of HNF-4\(_{\alpha}\)LBD for a non-fluorescent fatty acid, arachidonic acid, was determined. Increasing arachidonic acid also decreased the HNF-4\(_{\alpha}\)LBD Trp emission at 333 nm (upon excitation at 280 nm) and resulted in a hyperbolic, saturable binding curve (Fig. 9D). The linear plot of this curve resolved a single binding site with weak affinity as evidenced by \(K_d\) of 742 nM (Fig.9D, inset).

In summary, unlike the very high affinity of HNF-4\(_{\alpha}\)LBD for non-fluorescent fatty acyl CoAs (\(K_d\)s of 1-5 nM), HNF-4\(_{\alpha}\)LBD only weakly bound non-fluorescent free fatty acid (\(K_d\) of 742 nM). However, the presence of fluorophores in the free fatty acid increased this affinity by 5-7 fold. These data suggested that HNF-4\(_{\alpha}\)LBD’s ligand
binding specificity, especially for molecules that were not fatty acyl-CoAs, might be highly dependent on the individual structure of the potential ligand examined.

**HNF-4αLBD binds peroxisome proliferators (PPs) as CoA-thioesters or free fatty acids.** Because the structures of peroxisome proliferators such as Medica 16 and bezafibrate show some similarities to fatty acids (Table 1), the interaction of their CoA-thioesters with HNF-4αLBD and full length HNF-4α was recently examined (12). However, a radioligand competition assay yielded only low affinities for these drugs, i.e. K\(d\)s in the \(\mu\)M range (12). Since results with direct fluorescence binding assays presented herein showed that the radioligand binding assay underestimated the affinities of HNF-4αLBD for fatty acyl-CoAs by 3-orders of magnitude, HNF-4αLBD binding parameters for Medica 16-CoA, bezafibroyl-CoA, as well as for the free acid forms of these PPs were examined by exciting HNF-4αLBD Trp/Tyr at 280 nm and determining the effect on HNF-4αLBD Trp emission at 333 nm.

Medica 16-CoA, unlike all the other ligands tested, induced an increase in fluorescence emission intensity of Tyr/Trp of HNF-4αLBD. A titration curve of the Medica 16-CoA concentrations versus the Tyr/Trp fluorescence intensities fitted to a hyperbola, illustrating saturation binding (Fig. 10A). A reciprocal plot showed a straight line, consistent with a single binding site (Fig. 10A, inset), which yielded a K\(d\) of 2.6 nM (Table 4). Medica 16 in free acid form quenched HNF-4αLBD Trp fluorescence emission intensity. Again, the titration curve for Medica 16 in free acid form fitted a saturation binding curve (Fig. 10B), which when linearized indicated a single binding site (Fig. 10B, inset) with K\(d\) value of 33.6 nM (Table 4).

Titration of HNF-4αLBD with bezafibroyl-CoA induced quenching in the intrinsic fluorescence of the protein, resulting in a saturation binding curve (Fig. 10C) which in a double reciprocal plot suggested a single binding site (Fig. 10C, inset) and yielded a K\(d\) of 29.3 nM (Table 4). For bezafibrate in free acid form, linear plot of the binding curve (Fig. 10D) yielded a slightly higher K\(d\) (57.1 nM) (Table 4).

In summary, HNF-4αLBD has high affinity (K\(d\)s as low as 2.6 nM) for certain classes of peroxisome proliferator drugs, especially in their CoA thioester form. The order of affinities of the peroxisome proliferator drugs tested was Medica-16-CoA > Medica-16 > Bezafibroyl-CoA > Bezafibrate.
Secondary structure of HNF-4<sub>α</sub>LBD and effects of fatty acyl-CoAs: Circular dichroism. One mechanism whereby ligands are thought to affect the function of nuclear binding proteins is to induce a conformational change in the protein to allow further events important for transcription to occur. The observation that non-fluorescent ligands induced significant alterations in HNF-4<sub>α</sub>LBD Trp fluorescence emission (regardless of whether Trp/Tyr or Trp alone were excited) (Figs. 8,10) suggested that fatty acyl-CoAs altered the secondary structure and conformation of HNF-4<sub>α</sub>LBD. This possibility was further tested using circular dichroism.

The far UV circular dichroic spectrum of HNF-4<sub>α</sub>LBD exhibited two minima, at 208 nm and 220 nm and one maximum at 195 nm (Fig. 11, filled circles). Spectrum analysis using the CDsstr program as described in Methods, indicated that the polypeptide structure of HNF-4<sub>α</sub>LBD was comprised of 18.6% total helix, 27.3% β-strands, 1.5% turns and 38.7% other types of secondary structures (Table 5). Palmitoyl-CoA, a 16-carbon chain length, saturated fatty acyl-CoA, significantly changed the circular dichroic spectrum of HNF-4<sub>α</sub>LBD such that the minima at 208 nm and 220 nm significantly decreased to lower molar ellipticity values (Fig. 11, open triangles). Accordingly, a higher total helix percentage of 22% was calculated for HNF-4<sub>α</sub>LBD in the presence of palmitoyl-CoA (Table 5). Interestingly, arachidonoyl-CoA, a 20-carbon chain length, polyunsaturated fatty acyl-CoA also altered the secondary structure of HNF-4<sub>α</sub>LBD, but oppositely to palmitoyl-CoA. The molar ellipticity values of the minima at 208 and 220 nm exhibited by HNF-4<sub>α</sub>LBD in the presence of arachidonoyl-CoA were higher (Fig. 11, open squares) than in the absence of the fatty acyl-CoA (Fig. 11, filled circles). This change was reflected in the helical and the β-strand structure percentages: nearly 3-fold lower percentages of helical structures, in total only 6.4% (Table 5), but higher amount of turns (6.3%) and β-strands (35.7%) (Table 5) in the presence of arachidonoyl-CoA.

