Hepatocyte nuclear factor-4α (HNF-4α) activity is modulated by natural and xenobiotic fatty acid and fatty acyl-CoA ligands as a function of their chain length, unsaturation, and substitutions. The acyl-CoA site of HNF-4α is reported here to consist of the E-F domain, to bind long-chain acyl-CoAs but not the respective free acids, and to catalyze the hydrolysis of bound fatty acyl-CoAs. The free acid pocket, previously reported in the x-ray structure of HNF-4α, is nonhydrolyzable acyl-CoA ligands of HNF-4α that inhibit its thioesterase activity. Hence, HNF-4α tran-
scriptional activity is controlled by its two interrelated free fatty acid binding sites is abrogated by high affin-
ity, nonhydrolyzable acyl-CoA ligands of HNF-4α that inhibit its thioesterase activity. Hence, HNF-4α tran-
scriptional activity is controlled by its two interrelated acyl-CoA ligands and two binding sites interphased in tan-
dem by the thioesterase activity. The acyl-CoA/free-acid and receptor/enzyme duality of HNF-4α extends the paradigm of nuclear receptors.

Hepatocyte nuclear factor-4α (HNF-4α) is a member of the superfamily of nuclear receptors (reviewed in Ref. 1). It is expressed in liver, intestine, pancreas, and kidney and is required for tissue-specific expression of many of their respective traits (2,3, reviewed in 4). In analogy with other nuclear receptors of the superfamily, HNF-4α consists of an N-terminal A/B domain (aa 1–51), including a ligand-independent transactiva-
tion function (AF-1), C-domain (aa 51–106) consisting of the DNA binding domain, hinge D-domain (aa 106–185), ligand-binding domain (LBD) consisting of a ligand-dependent tran-
scriptional activation function (AF-2) within an E-domain (aa 185–368), and a C-terminal inhibitory F-domain (aa 368–455). Tran-
scriptional activation by HNF-4α is mediated by its binding as homodimer to DR-1 promoter sequences of target genes. Genes activated by HNF-4α encode some transcription factors (e.g. HNF-1, PXR, and SHP), enzymes and proteins involved in lipid and protein metabolism (e.g. apoA-I, -AII, -B, -CII, and -CIII and microsomal triglyceride transfer protein), carbohydrate metabolism (e.g. insulin, glut2, glucokinase, glucose-
6-phosphatase, phosphoenolpyruvate carboxykinase), hematopoiesis (e.g. erythropoietin and transferrin), blood coagulation (e.g. Factors VII, IX, X, and XI and fibrinogen), and others. The importance of HNF-4α is reflected by its extensive in vivo association with RNA polymerase II-transcribed genes in liver and pancreas (5). Its functional importance in regulating lipid and carbohydrate metabolism is reflected by the central role played by HNF-4α-responsive genes in controlling lipoprotein production and their plasma clearance, hepatic glucose production and utilization, and also pancreatic insulin production and secretion (1). Indeed, mutations in HNF-4α result in maturity onset diabetes of the young (MODY)-1 (6), and single nucleotide polymorphisms located in the HNF-4α promoter have recently been reported to show the strongest association with noninsulin-dependent diabetes mellitus in sibling pair families (7, 8).

Until recently, HNF-4α was considered to be a constitutively active orphan receptor. Various fatty acyl-CoA thioesters longer than C12 as well as xenobiotic acyl-CoA thioesters of hypolipidemic amphipathic carboxylates (e.g. Medica analogues) have now been reported by us to specifically bind to the full-length HNF-4α1, or its LBD (aa 132–455), with Keq values in the 1.0 nM range as compared with 10- to 100-fold lower binding affinities of the respective free acids (9–11). Binding of acyl-
CoAs results in conformational changes of HNF-4α and in activation or suppression of its transcriptional activity in transfected cells as function of chain length, degree of unsaturation and extent of substitution of respective acyl-CoA ligands (9–11). Activation of HNF-4α transcriptional activity by fatty acyl agonist ligands (e.g. C14:0, C16:0, and C16:1) is best exemplified by missense mutants of the ligand binding domain of HNF-4α (e.g. MODY-1 mutants), that fail to transactivate transcription due to their reduced binding affinities for fatty acyl agonist ligands of HNF-4α (12). These mutants may, however, be rescued in transfection assays by added C14:0, C16:0, or C16:1 fatty acids, yielding transcriptional activities in the wild type range (12). In contrast to activation by C14–C16 fatty acids, the transcriptional activity of HNF-4α is robustly suppressed by long-chain polyunsaturated fatty acids (e.g. C20:
Acyl-CoA Thioesterase Activity of HNF-4α

EXPERIMENTAL PROCEDURES

Plasmids and Recombinant Proteins—Full-length rat HNF-4α cDNA cloned into 6His-pET11d plasmid was from S. Malik (20). Rat C-terminal His-tagged HNF-4α(aa132–435) recombinant was prepared by PCR using the sense 5′-CGGGCCATATGGGATGAGGTCTTCCGCTTCTGCTGTTTTTCTGTTCTGCTCTGCTGAGG and antisense 5′-GAAGATCTCTAGGTGGACATCTCCGGCTCGAGGATGGCTTCCT-1 protein when cloned into the NdeI/XhoI site of pET21b plasmid (Novagen, Milwaukee, WI). Rat N-terminal His-tagged HNF-4α(aa132–410) recombinant was prepared by PCR using the sense 5′-CATGCCTAGGGGCCACCATCATCATCATACAGTGGCACGTCTGCTGAGG and antisense 5′-GAAGATCTCTAGGTGGACATCTCCGGCTCGAGGATGGCTTCCTGCTGAGG and primer pairs, followed by cloning the PCR product into pET11d plasmid. Rat N-terminal His-tagged HNF-4α(aa132–370) recombinant was prepared by PCR using the sense 5′-CATGCCTAGGGGCCACCATCATCATCATACAGTGGCACGTCTGCTGAGG and antisense 5′-CTCGAGTCACAGCTGCTTTTGCTT primers, followed by cloning the PCR product into pET11d plasmid. Recombinant plasmids were expressed in E. coli BL21(DE3)pLys strain, and the His-tagged proteins were purified by affinity chromatography on nickel nitroliaetric acid resin (Qiagen, Chatsworth, CA) and stored at −70 °C. Recombinant mPPARα and hRXRα proteins were prepared as previously described (18). Purity of recombinant proteins was assessed by SDS-PAGE and amounted to 70–90%.

Thioesterase Activity—Unless otherwise indicated, the thioesterase activity of HNF-4 recombinants was measured using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (21). Reaction mixtures contained 10 mM Hepes, pH 7.4, 200 mM KCl, 0.05 mM DTNB, 15–30 μg of the indicated HNF-4 recombinant, and the respective acyl-CoA as indicated in a final volume of 3.0 ml. The reaction was started with the addition of the acyl-CoA substrate and was followed spectrophotometrically (E412 = 13,600 M−1 cm−1) (21). The thioesterase activity of HNF-4 recombinants was also measured radioactively by following the hydrolysis of 1-[14C]palmitoyl-CoA. Reaction mixtures contained 10 mM Hepes, pH 7.4, 200 mM KCl, 0.2–1.0 μM 1-[14C]palmitoyl-CoA (specific activity, 55 mCi/mmol), and 0.1–1.0 μg of recombinant HNF-4 in a final volume of 100 μl. The reaction was started by the addition of 1-[14C]palmitoyl-CoA. Following 5-min incubation at room temperature, the reaction was stopped by the addition of 2.0 ml of Dole reagent (22). Thioesterase activity represents initial rates under conditions of linearity with protein concentration.

Analysis of Free Fatty Acids and Fatty Acyl Esters Content of HNF-4α—350 μg of HNF-4α protein samples were mixed with 5.0 nmol of C17:0 serving as internal standard and were extracted with 10 volumes of chloroform:methanol 2:1 (v/v). Free fatty acid content and composition were analyzed by reacting the N2-dried organic extract at room temperature in the dark for 30 min with a mixture containing 50 μl of methanol, 50 μl of 0.08 μm trimethylsilyldiazomethane (TMSD) in methanol, and 80 μl of ether. Following derivatization, the mixture was dried under Ne2, dissolved in chloroform and subjected to gas chromatography-mass spectrometry analysis. Alternatively, total fatty acid content and composition were analyzed by reacting the Ne2-dried organic extract for 90 min with 350 μl of 0.3 M H2SO4 in methanol at 95 °C, followed by adding 350 μl of H2O and 1 ml of heptane. The mixture was well mixed and centrifuged, and the upper phase was evaporated, dissolved in chloroform, and subjected to gas chromatography-mass spectrometry analysis. Gas chromatography-mass spectrometry analysis was carried out using a Quatro II Micromass quadrupole mass spectrometer coupled to a DB-5MS gas chromatograph (capillary column 30 m × 0.25 mm × 0.25 μm (J&W Scientific)), programmed for a 130 °C to 220 °C temperature increase at 8.4 °C/min. The mass spectrometer was operated with the electron impact mode at an ionization energy of 70 eV and a source temperature of 190 °C, using total ion current scanning mode and a scan range of 150–450 m/z. Methyl esters were identified by their respective retention time, molecular ion, and fragmentation pattern based on known standards.

Fatty Acids Displacement and Profiling—350 μg of HNF-4α protein samples were incubated for 30 min with fatty acid or fatty acyl-CoA as indicated in 1.5 ml of 20 mM Tris-HCl buffer, pH 8.0, containing 2 mM 5(α,3), C22:6(α,3) or by xenobiotic hypolipidemic amphipathic carboxylates (e.g. Medica analogues) (9, 11). Modulation of HNF-4α activity in transfection assays by amphipathic carboxylates is limited by their intracellular conversion to their respective acyl-CoAs, and amphipathic carboxylates that, due to structural constraints, do not serve as substrates for the acyl-CoA synthase fail to modulate HNF-4α transcriptional activity (9, 11). Activation/inhibition of HNF-4α transcriptional activity by acyl-CoA results from modulation of the binding affinity of HNF-4α to its DNA cognate enhancers, and, or shifting the equilibrium between active HNF-4α dimers and inactive monomers/oligomers, and or modulating its transactivation capacity (9, 11–13).

In apparent contrast to the above, the truncated rat and human HNF-4α(aa133–382) recombinants that lack the F-domain have recently been shown by x-ray crystallography combined with mass spectrometry analysis to consist of tightly bound nonexchangeable fatty acids, in a molar ratio of 1.0 per HNF-4α monomer, within a narrow pocket estimated at 370 Å3 (14, 15). Similarly, a fatty acid pocket, estimated at 626 Å3 and containing bound fatty acids in a molar ratio of 1.0 per monomer, has been reported for the HNF-4α(aa103–408) subtype, solved with its intact F-domain being unstructured in the electron density map (16). The rHNF-4α(aa133–382) dimer has been further reported to consist of an “open” and “closed” monomers having their H12 helices in the extended and clamped configurations, respectively, independently of their respective pockets being both occupied by fatty acids (14). The clamped configuration of the dimer was only enforced upon crystallizing the truncated hHNF-4α(aa133–382) protein in the presence of SRC1-derived coactivator peptide (15).