In summary, fatty acyl-CoA binding significantly altered the secondary structure of HNF-4<sub>α</sub>LBD with the direction of change highly dependent on the specific type of fatty acyl CoA.
DISCUSSION

Since hepatic nuclear factor-4α (HNF-4α) regulates expression of liver genes involved in both lipid and carbohydrate metabolism, investigations on its structure and mechanism of action impacts on our understanding of diabetes, obesity, and atherosclerosis. Despite this importance of HNF-4α, almost nothing is known regarding either its structure, especially that of the ligand binding domain (i.e. HNF-4αLBD), or how structure relates to function. In fact, until recently HNF-4α was considered an orphan nuclear receptor. However, in 1998 Hertz et al (15) demonstrated that various long chain fatty acyl-CoA (LCFA-CoA) thioesters bound to recombinant rat HNF-4α and affected its transcriptional activity, depending on their chain length and degree of saturation. While this exciting discovery first suggested that fatty acyl-CoAs may be the natural ligand for this nuclear receptor, the radioligand competition assay used to demonstrate binding of fatty acyl-CoAs to HNF-4α and HNF-4αLBD yielded low affinities, i.e. K_d’s in the µM range (15). Consequently, it has been suggested that this low affinity represents nonspecific binding and is insufficient for HNF-4α to significantly bind and be modulated by LCFC-CoAs in the nucleus (19). However, a significant potential problem with the radioligand competition assays is that they can seriously underestimate the affinities of binding proteins (20) or transcription factors (e.g., PPARα, (42)) for lipidic ligands. The purpose of the present investigation has been to resolve this issue of whether HNF-4αLBD binds fatty acyl-CoAs with µM or nM K_d, to determine the ligand specificity of HNF-4αLBD, and examine if fatty acyl-CoA binding alters the structure/conformation of HNF-4αLBD. As shown for the first time herein: (i) Forster fluorescence resonance energy transfer demonstrated close molecular interaction between fatty acyl-CoA and HNF-4αLBD; (ii) A series of three independent, direct fluorescence binding assays showed that HNF-4αLBD bound fatty acyl-CoAs with high affinity, K_d as low as 1 nM; (iii) Fatty acyl-CoA binding induced significant changes in HNF-4αLBD conformation as evidenced by intrinsic fluorescence and circular dichroism. The significance of these findings is detailed as follows:

First, the synthesis of cis-parinaroyl-CoA as ligand with excitation overlapping the emission of HNF-4αLBD allowed for the first time demonstration of their close
molecular interaction and determination of the intermolecular distance between the fluorescent ligand and its binding domain. FRET showed that the average intermolecular distance between HNF-4\(\alpha\)LBD Trp and bound cis-parinaroyl-CoA was in the range of 23 Å (no ligand-induced conformational change in HNF-4\(\alpha\)LBD) to 42 Å (ligand-induced conformational change in HNF-4\(\alpha\)LBD). These are average intermolecular distances since HNF-4\(\alpha\)LBD contains 2 Trp residues (43). As further studies with non-fluorescent fatty acyl CoAs and circular dichroism supported the conclusion that fatty acyl-CoAs induce conformational changes in HNF-4\(\alpha\)LBD, these data are consistent with the intermolecular distance between bound fatty acyl-CoA and HNF-4\(\alpha\)LBD Trp being < 42 Å. Further, the results demonstrate that fatty acyl-CoA binding with HNF-4\(\alpha\)LBD represent direct, molecular interaction rather than nonspecific coaggregation.