The crystallographic data taken together with the acyl-CoA binding and transfection data leave unanswered the following questions: (a) The entrapped fatty acids, claimed to be nonexchangeable with added fatty acids, the independence of HNF-4α active/clamped configuration on its fatty acid occupancy, and the lack of physical contact between the fatty acid pocket and the clamped H12 helix may all imply that the entrapped fatty acids may play the role of a fortuitous (17) or authentic (18) stabilizing cofactor rather than a ligand that may regulate the transcriptional activity of its nuclear receptor by on/off-induced conformational changes. (b) Nonexchangeability of the entrapped fatty acids does not conform to modulation of the transcriptional activity of HNF-4α by added fatty acids or xenobiotic amphipathic carboxylates in transfection assays (9, 11, 12). (c) Because both the entrapped fatty acids and the agonist ligands of HNF-4α consist of C14-C16 fatty acids (12, 14), transcriptional activation of HNF-4α by its agonist ligands does not conform with replacement of the entrapped fatty acids with the same fatty acids added in the medium. (d) The entrapped-fatty acid content remains essentially unaffected by MODY-1 missense mutations or in HNF-4α variants mutated in amino acids aligning the fatty acid pocket, despite robust differences in transcriptional activity between wild type HNF-4α and its respective mutants (12). The above discrepancies could perhaps be compromised by assuming that modulation of HNF-4α transcriptional activity by added free acids in transfection assays was accounted for by their endogenous conversion to respective acyl-CoAs of high binding affinity (nanomolar range) (9–11), followed by replacing the entrapped fatty acids with the respective acyl-CoAs. However, the fatty acid pocket of HNF-4α estimated at 370 Å3 is on the verge of accommodating long chain free fatty acids (estimated at 400–500 Å3) (19) and is apparently too limited in volume to accommodate respective acyl-CoAs. One is therefore left with the unsolved riddle of a fatty acid pocket of questionable relevance together with acyl-CoA effects mediated by yet unproved acyl-CoA site. The present report defines a distinctive acyl-CoA thioesterase site of HNF-4α and characterizes its functional relationship with the free fatty acid pocket of HNF-4α.
β-mercaptoethanol, followed by adding 70 μl of charcoal-coated dextran solution (3% charcoal and 0.3% dextran in 10 mM Tris-HCl, pH 7.4). The mixture was well mixed and centrifuged. The supernatant was mixed with 2–4 nmol of C17:0 serving as internal standard, lyophilized to dryness, derivatized by H$_2$SO$_4$, methanol, and subjected to fatty acid analysis by gas chromatography-mass spectrometry as described above. **Hexadecyl-CoA Binding—**Recombinant HNF-4a1 was desalted by Sephadex G-50 equilibrated with 20 mM ammonium acetate, pH 8.0, containing 2 mM mercaptoethanol. Protein samples of 200 μg and 10 μM hexadecyl-CoA were incubated for 25 min at room temperature in 20 mM ammonium acetate, pH 8.0, containing 2 mM mercaptoethanol in a final volume of 1 ml, followed by adding 70 μl of charcoal-coated dextran solution. The mixture was well mixed and centrifuged, and the supernatant was divided into 15-μg protein samples and lyophilized. For hexadecyl-CoA spiking, 0.25 nmol of hexadecyl-CoA was added to every other sample prior to lyophilization. Lyophilized samples were dissolved in acetonitrile:100 mM KH$_2$PO$_4$ (60:40) and centrifuged, and the supernatant was dried, dissolved in isopropanol:1 mM acetic acid (80:20), kept for 30 min at −70°C, and centrifuged, and then the second supernatant was dried and dissolved in 30 μl of acetonitrile:H$_2$O (60:40), containing 0.05% triethylamine and 0.3 nmol of C17:0-CoA as internal standard. Hexadecyl-CoA content was analyzed by negative ESI using Micromass Quattro II tandem mass spectrometer at one voltage of 35 V and electrospray source temperature at 85°C. The sample was pumped into the ESI source at 30 μl/min through a 10-μl injector loop connected to the ESI source via a fused silica capillary tube. The inflowing material was scanned within the range of 400–600 m/z at a scan duration of 1.3 s using Continuum Scan mode. Hexadecyl-CoA was identified by its m/z 495, corresponding to the (M-2H)/2 doubly charged molecular ion. C17:0-CoA (m/z 509) served as internal standard. Data processing was carried out using the Masslynx program.

**Acyl-CoA Biding Site and the Thioesterase Activity of HNF-4α**—The acyl-CoA thioesterase activity of HNF-4α was evaluated by the generation of free CoA using DTNB, or by following the hydrolysis of radioactively labeled 1-[14C]palmitoyl-CoA. C14:0-CoA (Fig. 1A) and C20:5-CoA (not shown), representing an agonist and antagonist fatty acyl-CoA ligands of HNF-4α, respectively (9), were both hydrolyzed by the full-length rHNF-4α in the absence of 1-[14C]palmitoyl-CoA. Mean ± S.E. of three independent experiments. C, thioesterase activity of recombinant rHNF-4α1(aa132–445) (15 μg) was analyzed with 4.0 μM of the indicated acyl-CoAs. Shown is a representative experiment.