Second, three types of direct fluorescence binding assays showed that previous radioligand competition assays underestimated the binding affinities of HNF-4\(\alpha\)LBD for fatty acyl-CoAs. Instead, the direct fluorescence binding assays showed that HNF-4\(\alpha\)LBD bound fatty acyl-CoAs with high affinities as indicated by \(K_d\)s as low as 1 nM. This underestimation is characteristic of lipidic radioligand competition binding assays which typically yield \(K_d\)’s that are 2-3 orders of magnitude higher (i.e. lower affinity) than \(K_d\)’s determined by direct fluorescence or titration microcalorimetry binding assays (rev. in (20)). In contrast, the direct fluorescence binding assays took advantage of three different intrinsic properties of ligand or protein including the fluorescence of cis-parinaroyl-CoA, the intrinsic fluorescence of HNF-4\(\alpha\)LBD, or the FRET sensitized emission of HNF-4\(\alpha\)LBD bound cis-parinaroyl-CoA. None of these direct fluorescence ligand binding assay required separation of bound from free ligand. Taken together, the present study demonstrates that i) HNF-4\(\alpha\)LBD exhibits saturable fatty acyl-CoA binding, (ii) HNF-4\(\alpha\)LBD has one ligand binding site, (iii) the ligand binding site of HNF-4\(\alpha\)LBD has \(K_d\)s for LCFA-CoAs (e.g., palmitoyl-CoA, stearoyl-CoA, linoleoyl-CoA, arachidonyl-CoA) in the very low nM range (1.6–4 nM), (iv) The respective free acids may bind, albeit with a significantly lower affinity (\(K_d\)s for C20:4-CoA and C20:4 differ by about 200 fold).
Third, fatty acyl-CoA binding was shown to differentially alter the structure of HNF-4αLBD. This was supported by two types of evidence: (i) A wide variety of fatty acyl-CoAs and peroxisome proliferator-CoAs significantly quenched HNF-4αLBD Trp fluorescence emission at 333 nm. (ii) Two LCFA-CoAs, a saturated one, C16:0-CoA and a polyunsaturated one, C20:4-CoA, were tested for ability to induce changes in the secondary structure of HNF-4αLBD. Although both ligands quenched HNF-4αLBD Trp fluorescence emission, they differentially altered circular dichroic spectra of HNF-4αLBD (Fig. 11). Very significant was the finding that palmitoyl-CoA increased the α-helix and turn content while arachidonoyl-CoA decreased the content of 310-helix and 31-helix, while increasing β-strands and turns. These very different changes induced by palmitoyl- vs arachidonoyl-CoAs in the secondary structure of HNF-4αLBD correlated to the opposite effect of saturated and polyunsaturated fatty acyl-CoAs in functional assays (15).

Fourth, the proposed role of LCFA-CoA as natural ligands of HNF-4α (15) was challenged by Bogan et al. (19) on the grounds of (i) computer modeling of HNF-4αLBD structure based on its 22% and 37% overall identity with the progesterone and RXR receptors LBD, respectively, and in particular its homology with helix 1 and the F domain of progesterone receptor LBD; this modeling predicted a ligand binding pocket of around 320 Å³ as compared with a volume of 850 Å³ apparently required for the CoA-thioester; (ii) previously reported μM Kᵦₛ for LCFA-CoA binding to HNF-4α as compared with nM Kᵦₛ for the more abundant cytosolic fatty acyl CoA binding protein such as ACBP (Kᵦ of 7 nM (40)), FABP (Kᵦₛ of 41–60 nM (26)), and SCP-2 (Kᵦ of 4.5 nM (27,29)); (iii) lack of induced conformational changes in HNF-4α as deduced from limited proteolysis (19). It is worth noting however that: (i) circular dichroic data presented here indicates a higher percentage of β-strand (27.3%) than helical secondary structure (3.3% α-helix, 6.0% 310-helix and 9.3% 31-helix) and is inconsistent with the predicted modeling. Furthermore, FRET results indicate direct interaction of LCFA-CoA with the ligand binding site of HNF-4α. (ii) While HNF-4α is localized in the nucleus (43,44), ACBP and L-FABP are essentially localized outside the nucleus (rev. in (16,20)) and SCP-2 is not detected in the nucleus (rev. in (45)). More important, the binding data
presented here indicates 1–4 nM $K_d$s for LCFA-CoA binding to HNF-4α, being in the range of other acyl-CoA binding proteins as well as in the range of unbound free fatty acyl-CoA concentrations estimated in the nucleus (18). It is possible, however, that these extranuclear fatty acyl-CoA binding proteins may alter the distribution of fatty acyl-CoAs to the nucleus. Alternately, some L-FABP (29,46,47) and ACBP (18) detected within the nucleus may compete with HNF-4α for fatty acyl-CoA binding therein. (iii) This study reports direct evidence for ligand-induced conformational change verified by differential quenching of HNF-4αLBD Trp fluorescence emission as well as by differential changes in helical content due to ligand binding. This evidence is consistent with previously reported protection from limited proteolysis of HNF-4α by C14:0-CoA (12). Taken together, the results presented here support the original proposal by Hertz et al. (15) that LCFA-CoAs may serve as endogenous ligands for HNF-4α.

This report further substantiates earlier findings by Hertz et al. (15) that CoA thioesters of hypolipidemic peroxisome proliferators such as Medica homologues or fibrate drugs bind to HNF-4α or HNF-4αLBD and suppress its transcriptional activity. It further confirms that Medica 16-CoA is a more effective ligand than bezafibroyl-CoA (order of magnitude difference in $K_d$s). Similarly to LCFA-CoAs, $K_d$s for the CoA-thioesters of hypolipidemic peroxisome proliferators measured by quenching of HNF-4αLBD Trp fluorescence emission were in the nM range as compared with µM $K_d$s previously reported using radioligand competition assays (12). However, in contrast to the free acid forms of LCFA which show low binding affinity to HNF-4αLBD ($K_d$s of 421–742 nM), binding affinities for the free acid forms of hypolipidemic peroxisome proliferators were in the low nM range (34–57 nM), albeit significantly lower as compared with the respective CoA-thioesters. The capacity of hypolipidemic peroxisome proliferators bound in their free acid form to suppress the transcriptional activity of HNF-4α still remains to be investigated. It is worth noting however that HNF-4α transcriptional activity was not suppressed by amphipathic carboxylates which due to a structural constraint did not endogenously yield the respective CoA-thioester.