**RESULTS**

**Acyl-CoA Biding Site and the Thioesterase Activity of HNF-4α**—The acyl-CoA thioesterase activity of HNF-4α was evaluated by the generation of free CoA using DTNB, or by following the hydrolysis of radioactively labeled 1-[14C]palmitoyl-CoA. C14:0-CoA (Fig. 1A) and C20:5-CoA (not shown), representing an agonist and antagonist fatty acyl-CoA ligands of HNF-4α, respectively (9), were both hydrolyzed by the full-length rHNF-4α in the absence of 1-[14C]palmitoyl-CoA. Mean ± S.E. of three independent experiments. C, thioesterase activity of recombinant rHNF-4α1(aa132–445) (15 μg) was analyzed with 4.0 μM of the indicated acyl-CoAs. Shown is a representative experiment.
4a1 recombinant, yielding $K_m$ values of 3.0–5.0 μM and $V_m$ values of 0.6–1.0 and 1.0–1.5 nmol of acyl-CoA/nmol rHNF-4a1/min for C14:0-CoA and C20:5-CoA, respectively.

Acyl-CoA hydrolysis by HNF-4a1 was catalyzed by HNF-4a1-LBD(aa132–455) (Fig. 1B). Thus, the $K_m$ and $V_m$ values for C14:0-CoA hydrolysis by rHNF-4a1(aa132–455), consisting of the E-F domains but lacking the N-terminal AF-1 domain as well as the DNA-binding domain, amounted to 1.5 ± 0.2 μM and 1.7 ± 0.4 nmol of acyl-CoA/nmol HNF-4a1-min, respectively, exceeding the activity of the full-length rHNF-4a1. Recombinant rHNF-4a1(aa132–455), further purified to homogeneity by ion exchange chromatography, catalyzed similar rates of acyl-CoA hydrolysis. Furthermore, truncated rHNF-4a1-LBD recombinants lacking the F-domain (e.g. HNF-4α(aa132–370) or HNF-4α(aa132–410)) were defective in hydrolyzing acyl-CoA, as verified by the DTNB method (Fig. 1B) or by the more sensitive 1-[14C]palmitoyl-CoA hydrolysis (not shown), indicating that the F-domain was required for the acyl-CoA thioesterase activity of HNF-4a1-LBD.

The substrate specificity of HNF-4α1 thioesterase conformed to the binding specificity of HNF-4α fatty acyl ligands (9). Thus, nonsubstituted fatty acyl-CoAs higher than C8, including saturated, monounsaturated, polyunsaturated, and dioic (e.g. C16:0DICA) fatty acyl-CoAs, served as substrates for HNF-4α1 thioesterase (Fig. 1C). CoA thioesters of substituted dioic acids of the Medica series, previously reported to bind to HNF-4α and inhibit its transcriptional activity (10, 11), did not serve as substrates for the HNF-4α thioesterase (not shown), despite their high binding affinity to HNF-4α (10).

The thioesterase specificity of HNF-4a1 was further verified by screening the thioesterase activity of related nuclear receptors. Acyl-CoA hydrolysis was catalyzed as well by rHNF-4a2(aa132–465) and human HNF-4α1(aa132–455) (not shown). Rate of acyl-CoA hydrolysis by HNF-4α under the quoted experimental conditions amounted to 5% as compared with that of full-length HNF-4α, thus further pointing to the strict requirement for the HNF-4α F-domain. Furthermore, PPARα was inactive in hydrolyzing its reported acyl-CoA antagonist ligand(s) (26, 27) within the time scale used for measuring HNF-4α or HNF-4γ thioesterase activities (not shown). Similarly, RXRα, having the highest LBD homology to HNF-4α-LBD among nuclear receptors (1), lacked thioesterase activity within the timescale used for measuring HNF-4α activity (not shown). Hence, the acyl-CoA thioesterase activity of HNF-4α is specific in terms of its catalytic protein entity as well as its ligand substrates.

The acyl-CoA thioesterase activity of HNF-4α1 was competitively inhibited by hexadecyl-CoA, representing a nonhydrolyzable long-chain acyl-CoA thioester (apparent $K_i$, 0.3 μM (Fig. 2A)), or by the CoA-thioester of Medica analogues (not shown), representing hindered long-chain acyl-CoAs (apparent $K_i$, 1.4 μM, 0.4 μM, and 0.2 μM for Medica 14, Medica 16, and Medica 18, respectively). Dead end inhibition of the acyl-CoA thioesterase activity by hexadecyl-CoA or by Medica 16-CoA specifically required the CoA-thioether or CoA-thioester configuration, whereas the respective free acids (e.g. hexadecanoic acid, β,β′-tetramethyl hexadecanedioic acid (Medica 16)) or free CoA were essentially inactive, having apparent $K_i$ higher than 30 μM (Fig. 2B). Also, the free fatty acid counterparts of respective acyl-CoA substrates of HNF-4a1 thioesterase (e.g. C14:0) were inactive as inhibitors (not shown). Similarly, C12-Bodipy did not inhibit the thioesterase activity of HNF-4a1 at concentrations up to 10 μM (not shown). Hence, the acyl-CoA thioesterase activity of HNF-4a defines an acyl-CoA binding site (ACS) that is essentially inaccessible to respective free acyl carboxylates.

**Fatty Acid Pocket of HNF-4α**—Because the acyl-CoA binding site (ACS) of HNF-4α1(aa132–455) is essentially inaccessible to the respective free acids, and in light of failure to crystallize HNF-4α1-LBD(aa132–455) (14), the characteristics of the free fatty acid pocket (FAP) of HNF-4α and its relationship with ACS in the context of the HNF-4α1(aa132–455) protein were verified here by employing mass spectrometry and fluorescence polarization analysis.