Suppression of HNF-4α activity by the CoA-thioesters of hypolipidemic peroxisome proliferators in the nM range may indicate that HNF-4α serves as target for
their therapeutic activity. This is consistent with HNF-4α involvement in controlling the expression of genes encoding proteins with role in production and clearance of plasma lipoproteins (e.g., MTP, apo B, apo C-III and others (7)). Since the human liver is not responsive to PPARα (rev. in (48,49)), the hypolipidemic activity induced by hypolipidemic PPARα agonists in humans must be independent of PPARα. In contrast, in rodents, activation of PPARα by hypolipidemic peroxisome proliferators may result in HNF4α displacement from DR-1 response elements shared by both transcription factors (50). Suppression of HNF-4α function in transactivation by the CoA-thioesters of hypolipidemic peroxisome proliferators offers an hypolipidemic mode of action of peroxisome proliferators in humans independent of liver PPARα and its proliferative-carcinogenic activity.

In conclusion, multiple fluorescence spectroscopic and circular dichroism approaches strongly demonstrate that fatty acyl-CoAs and certain peroxisome proliferator-CoAs are specific ligands for HNF-4αBD and that fatty acyl-CoA binding induces significant changes in HNF-4αBD secondary and overall conformational structure. While naturally-occurring fatty acids only weakly bind HNF-4αBD, some synthetic fluorescent fatty acids as well as certain peroxisome proliferators also bind as free acids to HNF-4αBD but with lower affinities than the respective acyl-CoAs. These data demonstrate for the first time that fatty acyl-CoAs and certain peroxisome proliferator drug thioesters may be natural and pharmacological HNF-4αBD ligands at physiological concentrations.
REFERENCES

1. Mangelsdorf, D. J. and Evans, R. M. (1995) Cell 83, 841-850.

2. Sladek, F. M. (1994) In Tronche, F. and Yaniv, M., editors. Liver Gene Expression, VRG Landes Co., Austin, TX, 207-230.

3. Jiang, G., Nepomuceno, L., Hopkins, K., and Sladek, F. M. (1995) Mol.Cell.Biol. 15, 5131-5134.

4. Nakshatri, H. and Chambon, P. (1994) J.Biol.Chem. 269, 890-902.

5. Li, J., Ning, G., and Duncan, S. A. (2000) Genes & Development 14, 464-475.

6. Hayhurst, G. P., Lee, Y. H., Lambert, G., Ward, J. M., and Gonzalez, F. J. (2001) Mol.Cell.Biol. 21, 1393-1403.

7. Zannis, V. I., Kan, H. Y., Kritis, A., Zanni, E., and Kardassis, D. (2001) Front.Biosci. 6, D456-D504.

8. Shih, D. W., Dansky, H. M., Fleisher, M., Assmann, G., Fajans, S., and Stoffel, M. (2000) Diabetes 49, 832-837.

9. Wang, H., Maechler, P., Antinozzi, P. A., Hagenfeldt, K. A., and Wollheim, C. B. (2000) J.Biol.Chem. 275, 35953-35959.

10. Stoffel, M. and Duncan, S. A. (1997) Proc.Natl.Acad.Sci 94, 13209-13214.

11. Jover, R., Bort, R., Gomez-Lechon, M. J., and Castell, J. V. (2001) Hepatology 33, 668-675.

12. Hertz, R., Sheena, V., Kalderon, B., Berman, I., and Bar-Tana, J. (2001) Biochem.Pharmacol. 61, 1057-1062.

13. Hadzopoulou-Cladaras, M., Kistanova, E., Evagelopoulou, C., Zeng, S., Cladaras, C., and Ladias, A. A. J. (1997) The Journal of Biological Chemistry 272, 539-550.
14. Carter, M. E., Gulick, T., Raisher, B. D., Caira, T., Ladias, A. A. J., Moore, D. D., and Kelly, D. P. (1993) *The Journal of Biological Chemistry* **268**, 13805-13810.

15. Hertz, R., Magenheim, J., Berman, I., and Bar-Tana, J. (1998) *Nature* **392**, 512-516.

16. Gossett, R. E., Frolov, A. A., Roths, J. B., Behnke, W. D., Kier, A. B., and Schroeder, F. (1996) *Lipids* **31**, 895-918.

17. Knudsen, J., Jensen, M. V., Hansen, J. K., Faergeman, N. J., Neergard, T., and Gaigg, B. (1999) *Mol.Cell.Biochem* **192**, 95-103.

18. Elholm, M., Garras, A., Neve, S., Tarnehave, D., Lund, T. B., Skorve, J., Flatmark, T., Kristiansen, K., and Berge, R. K. (2000) *J.Lip.Res.* **41**, 538-545.

19. Bogan, A. A., Dallas-Yang, Q., Ruse, M. D., Maeda, Y., Jiang, G., Nepomuceno, L., Scanlan, T. S., Cohen, F. E., and Sladek, F. M. (2000) *J.Mol.Biol.* **302**, 831-851.

20. McArthur, M. J., Atshaves, B. P., Frolov, A., Foxworth, W. D., Kier, A. B., and Schroeder, F. (1999) *J.Lip.Res.* **40**, 1371-1383.

21. Bar-Tana, J., Kahn-Rose, G., and Srebnik, B. (1985) *J.Biol.Chem.* **260**, 8404-8410.

22. Kawaguchi, A., Yohmura, T., and Okuda, S. (1981) *J.Biochem.* **89**, 337-339.

23. Frolov, A., Petrescu, A., Atshaves, B. P., So, P. T. C., Gratton, E., Serrero, G., and Schroeder, F. (2000) *J.Biol.Chem.* **275**, 12769-12780.

24. Juguelin, H. and Cassagne, C. (1984) *Anal.Biochem.* **142**, 329-335.

25. Hubbell, T., Behnke, W. D., Woodford, J. K., and Schroeder, F. (1994) *Biochemistry* **33**, 3327-3334.

26. Frolov, A., Cho, T. H., Murphy, E. J., and Schroeder, F. (1997) *Biochemistry* **36**, 6545-6555.
27. Frolov, A., Cho, T. H., Billheimer, J. T., and Schroeder, F. (1996) J.Biol.Chem. 271, 31878-31884.

28. Serrero, G., Frolov, A., Schroeder, F., Tanaka, K., and Gelhaar, L. (2000) Biochim.Biophys.Acta 1488, 245-254.

29. Schroeder, F., Frolov, A., Starodub, O., Russell, W., Atshaves, B. P., Petrescu, A. D., Huang, H., Gallegos, A., McIntosh, A., Tahotna, D., Russell, D., Billheimer, J. T., Baum, C. L., and Kier, A. B. (2000) J.Biol.Chem. 275, 25547-25555.

30. Nemecz, G., Hubbell, T., Jefferson, J. R., Lowe, J. B., and Schroeder, F. (1991) Arch.Biochem.Biophys. 286, 300-309.

31. Forster, T. (1967) Mechanism of energy transfer. In M.Florkin and E.H.Statz, editors. Comprehensive Biochemistry., Elsevier, New York, 61-77.

32. Frolov, A., Miller, K., Billheimer, J. T., Cho, T.-C., and Schroeder, F. (1997) Lipids 32, 1201-1209.

33. Lakowicz, J. F. (1999) Energy transfer. In Lakowicz, J. F., editor. Principles of Fluorescence Spectroscopy, Kluwer Academic/Plenum Publishers, New York, N.Y., 367-394.

34. Stryer, L. (1978) Ann.Rev.Biochem. 47, 819-844.

35. Parker, C. A. (1968) Photoluminescence in Solution., Elsevier Scientific Publishers B.V., Amsterdam

36. Wu, P. and Brand, L. (1994) Anal.Biochem. 218, 1-13.

37. Petrescu, A. D., Gallegos, A. M., Okamura, Y., Strauss, I. J. F., and Schroeder, F. (2001) J.Biol.Chem. 276, 36970-36982.

38. Hennessey, J. P. and Johnson, W. C. (1981) Biochemistry 20, 1085-1094.
39. Compton, L. A. and Johnson, W. C. Jr. (1986) *Analytical Biochemistry* **155**, 155-167.

40. Frolov, A. A. and Schroeder, F. (1998) *J. Biol. Chem.* **273**, 11049-11055.

41. Schroeder, F., Myers-Payne, S. C., Billheimer, J. T., and Wood, W. G. (1995) *Biochemistry* **34**, 11919-11927.

42. Lin, Q., Ruuska, S. E., Shaw, N. S., Dong, D., and Noy, N. (1999) *Biochem* **38**, 185-190.

43. Sladek, F. M., Zhong, W., Lai, E., and Darnell, J. E. (1990) *Genes & Development* **4**, 2353-2365.

44. Ktistaki, E., Ktistakis, N. T., Papadogeorgaki, E., and Taliandis, I. (1995) *Proc. Natl. Acad. Sci.* **92**, 9876-9880.

45. Gallegos, A. M., Atshaves, B. P., Storey, S. M., Starodub, O., Petrescu, A. D., Huang, H., McIntosh, A., Martin, G., Chao, H., Kier, A. B., and Schroeder, F. (2001) *Prog. Lipid Res* **40**, 498-563.

46. Bordewick, U., Heese, M., Borchers, T., Robenek, H., and Spener, F. (1989) *Biol. Chem. Hoppe-Seyler* **370**, 229-238.

47. Wolfrum, C., Borrmann, C. M., Borchers, T., and Spener, F. (2001) *Proc. Natl. Acad. Sci* **98**, 2323-2328.

48. Hertz, R. and Bar-Tana, J. (1998) *Toxicol. Lett.* **102-103**, 85-90.

49. Cattley, R. C., DeLuca, J., and Elcombe, C. (1998) *Regul. Toxicol. Pharmacol.* **27**, 47-60.

50. Hertz, R., Bishara-Shieban, J., and Bar-Tana, J. (1995) *Journal of Biological Chemistry* **270**, 13470-13475.
Table 1. Chemical structures of fatty acids and peroxisome proliferators whose CoA thioesters as well as free acid forms were tested for HNF-4αLBD binding.

| Chemical | Structure |
|----------|-----------|
| Cis-parinarate | ![Cis-parinarate](image) |
| Palmitate | ![Palmitate](image) |
| Arachidonate | ![Arachidonate](image) |
| Medica | ![Medica](image) |
| Bezafibrate | ![Bezafibrate](image) |

R = OH, free fatty acid form; R = CoA, thioester form.
Table 2. Binding affinity of HNF-4α-LBD for cis-parinaroyl-CoA, CoA and cis-parinaric acid.