In line with our previous report (12), recombinant rHNF-4a1(aa132–455) entraps C16:0, C16:1, C17:1, and C18:1 fatty acids (Fig. 3A), whereas other fatty acids are present at minute amounts. The entrapped fatty acids consist of their free acid form as verified by the similar stoichiometry derived by the free acid-selective TMSD method and the nonselective acidic methanalysis (Fig. 3A). However, the entrapped C18:1 consists of free as well as C18:1 ester/anhydride, as verified by the difference in C18:1 stoichiometry derived by the two methods. It is worth noting that the entrapped C16:1 amounts to 40% of the total entrapped free fatty acids, despite being a very minor

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2 R. Hertz, unpublished results.
FIG. 3. Exchange of HNF-4α-entrapped fatty acids with added free fatty acids. A, fatty acid composition of recombinant rHNF-4α(aa132–455). Free and esterified fatty acids (black bars) or free fatty acids only (gray bars) were determined by methylation with H$_2$SO$_4$-methanol or TMSD, respectively, as described under Experimental Procedures. *, significant as compared with the respective value derived by acidic methanolysis (p < 0.05). B–E, fatty acids content and composition of recombinant rHNF-4α(aa132–455) was determined as described under Experimental Procedures, prior to (black bars) and following incubation (gray bars) with added $^{13}$C16:1-COOH (4.0 μM) (B), C18:1trans-COOH (10.0 μM) (C), M16-COOH (10.0 μM) (D), or hexadecyl-CoA (10.0 μM) (E). Mean ± S.E. of three independent experiments. *, significant as compared with no added ligand (p < 0.05).
component of the free fatty acids pool of E. coli extract (12). The molar stoichiometry of entrapped fatty acids per HNF-4a1(aa132–455) protein amounted to 1:1 (Fig. 3A), indicating that the free fatty acid pocket of recombinant HNF-4a1(aa132–455) was essentially saturated by free fatty acids.

The entrapped free fatty acids of recombinant HNF-4a1(aa132–455) may exchange with free fatty acids added to the incubation mixture. That was verified by incubating the recombinant protein with added fatty acids, followed by removal of the nonbound fatty acids (e.g. displaced fatty acids together with excess fatty acid added to the incubation mixture) by charcoal, and re-profiling the bound fatty acids by mass spectrometry. It is noteworthy that, in light of the protein concentrations used (10 μM), concentrations of free fatty acids added to the incubation mixture were in the micromolar range. Added \(^\text{13}^\text{C}16:1\) free acid displaced 40%, 60, and 30% of the entrapped C16:0, \(^\text{13}^\text{C}16:1\), and C18:1 under the incubation conditions employed while replacing the originally entrapped acids by \(^\text{13}^\text{C}16:1\) (Fig. 3B). Similarly, added C18:1trans free acid displaced about 50% of the entrapped free fatty acids while replacing the endogenous acids by C18:1trans (Fig. 3C). Displacement of the entrapped fatty acids was similarly observed upon incubating the recombinant protein with C14:0 free acid (not shown), C16:0 free acid (Fig. 6B, see below), or C12-Bodipy (not shown). Hence, the entrapped free fatty acids of recombinant HNF-4a1(aa132–455) were exchangeable.

In contrast to monocarboxylic free acids, substituted (e.g. Medica 16, Fig. 3D) or nonsubstituted (Fig. 6D, see below) hexadecanedioic free acids added to the incubation mixture were ineffective in displacing the entrapped fatty acids of recombinant HNF-4a1(aa132–455). Furthermore, in contrast to free acids, acyl-CoA thioethers or nonhydrolyzable acyl-CoA thioesters were ineffective in displacing the entrapped free fatty acids of recombinant HNF-4a1(aa132–455). Thus, the entrapped fatty acid profile remained unaffected by added hexadecyl-CoA (Fig. 3E) or Medica 16-CoA (not shown), indicating that the free fatty acid pocket of recombinant HNF-4a1(aa132–455) could not accommodate acyl-CoAs. Medica 16-CoA must have been excluded due to both its dioic constraint as well as its CoA moiety.

Kinetic characteristics of the free fatty acid pocket of recombinant HNF-4a1(aa132–455) were analyzed by C12-Bodipy binding, followed by its displacement by free fatty acids added to the incubation mixture. Because C12-Bodipy does not compete with acyl-CoAs for the thioesterase site, and in light of its exchangeability (albeit limited) with the entrapped free acids, bound C12-Bodipy assayed by its fluorescence polarization was exploited as a fluorescent marker for FAP ligands. As shown in Fig. 4A, C12-Bodipy did bind to recombinant HNF-4a1(aa132–455), having an apparent \(K_d\) of 0.6 μM. Bound C12-Bodipy could be displaced by free C16:1, C14:0, or C20:5 monocarboxylic acids (apparent \(K_d\) values, 3-5 μM) (Fig. 4B), but not by free substituted (e.g. Medica 16) (Fig. 4B) or nonsubstituted (Fig. 6D, see below) hexadecanedioic acids. Also, C12-Bodipy could not be displaced by hexadecyl-CoA or by Medica 16-CoA, in line with results reported above (Fig. 3E). Hence, the fatty acid pocket of recombinant HNF-4a1(aa132–455) defines a binding site for free monocarboxylic acids that may exchange with added monocarboxylic acids but not with respective CoA-thioethers/thioethers or free dioic acids.

Because the specificity and respective binding affinities for free acids and acyl-CoAs of the ACS and FAP domains of recombinant HNF-4a1(aa132–455) are highly distinctive, the two domains appear to be noncongruent. Noncongruence of the two domains was further evaluated by the mutual saturation of FAP and ACS by their distinctive ligands. That was verified by incubating recombinant HNF-4a1(aa132–455) with hexadecyl-CoA, followed by removal of nonbound hexadecyl-CoA by charcoal and measuring the stoichiometry of bound hexadecyl-CoA by negative ESI, and of the entrapped fatty acids by gas chromatography/mass spectrometry. Because hexadecyl-CoA binds with high affinity to ACS (Fig. 2A), but is excluded from FAP (Fig. 3E and 4B), its stoichiometry may reflect its exclusive binding to ACS independently of FAP. The total amount (1.1 ± 0.1 nmol of free acids/nmol of HNF-4a1(aa132–455); Fig. 5A) and composition (Fig. 5B) of the entrapped free acids remained unaffected by added hexadecyl-CoA, whereas the recombinant protein was now associated with additional 0.8 ± 0.05 nmol of hexadecyl-CoA/nmol of HNF-4a1(aa132–455) (Fig. 5, A and B).

Moreover, a stoichiometry approaching 1.0 for hexadecyl-CoA binding to ACS required the E- domain of HNF-4a1 as verified by the robustly decreased stoichiometry of hexadecyl-CoA binding to the truncated HNF-4a1(aa132–370) (Fig. 5A). Hence, binding of acyl-CoAs to ACS and of free acids to FAP is not mutually exclusive, indicating that the two distinctive binding sites are noncongruent.

ACS/FAP Cross-talk—Cross-talk between the acyl-CoA and free fatty acid binding sites of HNF-4a1(aa132–455) has been
verified by evaluating the displacement of FAP-entrapped free fatty acids by hydrolyzable acyl-CoAs. Incubating recombinant HNF-4α(aa132–455) with 13C16:1-CoA (Fig. 6A), C16:0-CoA (Fig. 6B), or C14:0-CoA (not shown) resulted in displacement of FAP-entrapped fatty acids, being replaced by the free acid counterpart of the added acyl-CoA. Because the free fatty acid pocket excludes nonhydrolyzable acyl-CoAs (Fig. 3E), displacement of FAP-entrapped fatty acids by added acyl-CoAs must have involved their prior hydrolysis. Indeed, 13C16:1-CoA was essentially ineffective in displacing FAP-entrapped fatty acids of recombinant rHNF-4α(aa132–370), lacking the F-domain and its concomitant thioesterase activity, whereas added 13C16:1 free acid did displace FAP-entrapped fatty acids of rHNF-4α(aa132–370) (not shown). That is in contrast to rHNF-4α(aa132–455), consisting of a functional F-domain and its ACS thioesterase activity, where 13C16:1 free acid as well as 13C16:1-CoA were both effective in displacing FAP-entrapped fatty acids (Figs. 3B and 6A).

Furthermore, because the free acid pocket of recombinant HNF-4α(aa132–455) may be approximated by the solved x-ray structure of the truncated HNF-4α(aa133–382) (14), a putative hydrolytic active site within FAP was probed by mutating FAP amino acids that may form a Ser-His-Asp/Glu catalytic triad (28) in contact with the entrapped fatty acid. Of particular interest were Ser-181 and Glu-185 reported to be in immediate contact with the carbonyl function of the fatty acid entrapped in HNF-4α FAP (14). However, the thioesterase activity of the S181A, S256A, Q185K, and Q185A HNF-4α(aa132–455) mutants was comparable with that of wild type HNF-4α(aa132–455) (not shown), indicating that displacement of FAP-entrapped fatty acids by added hydrolyzable fatty acyl-CoAs was not accounted for by a putative Ser-His-Asp/Glu catalytic triad of FAP. Hence, in line with the inaccessibility of FAP to acyl-CoAs, prior hydrolysis of added acyl-CoAs by the thioesterase activity of ACS was obligatory for displacement of FAP-entrapped fatty acids by acyl-CoAs.

The requirement for prior acyl-CoA hydrolysis was further evaluated by studying displacement of FAP-bound C12-Bodipy by added acyl-CoAs. Bound C12-Bodipy was displaced by added C14:0-CoA (Fig. 6C) or C16:1-CoA (not shown). However, the displacement of FAP-entrapped fatty acids by added C14:0-CoA was inhibited in the presence of added Medica 16-CoA, whereas displacement by added C14:0 free acid remained unaffected (Fig. 6C). Hence, prior hydrolysis of acyl-CoA by ACS thioesterase was required for displacement of FAP-entrapped C12-Bodipy by added acyl-CoA.

Under conditions of short incubation times, displacement of FAP-entrapped fatty acids by added hydrolyzable acyl-CoAs was significantly more pronounced than that effected by the same concentrations of the respective free acid (Fig. 6B), indicating that the free acid product generated by acyl-CoA hydrolysis catalyzed by ACS thioesterase could directly exchange with FAP-entrapped free acids without being released first to the medium. Direct access of the free acid product of ACS thioesterase to FAP was further probed by searching for an acyl-CoA/free acid couple where the acyl-CoA would serve as substrate for ACS thioesterase while the concerned free acid, when added to the incubation mixture, would hardly exchange with FAP-entrapped fatty acids. Indeed, hexadecanediol-CoA (C16:0DICA-CoA) serves as substrate for ACS thioesterase (Fig. 6C), whereas the added free hexadecanediol acid (C16:0DICA-COOH) did not displace C12-Bodipy (Fig. 6D). However, despite the nonexchangeability of FAP-entrapped fatty acids with free hexadecanediol acid added in the medium, added hexadecanediol-CoA did displace C12-Bodipy (Fig. 6D). Nonexchangeability of FAP-entrapped fatty acids by added hexadecanediol acid, as contrasted with the free
Acid generated by ACS-catalyzed hydrolysis of the hexadecanedioyl-CoA substrate, may indicate proximity of ACS and FAP that may allow for binding to FAP of the free acid product of acyl-CoA hydrolyzed by ACS thioesterase, without being released first to the medium.

The functional relevance of ACS in controlling the transcriptional activity of HNF-4α was further evaluated by verifying the suppression of HNF-4α transcriptional activity by Medica 16, namely, a substituted amphipathic dicarboxylate inaccessible to FAP (Fig. 4B). In light of the low binding affinity of the free Medica 16 acid to ACS, as verified by its binding characteristics (10), as well as by its failure to inhibit ACS thioesterase activity (Fig. 2B), modulation of HNF-4α transcriptional activity by added Medica 16 must depend on its endogenous CoA-thioesterification. Indeed, the transcriptional activity of transfected HNF-4α was robustly suppressed by added Medica 16 (Fig. 7), in line with our previous report (11). However, suppression of HNF-4α activity by Medica 16 was fully abrogated by triacsin C that may prevent the generation of the acyl-CoA ligand (Fig. 7A), or upon replacing Medica 16 with an α,α′-substituted dicarboxylate (α,α′-dichlorotetradecane-α,α′-dioic acid) that fails to be thioesterified endogenously by CoA due to steric hindrance (29) (Fig. 7B). Hence, binding of a high affinity inhibitor to functional ACS is sufficient for suppressing HNF-4α activity independently of FAP.

DISCUSSION

The findings reported here may indicate that the HNF-4α monomer consists of two distinctive sites for acyl ligands, namely, the acyl-CoA (ACS) and the free acid (FAP) binding sites. FAP characteristics reported here in the context of HNF-4α-LBD(aa132–455) complement and extend those previously implied by the solved x-ray structure of the truncated HNF-4α-LBD(aa133–382) (14–16) as follows: (a) In contrast to previous
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reported (14–16), and despite their tight binding as reflected by their prevalence throughout the purification steps of the recombinant protein, FAP-entrapped fatty acids may exchange with added fatty acids. Exchangeability was verified here by profiling and quantifying the displaced FAP acids by mass spectrometry as well as by the displacement of FAP-entrapped C12-Bodipy by added fatty acids. The previous failure to exchange FAP-entrapped acids by added fatty acids may perhaps reflect differences in experimental conditions. The exchangeability of FAP acids may imply their revisited candidacy as ligands of HNF-4α. (b) Replacement of FAP-entrapped fatty acids was limited to added free monocarboxylic acids, whereas nonhydrolyzable acyl-CnA thioesters or thioethers were excluded by FAP. Hence, FAP defines a free acid pocket of HNF-4α. In contrast to FAP, ACS consists of an acyl-CoA active site characterized by the following: (a) high affinity, specific binding of long chain acyl-CoAs (10); (b) thioesterase activity resulting in hydrolysis of acyl-CoA ligands; (c) competitive inhibition of its thioesterase activity by binding of nonhydrolyzable long-chain acyl-CoA ligands; and (d) very low binding affinity or essential exclusion of free acids. Hence the two acyl binding sites of HNF-4α-LBD consist of a free acid pocket that excludes acyl-CoAs and an acyl-CoA active site that essentially excludes free acids.

The two acyl binding sites appear to occupy noncongruent domains of HNF-4α-LBD. Indeed, although FAP is contained within the E-domain of HNF-4α-LBD, as verified by the solved x-ray structure of the truncated LBD recombinant (14–16), ACS is proposed to be contained within the E-F domain of HNF-4α-LBD. The involvement of the F-domain in the acyl-CoA active site is inferred from its requirement for the ACS thioesterase activity (Fig. 1B). The involvement of the F-domain in the acyl-CoA active site may further be inferred from the binding affinities of the full-length HNF-4α or HNF-4α(aa132–455) for free acids and acyl-CoAs as compared with the truncated HNF-4α(aa132–370) (30). Thus, truncation of the F-domain results in robustly decreased affinity for acyl-CoAs and abrogation of conformational changes induced by acyl-CoAs, with concomitant increased affinity for the respective free acids. The involvement of the E-domain in the acyl-CoA active site may be inferred from loss of thioesterase activity in mutants of the E-domain. Hence, in contrast to FAP being contained within the E-domain of HNF-4α, high affinity acyl-CoA binding and its hydrolysis by ACS do both require a functional E-F domain, thus indicating that the two acyl binding sites of HNF-4α are noncongruent. This conclusion is further corroborated by the mutual saturation of ACS and FAP by their distinctive acyl-CoA and free acid ligands, indicating that the two binding sites are not mutually exclusive. The arrangement of the two acyl binding sites within the E-F domain would have to be verified by solving the x-ray structure of the nontruncated HNF-4α-LBD crystallized in the presence of nonhydrolyzable acyl-CoA.

It should be noted however that the affinities of added acyl ligands for their respective binding sites, in the context of the full-length HNF-4α or its nontruncated LBD, do differ extensively. Thus, $K_d$ values for free fatty acids amount to 0.1–0.7 µM (10, 30), implying relatively low binding affinities of free fatty acids to FAP. In contrast, $K_d$ values for fatty acyl-CoAs amount to 0.4–4.0 nm (10, 30), implying high binding affinities of fatty acyl-CoAs to ACS. Similar binding affinities of fatty acyl-CoAs to ACS may be deduced by dissecting the $K_m$ values of acyl-CoAs for the ACS thioesterase activity into their $K_d$ values and turnover components. Hence, the binding affinities of HNF-4α ACS for its preferred acyl-CoA ligands is about 200-fold higher compared with the binding affinity of HNF-4α FAP for its free acid ligands.

The duality of the distinctive acyl binding sites may allow for cross-talk that may control the transcriptional activity of HNF-4α. Such cross-talk would be dominated by the hierarchy of the two binding sites, whereby the free acid ligands of FAP may be generated by ACS-catalyzed hydrolysis of its acyl-CoA ligands, taken together with the 200-fold higher affinity of ACS for its acyl-CoA ligand as compared with the free acid ligands of FAP. This cross-talk may further be affected by the putative proximity of the two binding sites allowing, under specific conditions, for direct channeling into FAP of the free acid product of ACS-catalyzed acyl-CoA hydrolysis (Scheme 1). Such direct channeling may enable high efficiency binding of free acid ligands to FAP mediated by high affinity binding of acyl-CoA ligands to ACS followed by their hydrolysis to the free acid.
indeed, the composition of FAP-entrapped fatty acids in
products and their direct channeling into FAP without being
released and diluted in the medium. That is in contrast to low
affinity binding of free acid ligands to FAP mediated by ex-
change of FAP-entrapped fatty acids with medium fatty acids may result
in low affinity binding of medium free acid to FAP (Kd in the nanomolar
range). ACS activity may be blocked by high affinity binding of a
nonhydrolyzable acyl-CoA to ACS (e.g. Medica 16-CoA).

The duality of ACS/FAP of HNF-4α is of similar functional
relevance for HNF-4α suppression by its acyl antagonists. The
functional relevance of HNF-4α suppression by its natural or
xenobiotic antagonist ligands is reflected by their reported
effects in the context of lipid metabolism. Thus, increases in
blood lipids induced in human by dietary saturated fatty acids
(C14:0 and C16:0), as contrasted with decreases in blood lipids
by (n-3)polyunsaturated fatty acids (e.g. (n-3)C20:5 or (n-
3)C22:6) or by Medica 16, are correlated with activation or
suppression of HNF-4α transcriptional activity by the con-
cerned ligands, respectively (9, 11). Indeed, hydrolyzable acyl-
CoA antagonists of HNF-4α (e.g. (n-3)C20:5) (9) may bind with
high affinity to ACS followed by their hydrolysis by ACS thio-
esterase and their channeling into FAP with concomitant dis-
placement of FAP-entrapped fatty acid agonists. This seques-
tral may allow for high efficiency binding of antagonistic fatty acids
to FAP resulting in active or passive suppression of HNF-4α
transcriptional activity. Alternatively, displacement of FAP-
entrapped fatty acid agonists may be effected by low affinity
direct binding of free antagonistic fatty acids to FAP with
concomitant suppression of HNF-4α transcriptional activity.
Furthermore, high affinity binding of Medica 16-CoA to ACS
(10) (Fig. 7B) may exemplify an additional mode of suppression
of HNF-4α transcriptional activity, independently of FAP (Fig.
7). Because Medica 16 free acid is excluded from direct binding
to FAP, due to its dicarboxylate structure, and because its
acyl-CoA resists hydrolysis by ACS thioesterase, high affinity
binding of Medica 16-CoA to ACS may result in blocking ACS/
FAP cross-talk similarly to that affected by HNF-4α missense
mutants. Suppression of HNF-4α transcriptional activity by a
nonhydrolyzable high affinity acyl-CoA may however be allevi-
ated under conditions that may abrogate formation of the acyl-
CoA ligand (Fig. 7), thus reflecting the specific requirement for
ACS in suppressing HNF-4α by Medica 16.

The HNF-4α ACS/FAP reality extends the paradigm of nu-
clear receptors of the superfamily by the following two aspects:
(a) Nuclear receptors of the superfamily are usually considered
to be affected by unique ligands and their respective pocket.
The acyl-CoA/free acid duality of HNF-4α combined with its
ACS/FAP duality may imply that the transcriptional activity of
HNF-4α is mutually controlled by two ligands and two binding
sites operating in tandem. This combined duality may offer a
variety of modes by which HNF-4α transcriptional activity
could be modulated by physiological signals or drugs. (b) Li-
gands of nuclear receptors of the superfamily usually remain
stable in the course of conformational changes induced by their
binding. That is in contrast to enzymes where conformational
change induced by binding of their substrates is exploited for
driving formation or breakage of chemical bonds affecting their
substrates. This difference is well reflected in nominating su-
perfamily members as receptors and their low molecular
weight modulators as ligands, as opposed to enzymes and their
substrates. The HNF-4α mode of action implies a receptor/enzyme
and ligand/substrate duality. Moreover, it is noteworthy
that, in analogy with other thioesterases, transfer of the
acyl moiety of acyl-CoA to water may be complemented by
additional activities where the acyl moiety is transferred to a
variety of nucleophiles (31). Of particular interest could be the
putative autoacylation of HNF-4α or acylation of HNF-4α cog-
nate transcription factors (e.g. HNF-1, COUP, SP1, SMAD,
HIF-1, SHP, and SRC1) catalyzed by its ACS.

3 F. Schroeder, personal communication.
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