Cis-parinaroyl-CoA (cPNA-CoA); cis-parinaric acid (cPNA)a.

| Ligand       | Ex. b | Em c | Fluorophore    | Kd, (nM) | n  |
|--------------|-------|------|----------------|----------|----|
| cPNA-CoA     | 280   | 333  | Tyr/Trp        | 248.5    | 1.08 |
| cPNA-CoA     | 295   | 333  | Trp            | 238.2    | 1.42 |
| cPNA-CoA     | 280   | 430  | cPNA-CoA *     | 250.4    | 0.71 |
| cPNA-CoA     | 295   | 430  | cPNA-CoA *     | 758.9    | 1.02 |
| CoA          | 280   | 333  | Tyr/Trp        | 429.1    | 1.35 |
| CoA          | 295   | 333  | Trp            | 680.4    | 0.98 |
| cPNA         | 280   | 333  | Tyr/Trp        | 421.3    | 1.09 |
| cPNA         | 295   | 333  | Trp            | 588.9    | 0.82 |

aBinding parameters were obtained by Hill plot, Eq. 2, as described in Methods; bexcitation wavelength; cemission wavelength; * sensitized emission.
Table 3. Forster energy transfer from HNF-4αLBD Trp to bound cis-parinaroyl-CoA. J is the overlap integral. \( R_0 \) is the critical distance that allows 50% energy transfer efficiency and was calculated as described under “Experimental Procedures”. E is the energy transfer efficiency. \( R_{2/3} \) is the actual distance between HNF-4αLBD Trp and bound cis-parinaroyl-CoA.

| Energy donor  | Energy acceptor          | J         | \( R_0 \) | E  | \( R_{2/3} \) |
|---------------|--------------------------|-----------|-----------|----|--------------|
| HNF-4αLBD\(^a\) | cis-parinaroyl-CoA\(^a\) | 2.718 x 10\(^{14}\) | 30.1      | 12 | 42.0         |
| HNF-4αLBD\(^b\) | cis-parinaroyl-CoA\(^b\) | 2.718 x 10\(^{14}\) | 30.1      | 85 | 22.6         |

\(^a\) Both FRET and conformational change are induced by ligand binding to the protein.

\(^b\) FRET but no conformational change is induced by ligand binding to the protein.
Table 4. Specificity of HNF-4αLBD for non-fluorescent ligands.

| Ligand              | $K_d$ (nM) | $n^a$ |
|---------------------|------------|-------|
| Long chain fatty acyl-CoAs |            |       |
| C16:0-CoA           | 1.7        | 1.01  |
| C18:0-CoA           | 3.8        | 1.18  |
| C18:2-CoA           | 4.4        | -     |
| C20:4-CoA           | 4.0        | 1.38  |
| Long chain FFA      |            |       |
| C20:4               | 742        | 0.49  |
| Peroxisome proliferators |        |       |
| Medica-16-CoA       | 2.6        | 1.45  |
| Medica-16           | 33.6       | 1.57  |
| Bezafibroyl-CoA     | 29.3       | 1.63  |
| Bezafibrate         | 57.1       | 1.55  |

$^a$ n, the number of binding sites was determined from Hill plot, Eq. 2, as described in Methods.
Table 5. Effect of CoA thioesters of long-chain saturated and unsaturated fatty acids on the helical secondary structure of HNF4αLBD.

| Ligand      | α-Helix (H, %) | 3_10-Helix (G, %) | 3_1-helix (P, %) | Total α-helix (HJ %) | β-Strand (E, %) | Turns (T, %) | Others (O, %) |
|-------------|----------------|-------------------|------------------|----------------------|----------------|-------------|---------------|
| None        | 3.3 ± 0.003    | 6.0 ± 0.006       | 9.3 ± 0.021      | 7.7 ± 0.001          | 27.3 ± 0.025   | 1.5 ± 0.017 | 38.7 ± 0.032 |
| C16:0-CoA   | 6.0 ± 0.002    | 6.5 ± 0.001       | 9.5 ± 0.007      | 12.0 ± 0.002         | 27.5 ± 0.007   | 11.5 ± 0.007| 39.0 ± 0.014 |
| C20:4-CoA   | 3.0 ± 0.010    | 2.3 ± 0.015       | 1.1 ± 0.058      | 6.0 ± 0.004          | 35.7 ± 0.100   | 6.3 ± 0.025 | 43.0 ± 0.017 |

*C16:0-CoA is palmitoyl-CoA.

*C20:4-CoA is arachidonoyl-CoA.

* "Total α-helix (HJ %)" does not represent the sum of α 3_10 and 3_1 helix. Instead, “Total α-helix (HJ %)” is calculated using a different standard protein structure database with the same program as described in Methods.
FIGURE LEGENDS

Figure 1. Purity of recombinant rat HNF-4αLBD. **Panel A**: Ni-column purified His-HNF-4αLBD (lane 1) and molecular weight standards (lane 2) were run in SDS-PAGE and stained with Coomassie-Blue as described in Experimental Procedures. **Panel B**: Western blot of the recombinant His-HNF-4αLBD with rabbit polyclonal anti-HNF-4αLBD and alkaline phosphatase-labeled goat anti-rabbit IgG; lane 1, 5 ng purified recombinant HNF-4αLBD; lane 2, prestained molecular weight standards.

Figure 2. HPLC purification of cis-parinaroyl-CoA. **Panel A**: HPLC chromatogram indicating early exclusion of polar components in peaks 1, 2 and 3, and late exclusion of a hydrophobic component in peak 4 with retention time at 34 min. **Panel B**: Absorbance spectrum of a sample of partially purified cis-parinaroyl-CoA, before HPLC run. **Panel C**: the absorbance spectrum of a sample of peak 1, identical to free CoA (not shown). **Panel D**: The absorbance spectrum of HPLC purified peak 4 (from panel A) containing cis-parinaroyl-CoA.

Figure 3. Titration of HNF-4αLBD with cis-parinaroyl-CoA: binding curves derived from quenching in HNF-4αLBD fluorescence. **Panel A**: Emission spectra of HNF-4αLBD upon excitation of Tyr/Trp at 280 nm; spectrum 1, 170 nM HNF-4αLBD with no ligand added; spectra 2-9, HNF-4αLBD in the presence of 105 nM, 175 nM, 280 nM, 525 nM, 875 nM, 1400 nM, 2100 nM and 3500 nM cis-parinaroyl-CoA. **Panel B**: Plot of fluorescence emission maxima at 333 nm upon excitation at 280 nm, over the cis-parinaroyl-CoA concentrations. Inset: linear plot of the binding curve in panel B. **Panel C**: Emission spectra of HNF-4αLBD upon excitation of Trp only, at 295 nm; spectrum 1, 170 nM HNF-4αLBD in the absence of ligand; spectra 2-8, HNF-4αLBD in the presence of 105 nM, 280 nM, 525 nM, 875 nM, 1400 nM, 2100 nM and 3500 nM cis-parinaroyl-CoA. **Panel D**: Plot of fluorescence emission intensity maxima at 333 nm with excitation at 295 nm over increasing concentrations of cis-parinaroyl-CoA. Inset: linearization of the binding curve fits one exponential.
Figure 4. Spectral overlap of HNF-4αLBD Trp fluorescence emission with cis-parinaroyl-CoA absorption (excitation). Spectrum 1, the emission spectrum of HNF-4αLBD (170 nM in PBS); spectrum 2, the absorbance spectrum of cis-parinaroyl-CoA (10 μM in PBS).

Figure 5. Sensitized emission of HNF-4αLBD-bound cis-parinaroyl-CoA and binding curves as resulted from FRET data. Panel A: Emission spectra between 400 and 450 nm, showing increase in cis-parinaroyl-CoA fluorescence intensity with excitation of Tyr/Trp at 280 nm; spectra 1 to 9, as described in Fig.3 A. Panel B: Plot of fluorescence emission maxima of cis-parinaroyl-CoA at 430 nm upon excitation at 280 nm, over the cis-parinaroyl-CoA concentrations. Inset: linear plot of the binding curve in panel B. Panel C: Spectra portion from 400 to 500 nm showing increase in cis-parinaroyl-CoA fluorescence intensity from spectrum 1 to 8, with excitation of Trp at 295 nm; spectra 1-8, as described in Fig. 2 C. Panel D: Plot of fluorescence intensity maxima at 430 nm upon excitation of HNF-4αLBD Trp at 295 nm, over increasing concentrations of cis-parinaroyl-CoA. Inset: linearization of the binding curve fits one exponential. Spectra shown in panels A and C were obtained by subtracting control spectra of cis-parinaroyl-CoA in the absence of protein. When HNF-4αLBD was titrated with buffer only, no decrease in its fluorescence at 333 nm was detected.

Figure 6. Titration of HNF-4αLBD with CoA. Panel A: Fluorescence emission spectra of HNF-4αLBD (170 nM) upon excitation at 280 nm, in the absence (spectrum 1) and presence of increasing concentrations of CoA (spectra 2-8); 2, 250 nM; 3, 400 nM; 4, 750 nM; 5, 1250 nM; 6, 2 μM; 7, 3 μM; 8, 5 μM CoA. Panel B: The binding curve plotted with data in panel A. Inset: linearization of the binding curve fits to a single exponential. Panel C: Fluorescence emission spectra of HNF-4αLBD upon excitation at 295 nm, over increasing concentrations of CoA; spectrum 1, 170 nM HNF-4αLBD with no CoA added; spectra 2-8, CoA was added in concentrations from 250 nM to 5 μM. Panel D: The plot of fluorescence intensity maxima at 333 nm with excitation at 295 nm, over increasing concentrations of CoA. Inset: linear plot of the binding curve fits to a single exponential.
Figure 7. Titration of HNF-4αLBD with cis-parinaric acid. Panel A: Emission fluorescence spectra of 170 nM HNF-4αLBD in the absence (spectrum 1) and in the presence of 0.15-2.5 µM of cis-parinaric acid (spectra 2-6), with excitation at 280 nm. Inset: blow up of the emission spectra from 400 to 500 nm, showing no consistent increase in the fluorescence intensity at 440 nm. Panel B: The binding curve plotted as cis-parinaric acid concentration versus quenching in Tyr/Trp fluorescence of HNF-4αLBD protein with excitation at 280 nm. Inset: linear plot of the binding curve fits to a single exponential. Panel C: Emission fluorescence spectra of 400 nM HNF-4αLBD in the absence (spectrum 1) and in the presence of 0.25-2.5 µM cis-parinaric acid (spectra 2-6), with excitation of Trp only, at 295 nm. Inset: close up of the spectra from 400 to 500 nm, demonstrating no significant increase of the bound cis-parinaric acid, at 440 nm; Panel D: The binding curve plotted as cis-parinaric acid concentration versus quenching of Trp of HNF-4αLBD with excitation at 295 nm. Inset: linear plot of the binding curve fits to a single exponential.

Figure 8. Binding of long chain fatty acyl-CoAs to HNF-4αLBD. Panel A: Titration of 170 nM HNF-4αLBD with palmitoyl-CoA. Inset: linear plot of the binding curve fits to one exponential. Panel B: Titration of 170 nM HNF-4αLBD with stearoyl-CoA. Inset: linear plot of the binding curve fits to a single exponential. Panel C: Titration of HNF-4αLBD (170 nM) with linoleoyl-CoA. Inset: linear plot of the binding curve fits to one exponential. Panel D: Titration of HNF-4αLBD (170 nM) with arachidonoyl-CoA. Inset: linear plot of the binding curve fits to a single exponential.

Figure 9. Binding of long chain free fatty acids to HNF-4αLBD. Panel A: Emission fluorescence spectra of NBD-stearic acid (250 nM) when titrated with increasing concentrations of HNF-4αLBD; protein was added in following concentrations: 1, 0 nM; 2, 42.5 nM; 3, 85 nM; 4, 128 nM; 5, 170 nM; 6, 255 nM; 7, 340 nM; 8, 390 nM. Panel B: Sensitized emission of NBD-stearic acid upon excitation of Tyr/Trp of HNF-4αLBD at 280 nm; 1, NBD-stearic acid only; 2, NBD-stearic acid plus 43 nM HNF-4αLBD; 3,
NBD-stearic acid plus 400 nM HNF-4αLBD. Panel C: Binding of NBD-stearic acid to HNF-4αLBD measured as increase in NBD-stearic acid fluorescence at 530 nm upon excitation at 480 nm. Inset: linear plot of the binding curve fits to a single exponential. Panel D: Binding of arachidonic acid to HNF-4αLBD measured as quenching in Tyr/Trp fluorescence of HNF-4αLBD, upon excitation at 280 nm. Inset: linear plot of the binding curve fits to a single exponential.

**Figure 10. Binding of peroxisome proliferators as CoA thioesters or free fatty acids, to HNF-4αLBD.** Panel A: Titration of 170 nM HNF-4αLBD with Medica-16-CoA, measured as quenching in Tyr/Trp fluorescence of HNF-4αLBD upon excitation at 280 nm. Inset: linear plot of the binding curve fits to one exponential. Panel B: Titration of 170 nM HNF4αLBD with Medica-16, measured as increase in Tyr/Trp fluorescence of HNF-4αLBD. Inset: linear plot of the binding curve fits to a single exponential. Panel C: Titration of 170 nM HNF-4αLBD with bezafibroyl-CoA, measured as quenching in Tyr/Trp fluorescence of HNF-4αLBD upon excitation at 280 nm. Insert: linear plot of the binding curve fits to one exponential. Panel D: Titration of 170 nM HNF-4αLBD with bezafibrate, measured as quenching in Tyr/Trp fluorescence of HNF-4αLBD upon excitation at 280 nm. Inset: linear plot of the binding curve fits to one exponential.

**Figure 11. Circular dichroic (CD) spectral changes induced by fatty acyl-CoAs in HNF-4αLBD.** The far UV CD spectrum of 2 μM HNF-4αLBD in the absence (filled circles) and in the presence of 50 μM arachidonoyl-CoA (empty squares) or palmitoyl-CoA (empty triangles).
Fig. 1

A

B

HNF4α-LBD

1  2

45  29

19  15

77 kD

45 kD

1  2

29

19  15  5.5

HNF4α-LBD
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 7
Fig. 8
Fig. 9
Fig. 10

A. Fluorescence intensity (a.u., ex. 280 nm, em. 333 nm) vs. [Med16-CoA]/(Fi/Fmax), nM

B. Fluorescence intensity (a.u., ex. 280 nm, em. 333 nm) vs. [Med16]/(Fi/Fmax), nM

C. Fluorescence intensity (a.u., ex. 280 nm, em. 333 nm) vs. [BZF-CoA]/(Fi/Fmax), nM

D. Fluorescence intensity (a.u., ex. 280 nm, em. 333 nm) vs. [BZF]/(Fi/Fmax), nM
Fig. 11
Ligand specificity and conformational dependence of the hepatic nuclear factor-4α (HNF-4 α)
Anca D. Petrescu, Rachel Hertz, Jacob Bar-Tana, Friedhelm Schroeder and Ann B. Kier
J. Biol. Chem. published online April 8, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201241200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts