Role of Regulatory F-domain in Hepatocyte Nuclear Factor-4α Ligand Specificity*

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The F-domain of rat HNF-4α has a crucial impact on the ligand binding affinity, ligand specificity and secondary structure of HNF-4α. (i) Fluorescent binding assays indicate that wild-type, full-length HNF-4α (amino acids 1–455) has high affinity (Kd = 0.06–12 nM) for long chain fatty acyl-CoAs (LCFA-CoA) and low affinity (Kd = 58–296 nM) for unesterified long chain fatty acids (LCFAs). LCFA-CoA binding was due to close molecular interaction as shown by fluorescence resonance energy transfer (FRET) from full-length HNF-4α tryptophan (FRET donor) to bound cis-parinaroyl-CoA (FRET acceptor), which yielded an intermolecular distance of 33 Å, although no FRET to cis-parinaric acid was detected. (ii) Deleting the N-terminal A-D-domains, comprising the AF1 and DNA binding functions, only slightly affected affinities for LCFA-CoAs (Kd = 0.9–4 nM) and LCFAs (Kd = 93–581 nM). (iii) Further deletion of the F-domain robustly reduced affinities for LCFA-CoA and reversed ligand specificity (i.e. high affinity for LCFAs (Kd = 1.5–32 nM) and low affinity for LCFA-CoAs (Kd = 54–302 nM)). No FRET from HNF-4α-E (amino acids 132–370) tryptophan (FRET donor) to bound cis-parinaroyl-CoA (FRET acceptor) was detected, whereas an intermolecular distance of 28 Å was calculated from FRET between HNF-4α-E and cis-parinaric acid. (iv) Circular dichroism showed that LCFA-CoA, but not LCFAs, altered the secondary structure of HNF-4α only when the F-domain was present. (v) cis-Parinaric acid bound to HNF-4α with intact F-domain was readily displaceable by S-hexadecyl-CoA, a nonhydrolyzable thioester analogue of LCFA-CoA. Truncation of the F-domain significantly decreased cis-parinaric acid displacement. Hence, the C-terminal F-domain of HNF-4α regulated ligand affinity, ligand specificity, and ligand-induced conformational change of HNF-4α. Thus, characteristics of F-domain-truncated mutants may not reflect the properties of full-length HNF-4α.

The abbreviations used are: HNF-4α, hepatocyte nuclear factor-4α; aa, amino acid(s); AR, androgen receptor; CoA, coenzyme A; FRET, fluorescence resonance energy transfer; LCFA, long chain fatty acid; LCFAs, long chain fatty acids; TFE, trifluoroethanol; PR, progesterone receptor; RXRα, retinoid X receptor α; PBS, phosphate-buffered saline; TBE, tris-borate-EDTA; FRET, fluorescence resonance energy transfer; LCFA, long chain fatty acid; LCFAs, long chain fatty acids; AR, androgen receptor; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; HNF, hepatocyte nuclear factor; WIF, Wnt inhibitory factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; COOH, carboxy; NH2, amino; d, distance; aff, affinity; spec, specificity; AF1, activation function 1; DNA, deoxyribonucleic acid; FRET, fluorescence resonance energy transfer; TBE, Tris-borate-EDTA; LCFA, long chain fatty acid; LCFAs, long chain fatty acids; AF1, activation function 1; DNA, deoxyribonucleic acid.

Hepatocyte nuclear factor-4α (HNF-4α)1 (1) is a member of the superfamily of nuclear receptors highly expressed in liver, intestine, pancreas, and kidney, where it modulates transcription of multiple genes involved in lipid and glucose homeostasis (2–4). Furthermore, missense mutations in the HNF-4α gene are associated with maturity onset diabetes of the young (5–10). HNF-4α exhibits constitutive but not full activity in the absence of added exogenous ligands (reviewed in Ref. 1) (8, 11–16). The molecular basis for this constitutive activity remains to be determined but may include ligand-based mechanisms involving dietary or endogenous long chain fatty acids (LCFA) (17, 18), endogenously synthesized coenzyme A (CoA) thioesters of long chain fatty acids (LCFA-CoAs) (8, 11, 14, 19), and/or the F-domain itself functioning as a modulator (16).

Whether LCFA-CoAs or LCFAs represent the endogenous functional ligands accounting for partial constitutive activity of HNF-4α is as yet unresolved. Although HNF-4α shares overall similar domain structure (i.e. A/B, C, D, E) with many other nuclear transcription factors, it is unique in exhibiting a very large (85-aa) C-terminal F-domain that has negative regulatory activity (Fig. 1). Previous reports of HNF-4α structure (17–20) and function (8, 11, 12, 14, 19–21) examined N- and/or C-terminal truncation forms of this protein (Fig. 1). Modular structures of most frequently employed HNF-4α truncation mutants are schematically shown in comparison with the full-length HNF-4α molecule (Fig. 1). (i) HNF-4α-E-F (aa 132–455) is an N-terminal truncation form, lacking the A/B (including activation function 1 (AF1)), C (DNA binding domain), and D (hinge) domains; (ii) the shortest truncation forms, HNF-4α-E-0.1F (aa 133–382) and HNF-4α-E (aa 132–370), contain mainly the ligand binding domain (E). Whether HNF-4α has an endogenous ligand with a role in transactivation regulation has been widely debated, and controversial conclusions have been made based on experimental data obtained with different truncation forms of HNF-4α rather than full-length HNF-4α. For example, recent x-ray crystallography data revealed the presence of a free fatty acid molecule within the binding pocket of HNF-4α-E-0.1F (aa 133–382), suggesting that this was a constitutive, unplaceable ligand (18). In contrast, fluorescence and circular dichroic investigations of another truncated form that contained the complete F-domain (i.e. HNF-4α-E-F (aa 132–455)) showed that HNF-4α preferentially binds fatty acyl-CoAs with much higher affinity than fatty acids (19). The basis for this discrepancy may lie in the presence or absence of the complete C-terminal F-domain. Consistent with this possibility, it is known that deletion of the F-domain converts the partially repressed HNF-4α into fully active HNF-4α in transactivation assays (8, 12, 22).

In the present study, the ability of various long chain fatty...
acids as free acids or CoA-thioesters to interact with and induce conformational changes into various truncation mutants of rat HNF 4α was examined. The main objectives were to resolve the role of the HNF-4α C-terminal F-domain in determining (i) ligand affinity (Kd), (ii) ligand specificity (LCFA-CoA versus LCFA), and (iii) F-domain-dependent conformational responsiveness of HNF-4α to ligand (LCFA-CoA versus LCFA).

**EXPERIMENTAL PROCEDURES**

**Chemicals—**Nonfluorescent long chain fatty acids (C14:0, myristic; C16:0, palmitic; C16:1, palmitoleoyl-CoA; C20:4, arachidonic) and long chain fatty acyl-CoAs (C14:0-CoA, myristoyl-CoA; C16:0-CoA, palmitoyl-CoA; C16:1-CoA, palmitoleoyl-CoA; C20:4-CoA, arachidonyl-CoA) were purchased from Sigma. The 18 carbon chain length fluorescent fatty acids (cis-parinaric acid and NBD-stearic acid) were obtained from Molecular Probes, Inc. (Eugene, OR). Fluorescent acyl-CoA (i.e. cis-parinaroyl-CoA) was prepared in our laboratory as previously described (19).

**Expression and Purification of Recombinant Full-length and Deletion Mutant Forms of Rat HNF-4α**—The full-length rat HNF-4α (1–455) and the N-terminal deletion mutant HNF-4α-E-F (aa 132–455) recombinant proteins were obtained as previously described (11, 19). The cDNA of the C-terminal truncation mutant lacking the entire F-domain, HNF-4α-E-E (aa 132–370), was obtained by PCR using the sense 5′-CATGCCATGGGACGAGGCTATCATGACGTTACAGCTACGAG and antisense 5′-GAAGACTTCTAGGCGAGCCCTCCAGGG primers. The PCR product was cloned into pet11d plasmid. The recombinant plasmids were expressed in Escherichia coli BL21 (DE3)pLy8 strain, and the His-tagged proteins were purified by affinity chromatography on nickel-nitrilotriacetic resin (Qiagen, Chatsworth, CA) and stored at −70 °C. The purity of recombinant proteins was assessed by SDS-PAGE.

**Ligand Binding Assay: Fluorescent Ligands—**Ligand (LCFA, LCFA-CoA) binding to HNF-4α and HNF-4α mutants was determined by fluorescence ligand binding assays as described earlier (8, 19). Since binding of fluorescent ligands (cis-parinaric acid, NBD-stearic acid, cis-parinaroyl-CoA) to HNF-4α (or HNF-4α truncation mutants) induced a measurable increase in ligand fluorescence intensity, direct excitation of ligand and simultaneous monitoring of ligand fluorescence emission intensity was performed in the presence of increasing ligand concentration. Fluorescence emission of fluorescent ligands was detected with a PCl1 photon counting fluorimeter (ISS Inc., Urbana, IL) (8, 19). cis-Parinaric Acid and cis-parinaroyl-CoA were excited at 317 nm, and fluorescence emission intensity was measured from 400 to 500 nm. For NBD-stearic acid/HNF-4α (or HNF-4α mutant) binding assays, the ligand was excited at 480 nm, and emission spectra were recorded from 500 to 600 nm. All fluorescent ligand binding assays were performed at 22 °C, in L&K buffer (20 mM Tris-HCl, pH 8, containing 300 mM NaCl, 10% glycerol) with moderate stirring and a short (1-min) protein/ligand mixing time to allow for equilibration before measuring fluorescence emission intensities. Controls for the emission intensity of fluorescent ligands in the absence of the respective proteins were run for all concentrations of ligand used in the titration experiments. In order to avoid inner filter effects, sample absorbance at excitation wavelength was kept at <0.05.

The binding parameters (i.e. dissociation constant, Kd, and n (number of binding sites)) were calculated from the titration curves plotted as ligand fluorescence intensity as a function of ligand concentration in nm at each titration point as follows (8, 19):

\[
1/(1 - F/F_{\text{max}}) = (nE_0/K) - (nE_0/K)F
\]

where \(F\) and \(F_{\text{max}}\) represented the corrected measured fluorescence intensity at each titration point and maximal fluorescence intensity of ligand, respectively, [L] was the total ligand concentration, and \(E_0\) was the protein concentration used in the assay. A plot of \(1/(1 - F/F_{\text{max}})\) versus \([L]/(F/F_{\text{max}})\) yielded a linear function with a slope of \(1/K_d\) and an intercept of \((nE_0)/K_d\). Since the n values calculated by this method were around 1, curve fitting to one ligand binding site model by nonlinear regression was also performed, and \(K_d\) values were calculated. Basically, similar values were obtained.

**Ligand Binding Assay: Protein Intrinsic Fluorescence Measurements—**Nonfluorescent ligand (LCFA, LCFA-CoA) binding to full-length HNF-4α and HNF-4α truncation mutants was determined from the change in protein tryptic fluorescence in the presence of nonfluorescent LCFA or LCFA-CoA ligands as described (8, 19). These titration curves allowed calculation of dissociation constants (Kd) for each type of ligand interacting with HNF-4α and HNF-4α mutant proteins. In this type of experiment, Tyr and Trp residues within the respective proteins were excited at 280 nm, and their fluorescence emission was scanned from 300 to 400 nm. Controls were run with the respective proteins in the absence of ligands, and the fluorescence intensity was used as a reference to determine the contribution of small volumes of solvent to protein tryptic fluorescence quenching. Calculation of binding parameters (i.e. dissociation constant, Kd, and n (number of binding sites)) was performed from the titration curves plotted as quenching in protein fluorescence intensity (\(F/F_{\text{max}}\), where \(F\) and \(F_{\text{max}}\) represented the HNF-4α Tyr/Trp fluorescence intensities in the absence and presence of ligand, respectively, at each titration point) as a function of ligand concentration. Linear plots of \(1/(1 - F/F_{\text{max}})\) versus \([\text{ligand}]/(F/F_{\text{max}})\) according to Equation 1 were used for calculation of Kd and n values. In this case, F and \(F_{\text{max}}\) measured the decrease in protein fluorescence intensity (i.e. quenching) in the presence of ligand at each titration point and at saturation, respectively (19). Since the n values calculated by this method were within a range around 1, curve fitting to one ligand binding site model by nonlinear regression was also performed, and Kd values were calculated. Sigmoidal curves have been also fit to the Hill equation,

\[
y = ax^b/c + X^b
\]

where \(y\) and \(x\) represented (\(F_{\text{max}} - F\)) and ligand concentration at each titration point, whereas the a, b, and c parameters were used to estimate \(B_{\text{max}}\) or the maximum binding, n (the number of binding sites), and the Kd value, respectively.

**Displacement of Bound Fluorescent LCFA—**To determine whether an exogenously added LCFA-CoA could displace bound LCFA, a displacement assay was employed utilizing the fluorescence of naturally occurring LCFA, cis-parinaric acid. Displacement was determined in the presence of increasing concentrations of S-hexadecyl-CoA, a nonhydrolyzable and nonfluorescent LCFA-CoA ether analogue. The latter was chosen to preclude potential interference from nonfluorescent LCFA arising upon degradation/hydrolysis of the LCFA-CoA. Briefly, HNF-4α protein (150 nm) was saturated with cis-parinaric acid and then titrated with increasing concentrations of S-hexadecyl-CoA (0–75 nm). Fluorescence emission spectra of cis-parinaric acid were recorded from 400 to 480 nm (excitation at 317 nm) to monitor HNF-4α saturation with S-hexadecyl-CoA followed by displacement during titration with S-hexadecyl-CoA. The decrease in maximum fluorescence intensity of cis-parinaric acid at 420 nm was plotted versus the S-hexadecyl-CoA concentrations. From the plot, the I50 value was determined, and then the equation, \(I = [L]/(1 + [L]/K_d)\), was used to calculate the dissociation constant (Kd) for S-hexadecyl-CoA; [L] was the cis-parinaric acid concentration used in the experiment, and Kd was the dissociation constant for cis-parinaric acid and HNF-4α binding.
Reverse Titration of NBD-Stearic Acid with Full-length HNF-4a versus Deletion Mutants HNF4a-E/F (aa 122–455) and HNF-4a-E (aa 122–370)—In the HNF-4a/NBD-stearic acid reverse titration experiments, NBD-stearic acid (100–250 nM) was titrated with increasing concentrations of HNF-4a protein up to a plateau (binding saturation). The NBD fluorescent group was excited at 480 nm, and its fluorescence emission spectra were recorded from 500 to 600 nm for each titration point as previously described (8, 19). The buffer used in these experiments was 20 mM Tris-HCl, pH 8, containing 300 mM NaCl, 10% glycerol in order to ensure the best solubility of HNF-4a. The ligand/protein molar ratio (i.e. the number of ligand binding sites per HNF-4a molecule) was estimated as previously described for cis-parinaric acid binding to albumin (23). Additionally, in cases where the binding curves had sigmoidal shape, curve fitting to a Hill plot (Eq. 2) was performed for estimation of $K_d$ and n values.

Fluorescence Resonance Energy Transfer (FRET) to Determine Intermolecular Distance between HNF-4a Constructs and Bound Ligands—To further establish the close molecular interaction between a bound LCFA-CoA and the various HNF-4a constructs, Forster FRET (24) was measured between HNF-4a tryptophan and bound cis-parinaroyl-CoA or cis-parinaric acid basically as described earlier (19). HNF-4a tryptophan was excited at 285 nm to avoid contributions from Tyr. The emission of Trp in proteins at 330 nm exhibits significant overlap with cis-parinaroyl-CoA absorption (24, 25), a requisite condition for Forster FRET (24). Since efficiency of FRET varies as (intermolecular distance)$^6$(sufrax.1,6), donor (Trp in HNF-4a variants) and acceptor (bound cis-parinaroyl-CoA of cis-parinaric acid) must be in very close proximity (up to 100 Å) for efficient energy transfer (Fig. 2). The full-length HNF-4a exhibited saturation binding curves with all LCFA-CoAs examined, thereby allowing calculation of binding parameters that are summarized in Table I. Full-length HNF-4a exhibited high affinity for saturated LCFA-CoAs (i.e. myristoyl-CoA (Fig. 2A) and palmitoyl-CoA (Fig. 2B)) characterized by $K_d$ values in the low nanomolar range ($K_d$ of 0.4 and 12 nM, respectively) (Table I). The affinity for myristoyl-CoA (C14:0-CoA) was significantly higher than for palmitoyl-CoA (C16:0-CoA), suggesting a preferential binding of the 14-carbon-long saturated acyl-CoA. The binding curves of palmitoleoyl-CoA (16:1-CoA), a monounsaturated acyl-CoA, and arachidonoyl-CoA (C20:4-CoA), a polyunsaturated acyl-CoA (Fig. 2, C and E, respectively) also suggested high affinity binding and yielded $K_d$ values of 0.7 and 4 nM for C16:1-CoA and C20:4-CoA, respectively (Table I). The affinity of HNF-4a for cis-parinaroyl-CoA (Fig. 2D), with $K_d$ of 70 nM, also demonstrated binding that was good but weaker than for all of the other tested LCFA-CoAs. Both saturated and unsaturated LCFA-CoAs were bound by full-length HNF-4a at one binding site per protein molecule as demonstrated by the linear plots shown in the insets of Fig. 2 as well as by nonlinear regression analysis, which demonstrated best fit to one binding site model (not shown).

Ligand Affinities of Full-length HNF-4a (aa 1–455) for LCFA-CoAs—To date, with one exception, almost nothing is known regarding the ligand specificity of full-length HNF-4a. An early radioligand competition binding assay showed that full-length HNF-4a specifically binds LCFA-CoA, but not LCFA (11). The reported affinities based on this radioligand competition binding assay were low (i.e. micromolar $K_d$ values), thereby suggesting that neither LCFA-CoA nor LCFA were physiologically significant ligands for HNF-4a (12, 13, 16). However, radioligand competition binding assays may underestimate the affinities of LCFA (revised in Ref. 33) (34, 35) and LCFA-CoA (36, 37) binding proteins by 100–1000-fold. Therefore, a direct fluorescence binding assay was applied to determine the affinity of full-length HNF-4a for naturally occurring LCFA-CoA (8, 19). Details of this assay, based on quenching of HNF-4a intrinsic aromatic amino acid fluorescence, were provided under “Experimental Procedures.”

Representative binding curves with naturally occurring saturated and unsaturated LCFA-CoAs were obtained by following the decrease in Tyr/Trp intrinsic fluorescence of full-length HNF-4a upon titration with increasing amounts of LCFA-CoA (Fig. 2). The full-length HNF-4a exhibited saturation binding curves with all LCFA-CoAs examined, thereby allowing calculation of binding parameters that are summarized in Table I. Full-length HNF-4a exhibited high affinity for saturated LCFA-CoAs (i.e. myristoyl-CoA (Fig. 2A) and palmitoyl-CoA (Fig. 2B)) characterized by $K_d$ values in the low nanomolar range ($K_d$ of 0.4 and 12 nM, respectively) (Table I). The affinity for myristoyl-CoA (C14:0-CoA) was significantly higher than for palmitoyl-CoA (C16:0-CoA), suggesting a preferential binding of the 14-carbon-long saturated acyl-CoA. The binding curves of palmitoleoyl-CoA (16:1-CoA), a monounsaturated acyl-CoA, and arachidonoyl-CoA (C20:4-CoA), a polyunsaturated acyl-CoA (Fig. 2, C and E, respectively) also suggested high affinity binding and yielded $K_d$ values of 0.7 and 4 nM for C16:1-CoA and C20:4-CoA, respectively (Table I). The affinity of HNF-4a for cis-parinaroyl-CoA (Fig. 2D), with $K_d$ of 70 nM, also demonstrated binding that was good but weaker than for all of the other tested LCFA-CoAs. Both saturated and unsaturated LCFA-CoAs were bound by full-length HNF-4a at one binding site per protein molecule as demonstrated by the linear plots shown in the insets of Fig. 2 as well as by nonlinear regression analysis, which demonstrated best fit to one binding site model (not shown).
by a direct effect of NBD bulky group influencing the protein/ligand binding. The binding curves for full-length HNF-4α and H9251 with unsaturated LCFA (i.e., palmitoleic acid (C16:1), cis-parinaric acid (cis-C18:4), and arachidonic acid (C20:4)) (Fig. 3, D–F) yielded $K_d$ values of 78, 206, and 296 nM, respectively, again indicating that full-length HNF-4α and H9251 had lower affinities for these free fatty acids (111-, 52-, and 4-fold, respectively) than for their CoA-thioester counterparts. Saturated and unsaturated LCFA were bound by full-length HNF-4α and H9251 at one binding site per protein molecule as demonstrated by the linear plots shown in the insets of Fig. 3, as well as by nonlinear regression analysis, in which the best fit was obtained for one binding site model (data not shown).

Effect of N-terminal Deletion of the DNA Binding Domain on Ligand Specificity of HNF-4α and H9251 (aa 132–455)—We previously examined ligand affinity and specificity of HNF-4α and H9251 (aa 132–455), including the ligand binding domain (E) and the C-terminal negative regulatory domain (F), and demonstrated that the affinity of the truncated HNF-4α-E-F mutant for saturated and unsaturated LCFA-CoA was higher than for free LCFA (19). In the present study, additional ligands were tested, namely C14:0-CoA, C16:1-CoA, C14:0, and C16:0, resulting in $K_d$ values of 0.9, 1, 165, and 150 nM, respectively (plots not shown). These ligand binding parameters of HNF-4α-E-F are very similar to those exhibited by full-length HNF-4α (Figs. 2 and 3; Table I). Thus, N-terminal deletion of the DNA binding domain had little effect on ligand binding affinities and specificity of HNF-4α.

Effect of C-terminal F-domain Truncation on LCFA-CoA Binding: Fluorescence Binding Assays—Due to the difficulty in crystallizing full-length nuclear receptors, to date x-ray crystallographic studies of HNF-4α structure utilized HNF-4 constructs, wherein not only the N-terminal DNA binding domain was deleted, but also most of the C-terminal F-domain was truncated, including HNF-4α (aa 132–382) (18) and HNF-4α (aa 103–408) (17). When isolated from recombinant bacteria, all such N- and C-terminal truncation mutants contained entrapped LCFA (17, 18), especially palmitoleic acid (C16:1). However, the C-terminal F-domain of HNF-4α is known to be a negative regulator of HNF-4α transactivation in intact cells (8, 16, 22, 38), and the assumption that its deletion had no effect on HNF-4α structure or LCFA/LCFA-CoA binding was never tested before. In order to resolve whether ligand affinity was affected by deleting the negative regulatory F-domain from the C terminus of HNF-4α, we expressed a truncated mutant lacking N-terminal domains A–D and F-domain (i.e. HNF-4α-E (aa 132–370)) in E. coli and purified. Fluorescent binding assays were performed with this truncated HNF-4α.

**Fig. 2.** Fatty acyl-CoA binding curves for full length HNF-4α as determined by quenching in HNF-4α Tyr/Trp fluorescence upon ligand binding. Full-length HNF-4α was titrated with increasing concentrations of myristoyl-CoA (A), palmitoyl-CoA (B), palmitoleoyl-CoA (C), cis-parinaroyl-CoA (D), and arachidonoyl-CoA (E). $F_0 - F$ (where $F_0$ is the Tyr/Trp fluorescence intensity in the absence of ligand, and $F$ is the Tyr/Trp fluorescence intensity in the presence of ligand at each titration point) represents quenching in fluorescence of Tyr/Trp at each titration point, measured at 330 nm with excitation at 280 nm. The insets present linear plots of the corresponding hyperbolic titration curves, obtained as described under “Experimental Procedures”; $F_i$ and $F_{max}$ measured the decrease in HNF-4α fluorescence intensity in the presence of ligand at each titration point and at saturation, respectively.
breviated as R-CoAs, where R- represents the acyl-group. S-C16-CoA, arachidonic acid. The coenzyme thioesters of these fatty acids are ab-

A–C

molecule (Fig. 6, A–C). The \( K_d \) values for all tested LCFA-CoAs were in the range of 54–236 nM (Table I). These values indicated that HNF-4α-E mutant exhibited considerably lower affinity for LCFA-CoAs as compared with the full-length protein HNF-4α or HNF-4α-E-F (aa 132–455). Thus, the affinity of HNF-4α-E for C14:0-CoA was 243- and 108-fold lower than the affinity of full-length HNF-4α and HNF-4α-E-F (aa 132–455) (Fig. 4A, Table I) for this ligand, respectively. For C16:0-CoA (Fig. 4B), the discrepancy between HNF-4α-E-F and HNF-4α-E truncated mutant amounted to 32-fold lower affinity of the HNF-4α-E. When binding of C20:4-CoA was tested, the affinity of HNF-4α-E was 48- or 51-fold lower than the affinity of full-length HNF-4α or HNF-4α-E-F (aa 132–455) (Fig. 4C, Table I) for the same ligand, respectively. All of these data indicated that deletion of the F-domain of HNF-4α significantly decreased the affinity of HNF-4α-E full-length or of HNF-4α-E-F for LCFA-CoAs.

Effect of C-terminal F-domain Truncation on LCFA Binding: Fluorescence Binding Assays—Since deletion of the F-domain resulted in significant reduction in binding affinity for LCFA-CoAs, the affinity of HNF-4α-E (aa 132–370) truncated mutant for free long chain fatty acids was also tested. The binding curves of HNF-4α-E with saturated (C14:0 and C16:0) and unsaturated (C16:1 and C20:4) LCFA-CoAs (Fig. 4, D–F) yielded \( K_d \) values indicating significantly enhanced binding of LCFA-CoAs (Table I). The \( K_d \) values for tested saturated LCFA-CoAs were within an 11–13 nM range (Table I), demonstrating 4–16-fold increased affinity for free, nonesterified LCFA-CoAs upon F-domain deletion (i.e. HNF-4α-E). The \( K_d \) values for unsaturated LCFA-CoAs were in the range of 1.5–32 nM (Table I), indicating a 9–52-fold increase in affinity for these LCFA-CoAs upon deleting the F-domain (i.e. HNF-4α-E). Thus, these data suggested that truncation of the C-terminal F-domain (i.e. HNF-4α-E) increased the affinities for LCFA-CoAs as compared with those exhibited by HNF-4α-E-F and full-length HNF-4α (Table I). When taken together with the 32–243-fold decreased affinity for LCFA-CoAs upon deletion of the C-terminal F-domain (see above), these data indicated that deletion of the F-domain also reversed the ligand specificity of HNF-4α.

Effect of C-terminal F-domain Truncation on HNF-4α-bound cis-Parinaric Acid: Displacement by LCFA-CoA—Although the above studies demonstrated that the 2–3-order greater affinity of full-length HNF-4α for LCFA-CoA than LCFA was dramatically altered by truncation of the F-domain, relatively little is known regarding the relative ability of LCFA-CoA to displace bound LCFA from full-length HNF-4α or HNF-4α-E. To address this issue, a displacement assay was developed taking advantage of fluorescence properties of cis-parinaric acid (bound ligand) and S-hexadecyl-CoA (displacing ligand) as described under “Experimental Procedures.” In this assay, cis-parinaric acid bound to HNF-4α proteins was displaced by an LCFA-CoA nonhydrolyzable analogue (S-hexadecyl-CoA). To avoid the possibility of LCFA-CoA degradation/hydration, the nonhydrolyzable analogue of LCFA-CoA (i.e. S-hexadecyl-CoA ether) was chosen as the displacing ligand. Qualitatively, S-hexadecyl-CoA displaced bound cis-parinaric acid from full-length HNF-4α (Fig. 5A) as well as from truncation mutants HNF-4α-E-F (Fig. 5B) and HNF-4α-E (Fig. 5C). However, quantitative analysis revealed that the amount of S-hexadecyl-CoA required to displace half of the protein-bound cis-parinaric acid (i.e. \( I_{50} \)), differed significantly, indicating different affinities of the three HNF-4α protein forms for hexadecyl-S-CoA. Indeed, the \( I_{50} \) values for full-length HNF-4α and HNF-4α-E-F were very low (0.12 and 2.5 nm, respectively), yielding \( K_d \) values of 0.06 and 0.96 nm, respectively. In contrast, C-terminal deletion of the F-domain significantly increased the \( I_{50} \) and \( K_d \) values for HNF-4α-E-F to 273 and 83 nm, respectively. In summary, these data clearly demonstrated the following. (i) LCFA-CoA readily displaced bound LCFA from full-length HNF-4α. (ii) N-terminal truncation of the DNA binding domain (i.e. HNF-4α-E-F) had some effect on this displacement (i.e. a 1-order of magnitude decrease in \( K_d \) of cis-parinaric displacement by S-hexadecyl-CoA). (iii) For LCFA-CoAs, \( K_d \) values from displacement assays and \( K_d \) values from direct binding assays were in the same range, indicating much higher affinity for LCFA-CoA than LCFA by full-length HNF-4α and HNF-4α-E-F. (iv) C-terminal truncation of F-domain, in addition to the N-terminal deletion, increased the \( K_d \) for displacement of bound LCFA by 692-fold, consistent with the high \( K_d \) (low affinity) for LCFA-CoA binding by HNF-4α-E in direct binding assays.

Reverse Titration of NBD-Stearic Acid with Full-length HNF-4α Versus Deletion Mutants HNF-4α-E-F (aa 132–455) and HNF-4α-E (aa 132–370): Differences in Protein Dimer Formation—Reverse titration experiments (i.e. increasing protein, constant ligand) may yield additional insights to the process of protein-ligand complex formation. When NBD-stearic acid was titrated with increasing concentrations of full-length HNF-4α, truncated HNF-4α-E-F (aa 132–455) or HNF-4α-E (aa 132–370), enhancement in the NBD-stearic acid fluorescence intensity was observed up to about a 1:1 molar ratio of protein versus ligand, demonstrating one ligand binding site per monomeric HNF-4α molecule (Fig. 6, A–C). However, the binding curves exhibited distinctly different shapes. The NBD-stearic acid/full-length HNF-4α binding curve showed an isosbestic point and sigmoid shape (Fig. 6A, inset). Likewise, the titration of NBD-stearic acid with HNF-4α-E-F (aa 132–455) resulted in a sigmoid curve (Fig. 6B, inset). In contrast, the truncated HNF-4α-E protein exhibited a hyperbolic binding curve (Fig. 6C, inset) with no isosbestic points. The differential binding curves obtained for the three forms of HNF-4α in reverse titrations are

| Ligand                  | Full-length HNF-4α \( K_d \) | HNF-4α-E (aa 132–370) \( K_d \) |
|------------------------|-------------------------------|----------------------------------|
| Saturated CoAs         |                               |                                  |
| C14:0-CoA              | 0.4 ± 0.05                    | 97 ± 10                          |
| C16:0-CoA              | 12 ± 3                        | 54 ± 7                           |
| S-C16:0-CoA            | 0.06 ± 0.01                   | 302 ± 25                         |
| Unsaturated CoAs       |                               |                                  |
| C16:1-CoA              | 0.7 ± 0.2                     | 265 ± 31                         |
| C20:4-CoA              | 4 ± 0.7                       | 236 ± 67                         |
| cis-C18:4-CoA          | 70 ± 6                        |                                  |
| Free fatty acids       |                               |                                  |
| Saturated CoAs         |                               |                                  |
| C14:0                  | 109 ± 21                      | 11 ± 3                           |
| C16:0                  | 182 ± 19                      | 11 ± 2                           |
| NBD-C18:0              | 58 ± 4                        | 13 ± 3                           |
| Unsaturated CoAs       |                               |                                  |
| C16:1                  | 78 ± 12                       | 1.5 ± 0.2                        |
| C20:4                  | 206 ± 17                      | 4 ± 0.8                          |
| cis-C18:4              | 296 ± 21                      | 32 ± 4                           |

Table I Binding affinities of full-length HNF-4α (aa 1–455) versus truncated mutant HNF-4α-E (aa 132–370) for acyl-CoA-thioesters and free fatty acids

HNF4α (aa 1–455) is the full-length HNF4α. HNF-4α-E (aa 132–370) is the truncated mutant that contains E-domain only. Ligand binding assays were determined as described under “Experimental Procedures.” C14:0, myristic acid; C16:0, palmitic acid; NBD-C18:0, NBD-labeled stearic acid; C16:1, palmitoleic acid; cis-C18:4, cis-parinaric acid; C20:4, arachidonic acid. The coenzyme thioesters of these fatty acids are abbreviated as R-CoAs, where R- represents the acyl-group. S-C16-CoA, S-hexadecyl-CoA, a nonhydrolyzable thioester, homolog to the CoA-thioester of hexadecanoic acid. Values for \( K_d \), the dissociation constant in nm, are expressed as the mean ± S.E.
**Intrinsic Fluorescence Characteristics of Full-length HNF-4α**

The intrinsic aromatic amino acid emission properties were examined. Since Trp residues typically have a much higher quantum yield and Tyr residues close to Trp also internally transfer energy to Trp in proteins, the emission spectra of most proteins is dominated by Trp emission. In aqueous buffers, the fluorescence emission maximal wavelength of aromatic amino acids in proteins represents a measure of the relative exposure of Trp (and less so Tyr) to the aqueous accessible surface of the proteins (19). The full-length HNF-4α protein contains 2 Trp and 12 Tyr residues. The distribution of these fluorescent residues throughout the various domains of HNF-4α is as follows: (i) 1 Trp at position 340 in the amino acid sequence, within the E (LBD) domain; (ii) 1 Trp at position 411 within the F-domain; (iii) 5 Tyr residues within the A/B-domain; (iv) 6 Tyr residues within E-domain (LBD); (v) 1 Tyr within F-domain. To determine whether N- and C-terminal truncations of HNF-4α affected the relative proportion of aqueous exposed Trp, emission spectra of full-length HNF-4α (top spectrum in Fig. 7, A and B) and HNF-4α-E (aa 132–370) (top spectrum in Fig. 7, C and D) were obtained upon excitation at 295 nm (Fig. 7) and at 280 nm (not shown). The lower content of Tyr and Trp in HNF-4α-E (aa 132–370) truncation mutant than in full-length HNF-4α protein was reflected in 2-fold higher fluorescence intensity of protein was reflected in 2-fold higher fluorescence intensity of

**Fig. 3.** Free long chain fatty acid binding curves for full-length HNF-4α as determined by quenching in HNF-4α Tyr/Trp fluorescence and/or by increase in ligand fluorescence upon protein binding at each titration point and at saturation, respectively. In A, B, D, and F, F_max and F_max values measure quenching in protein fluorescence intensity in the presence of ligand, at each titration point and at saturation, respectively. In C and E, the F_max values measure ligand fluorescence intensities upon protein binding at each titration point and at saturation, respectively. In the case of full-length HNF-4α dimers may have higher affinity for ligand than the monomers (39, 40). However, the potential effect of HNF-4α stable homodimers in the absence or presence of DNA or ligands exists, being characterized by three different dissociation constants: one for protein-protein interactions and dimer formation, one for ligand binding to monomers, and one for ligand binding to dimers. Since sigmoidal curves are usually indicative of cooperative interactions, one possible explanation is that in the reverse titrations, the more protein is added to a constant amount of ligand, the more dimers are formed, and the dimers may have higher affinity for ligand than the monomers in the case of full-length HNF-4α or HNF-4α-E-F (insets of Fig. 6, A and B), but not for HNF-4α-E (inset of Fig. 6C), which lacks the regulatory F-domain. Previous in vitro gel shift studies suggested that full-length and truncated mutants of HNF-4α formed stable homodimers in the absence or presence of DNA or ligands (39, 40). However, the potential effect of HNF-4α dimerization on ligand binding was not considered before. The differential ligand binding curves observed in reverse titration experiments may indicate that only in the presence of F-domain, a cooperative mechanism (between the monomeric units of a dimer) may occur and play a role in a regulatory function of HNF-4α.

**Intrinsic Fluorescence Characteristics of Full-length HNF-4α and Deletion Mutants HNF-4α-E-F (aa 132–455) and HNF-4α-E (aa 132–370): Effect of cis-Parinaroyl-CoA and cis-Parinaric Acid Binding**—To understand the differential effects of N- versus both N- and C-terminal truncations on ligand affinity and specificity of HNF-4α constructs, the intrinsic aromatic amino acid emission properties were examined. Since Trp residues typically have a much higher quantum yield and Tyr residues close to Trp also internally transfer energy to Trp in proteins, the emission spectra of most proteins is dominated by Trp emission. In aqueous buffers, the fluorescence emission maximal wavelength of aromatic amino acids in proteins represents a measure of the relative exposure of Trp (and less so Tyr) to the aqueous accessible surface of the proteins (19). The full-length HNF-4α protein contains 2 Trp and 12 Tyr residues. The distribution of these fluorescent residues throughout the various domains of HNF-4α is as follows: (i) 1 Trp at position 340 in the amino acid sequence, within the E (LBD) domain; (ii) 1 Trp at position 411 within the F-domain; (iii) 5 Tyr residues within the A/B-domain; (iv) 6 Tyr residues within E-domain (LBD); (v) 1 Tyr within F-domain. To determine whether N- and C-terminal truncations of HNF-4α affected the relative proportion of aqueous exposed Trp, emission spectra of full-length HNF-4α (top spectrum in Fig. 7, A and B) and HNF-4α-E (aa 132–370) (top spectrum in Fig. 7, C and D) were obtained upon excitation at 295 nm (Fig. 7) and at 280 nm (not shown). The lower content of Tyr and Trp in HNF-4α-E (aa 132–370) truncation mutant than in full-length HNF-4α protein was reflected in 2-fold higher fluorescence intensity of protein was reflected in 2-fold higher fluorescence intensity of
132–370) exhibited maxima at 328 and 342 nm, respectively, with excitation at 280 nm (data not shown). Thus, truncation of both N- and C-terminal domains (i.e. HNF-4a/E (aa 132–370)), resulted in a 14-nm red shift in maximal fluorescence emission wavelength, indicating a greater exposure of Tyr and Trp residues to aqueous in HNF-4a/E than in full-length HNF-4a. With selective excitation of Trp at 295 nm, only a small red shift of 4 nm was detected (Fig. 7, A and C), suggesting that Tyr residues rather than Trp gained a larger exposure to aqueous in HNF-4a/E truncation form as compared with full-length HNF-4a. Full-length HNF-4a and HNF-4a/E differentially responded to the addition of cis-parinaroyl-CoA. With increasing cis-parinaroyl-CoA concentrations over the range of 0–1.20 μM, the maximal fluorescence emission intensity was decreased by 64% in full-length HNF-4a and by 30% in HNF-4a/E (aa 132–370) when the proteins were excited at 280 nm (data not shown). However, with excitation of Trp only, at 295 nm over a similar cis-parinaroyl-CoA concentration range, the emission fluorescence of full-length HNF-4a and HNF-4a/E (aa 132–370) was similarly quenched by 35 and 28%, respectively (Fig. 7, A and B). Earlier data on cis-parinaroyl-CoA-induced quenching of HNF-4a/E-F (aa 132–455) indicated a maximal decrease in fluorescence of this truncation mutant by 83% (19). These results suggested that cis-parinaroyl-CoA elicited quenching in the emission intensity of Tyr rather than Trp residues in the F-domain. This quenching can be explained by (i) cis-parinaroyl-CoA binding at the E LBD and inducing a conformational change of the F-domain to result in a Tyr fluorescence change and (ii) direct interaction and possibly FRET of cis-parinaroyl-CoA with F-domain Tyr/Trp. This observation demonstrates that the long F-domain of HNF-4a is not exclusively separated from the E-domain (LBD) but is rather in very close proximity and interaction with it. The effect of free, non-esterified cis-parinaric acid on the full-length HNF-4a and HNF-4a/E (aa 132–370) (Fig. 7, B and D) was compared with that of HNF-4a/E-F (aa 132–455) described previously (19). cis-Parinaric acid quenched the full-length HNF-4a, HNF-4a/E-F (aa 132–455), and HNF-4a/E (aa 132–370) by 49, 59, and 55%, respectively, when Tyr and Trp residues were excited at 280 nm. A closer distribution of quenching percentages for cis-parinaric acid when compared with cis-parinaroyl-CoA, suggested that the free fatty acid interacted mostly with Tyr residues present in the E-domain, whereas the fatty acyl-CoA interacted with Tyr residues in both the E- and F-domains.

**Fig. 4.** Acyl-CoA and free fatty acid binding curves for HNF-4a/E (aa 132–370) truncated mutant as determined by quenching in Tyr/Trp fluorescence upon ligand binding. HNF-4a/E protein was titrated with increasing concentrations of myristoyl-CoA (A), palmitoyl-CoA (B), arachidonoyl-CoA (C), myristic acid (D), palmitic acid (E), and arachidonic acid (F). The insets show the linear plots (according to Equation 1). $F_0$ and $F_{\text{max}}$ represent HNF-4a/E Tyr/Trp fluorescence quenching (calculated from $F_0/F_{\text{max}}$) in the presence of ligand at each titration point and at saturation, respectively.
FRET Confirms Close Molecular Interaction between HNF-4α Constructs and Bound Long Chain Fatty Acyl-CoA—To further establish the close molecular interaction between bound LCFA-CoA and HNF-4α, FRET was measured between HNF-4α tryptophan and bound cis-parinaroyl-CoA as established under “Experimental Procedures.” The Trp/cis-parinaroyl-CoA was chosen as a donor/acceptor pair because emission of Trp in proteins exhibits significant overlap with cis-parinaroyl-CoA absorption (19, 25). Upon excitation of Trp in HNF-4α variants at 295 nm, FRET was detected both as a decrease in donor fluorescence intensity (at 328 nm for both full-length HNF-4α and HNF-4α-E) and an increase in acceptor-sensitized emission (at 420 nm for both cis-parinaric acid and cis-parinaroyl-CoA) with increasing acceptor concentration (Fig. 7). Because high affinity ligand binding altered the conformation of HNF-4α variants, it was not possible to accurately determine FRET efficiency and intermolecular distance from quenching of donor emission (i.e. HNF-4α Trp). Instead, FRET efficiency was calculated from sensitized emission of energy acceptor as described under “Experimental Procedures.” The spectral overlap of HNF-4α Trp emission fluorescence spectrum (Fig. 8, dashed and dotted line) with the absorption (excitation) spectrum of cis-parinaric acid and cis-parinaroyl-CoA (Fig. 8, continuous line) indicated a slightly lower overlap integral for HNF-4α-E than for full-length HNF-4α. The overlap integrals were calculated and used to determine R0 (distance between protein Trp and bound ligand fluorophore for 50% FRET efficiency): 28.5 Å for full-length HNF-4α and 25.4 Å for HNF-4α-E truncation mutant (Table II). R0, the actual distance between protein Trp and bound ligand was 33 Å for the full-length HNF-4α/cis-parinaroyl-CoA pair and so large that it could not be measured for the full-length HNF-4α/cis-parinaric acid pair (Table II). Thus, the cis-parinaroyl-CoA bound in much closer proximity to the binding site than cis-parinaric acid, consistent with the higher affinity of the full-length HNF-4α for LCFA-CoA than LCFA. In contrast, the HNF-4α-E mutant bound cis-parinaric acid much closer than cis-parinaroyl-CoA (i.e. within 28 Å versus more than 100 Å), again consistent with the higher affinity of this deletion mutant for LCFA than for LCFA-CoA (Table II).

Full-length HNF-4α has two Trp residues at positions 340 (within E, the ligand binding domain) and 411 (within F, the negative regulatory domain), respectively. cis-Parinaric acid and its CoA-thioester caused 24–35% quenching in Trp fluorescence of full-length HNF-4α and HNF-4α-E (Fig. 7, Table II), whereas CoA decreased Trp fluorescence emission intensity by less than 10% in both HNF-4α proteins (data not shown). cis-Parinaric acid exhibited significant sensitized emission due...
F-domain Determines HNF4α Ligand Specificity

Fig. 7. cis-Parinaroyl-CoA and cis-parinaric acid-induced changes in fluorescence emission spectra of full-length HNF-4α and its deletion mutant HNF-4α-E (aa 132–370): FRET. Fluorescence emission spectra of full-length HNF-4α with increasing concentrations of cis-parinaroyl-CoA (A) and cis-parinaric acid (B) were scanned from 300 to 450 nm with excitation at 295 nm. The top lines (I) represent the spectra of 100 nM HNF-4α in the absence of ligand, whereas the following lines to the bottom (2–7) are the spectra of the same concentration of HNF-4α with 2 nM, 35 nM, 170 nM, 500 nM, 1 μM, and 1.2 μM (bottom dotted line) cis-parinaroyl-CoA (A) or cis-parinaric acid (B). Sensitized emission of cis-parinaroyl-CoA at 420 nm upon excitation of HNF-4α at 285 nm was detected (A), suggesting FRET from Trp (FRET donor) to the cis-parinaroyl-CoA group (FRET acceptor). No FRET occurred between full-length HNF-4α and cis-parinaric acid (B). Results of the same experiment with HNF-4α-E mutant instead are shown in C and D. No FRET was detected from HNF-4α-E to cis-parinaroyl-CoA (C), whereas excitation of HNF-4α-E at 295 nm resulted in high sensitized emission of cis-parinaric acid (D).

Fig. 8. Spectral overlap of full-length HNF-4α and HNF-4α-E fluorescence emission with cis-parinaric and cis-parinaroyl-CoA absorption (excitation). Solid line, cis-parinaric acid and cis-parinaroyl-CoA absorption (excitation) spectrum. Dashed line, full-length HNF-4α (aa 1–455) emission fluorescence spectrum, with excitation at 295 nm. Dotted line, HNF-4α-E (aa 132–370) emission fluorescence spectrum, with excitation at 295 nm.

to FRET from Trp340 in HNF-4α-E (Fig. 7D), in the absence of F-domain only, and did not interact with Trp340 and/or Trp411 residues within full-length HNF-4α (Fig. 7B). In contrast, cis-parinaroyl-CoA exhibited sensitized emission upon excitation of Trp340 and/or 411 with 295-nm light in the presence of full-length HNF-4α (Fig. 7A) but not with HNF-4α-E (Fig. 7C). This suggested that, when the F-domain was missing (in HNF-4α-E), cis-parinaric acid but not cis-parinaroyl-CoA-thioester had access to Trp340, whereas oppositely, in the presence of F-domain (in full-length HNF-4α) cis-parinaroyl-CoA but not free cis-parinaric acid was in close proximity of Trp340 and/or 411. This further indicated that the F-domain was critical for parinaroyl-CoA to be recognized as a specific high affinity ligand by E-domain and that the access of the cis-parinaroyl-CoA acyl group to Trp340 was through the F-domain rather than directly to the E-domain.

Effect of N- and C-terminal Truncation on Secondary Structure of HNF-4α: Circular Dichroism—To assess effects of N- and C-terminal truncation on secondary structure, CD spectra were obtained for full-length HNF-4α (HNF-4α-E-F (aa 132–455)) and HNF-4α-E (aa 132–370), followed by analysis for relative contributions of helix, β-sheet, and random coil. The far UV CD spectra of HNF-4α proteins were dependent on the polarity of the aqueous buffer used for CD experiments. In high polarity buffer (150 mM PBS, pH 7.4), full-length HNF-4α and HNF-4α-E exhibited larger molar ellipticities as compared with HNF-4α-E-F (Fig. 9A). Full-length HNF-4α contained 45% helix, 13% β-strand, and 22% turns (Fig. 9B). HNF-4α-E-F had a predominant β-strand secondary structure, containing only 22% helix but 29% β-strands and 21% turns (Fig. 9B). HNF-4α-E was mostly helical with 36% helix, 18% β-strand, and 21% turns (Fig. 9B). Thus, in a highly ionic environment, HNF-4α-E-F had the highest content of β-strand and the lowest percentage of helix when compared with the other two HNF-4α forms. When a low ionic buffer was used (L&C buffer; 0.5 mM Tris-HCl with 7.5 mM NaCl and 0.25% glycerol), all three proteins exhibited CD spectra typical for highly helical secondary structures (Fig. 9C). In low ionic environment, helix was predominant in all three forms of HNF-4α; (i.e. 54–72%), β-strand was the lowest fraction (i.e. 3–4%), whereas turns and random coil were within a 10–16 and 15–27% range, respectively (Fig. 9D).

This was also the case when the circular dichroic data were expressed in terms of the number of amino acids contributing to each type of secondary structure (Table III). The higher helical content of HNF-4α forms in the lower polarity buffer was consistent with the highly helical structures of HNF-4α truncation forms (e.g. aa 132–380) observed in x-ray studies of HNF-4α (17, 18). This may be explained by the fact that in crystals, protein molecules are depleted of much of the surrounding water, and in this sense a crystal favors protein structures characteristic for a hydrophobic rather than aqueous environment.

To determine whether the decrease in the helical content of domains EF relative to that of domain E was more or less...
F-domain Determines HNF4α Ligand Specificity

The overlap integral (\( J \)), the critical distance (\( R_0 \)) that allowed 50% energy transfer efficiency (\( E \)), and the actual distance (\( R_{90} \)) between HNF-4α Trp and the fluorophore in the bound ligand were calculated as described under “Experimental Procedures” for both full-length HNF-4α (aa 1–455) and its N- and C-terminal truncation mutant HNF-4α-E (aa 132–370). The amount of quenching measured upon ligand binding saturation, \( Q \), represents percentage decrease from the initial fluorescence intensity of Trp in HNF-4α proteins upon excitation with 295 nm. The \( Q, E, \) and \( R_{90} \) values are expressed in mean ± S.D.

| FRET donor | FRET acceptor | \( Q \) | \( J (\AA^2 \cdot \text{cm}^{-2} \cdot \text{nm}^{-1}) \) | \( R_0 \) | \( E \) | \( R_{90} \) |
|------------|--------------|--------|-----------------------------------|--------|--------|--------|
| HNF-4α     | cis-C18:4/CoA| 35 ± 3 | 2.86 × 10^{-14}                   | 28.5   | 30 ± 2 | 33 ± 0.5|
| HNF-4α     | cis-C18:4    | 28 ± 2 | 2.86 × 10^{-14}                   | 28.5   | 0.00   |
| HNF-4α-E   | cis-C18:4/CoA| 28 ± 3 | 2.54 × 10^{-14}                   | 25.4   | 0.00   |
| HNF-4α-E   | cis-C18:4    | 24 ± 2 | 2.54 × 10^{-14}                   | 25.4   | 38 ± 2 | 28 ± 1.4|

**Fig. 9.** Secondary structure composition of full-length HNF-4α and its truncation mutants as determined by circular dichroism. A, CD spectra of full-length HNF-4α (black circles), HNF-4α-E-F (white circles), and HNF-4α-E (white triangles) in highly polar solution (PBS). B, percentages of helix, \( \beta \)-strand, turn, and random coil secondary structures of full-length HNF-4α (black columns), HNF-4α-E-F (light gray columns), and HNF-4α-E (dark gray columns) as determined by CDPRO analysis of spectra in A. C, CD spectra of full-length HNF-4α (black circles), HNF-4α-E-F (white circles), and HNF-4α-E (white triangles) in low ionic solution (diluted L&C buffer; see “Experimental Procedures”). D, percentages of helix, \( \beta \)-strand, turn, and unordered secondary structures of full-length HNF-4α (black), HNF-4α-E-F (light gray), and HNF-4α-E (dark gray) as determined by CDPRO analysis of spectra in C. *Statistically significant changes with \( p < 0.0001 \) as determined by Student’s \( t \) test.

**Table II**

| Ligand Specificity | E | \( R_{90} \) |
|-------------------|---|-----------|
| Full-length HNF-4α (455 aa) | 205 ± 10 | 100 ± 16 | 91 ± 9 |
| HNF-4α-E-F (323 aa) | 71 ± 10 | 65 ± 4° | 90 ± 6 |
| HNF-4α-E (238 aa) | 85 ± 16 | 59 ± 4° | 60 ± 7° |
| L&C buffer | 59 | 59 | 59 |
| Full-length HNF-4α (455 aa) | 277 ± 14 | 68 ± 7 | 96 ± 10 |
| HNF-4α-E-F (323 aa) | 174 ± 14° | 52 ± 7° | 84 ± 5 |
| HNF-4α-E (238 aa) | 171 ± 19° | 24 ± 8° | 36 ± 12° |

a Significant difference for HNF-4α-E-F as compared with full-length HNF-4α. The statistical significance was calculated by \( t \) test for \( p < 0.04 \).

b Significant difference for HNF-4α-E as compared with full-length HNF-4α. The statistical significance was calculated by \( t \) test for \( p < 0.04 \).

c Significant difference for HNF-4α-E as compared with HNF-4α-E-F. The statistical significance was calculated by \( t \) test for \( p < 0.04 \).

The number of amino acids that participated in each type of secondary structure for full-length HNF-4α, N-terminal truncation mutant HNF-4α-E-F, and N- and C-terminal truncation mutant HNF-4α-E are presented. The number of amino acids involved in each type of secondary structure was calculated based on the percentage composition of each secondary type of structure (shown in Fig. 9) and the total number of amino acids of each of the three forms of HNF-4α.

| Buffer/HNF-4α | α-Helix | β-Strand | Turn | Unordered |
|---------------|---------|----------|------|-----------|
| PBS           |         |          |      |           |
| Full-length HNF-4α (455 aa) | 205 ± 10 | 59 ± 7 | 100 ± 16 | 91 ± 9 |
| HNF-4α-E-F (323 aa) | 71 ± 10 | 65 ± 4° | 90 ± 6 |
| HNF-4α-E (238 aa) | 85 ± 16 | 59 ± 4° | 60 ± 7° |
| L&C buffer | 59 | 59 | 59 |
| Full-length HNF-4α (455 aa) | 277 ± 14 | 68 ± 7 | 96 ± 10 |
| HNF-4α-E-F (323 aa) | 174 ± 14° | 52 ± 7° | 84 ± 5 |
| HNF-4α-E (238 aa) | 171 ± 19° | 24 ± 8° | 36 ± 12° |
polar (reduced by 32% from 100 to 68 aa) and in hydrophobic (reduced by 23.5% from 68 to 52 aa) environments. Oppositely, the number of amino acids within β-strands was 37% larger in the HNF-4α-E-F truncation mutant than in full-length HNF-4α in a polar environment. In the HNF-4α-E truncation mutant, in which the F-domain was missing in addition to the deletion of N-terminal A–D-domains, the number of amino acids involved in β-strands, turns, and unordered structures was significantly reduced as compared with HNF-4α-E-F mutant, in both polar and hydrophobic buffers, whereas the amino acid number contained in helical structures was not changed (Table III).

In summary, these data suggest that the F-domain contributed to the overall secondary structure of HNF-4α, especially by adding an increased proportion of β-strand, turn, and unordered fractions, in both polar and hydrophobic environments.

**Effect of Trifluoroethanol (TFE) on Secondary Structure of Full-length HNF-4α and Its Deletion Mutants: Circular Dichroism**—TFE is known to induce helical secondary structure in proteins that contain unordered structures and have potential for developing helices under a hydrophobic environment (41, 42). Since the long F-domain in HNF-4α molecule is random coil-proline-rich with very low helix content (22, 38), the potential of the three forms of HNF-4α to form helix structure in the presence of TFE and the influence of F-domain were examined (Fig. 10). The far-UV-CD spectra of full-length HNF-4α, HNF-4α-E-F, and HNF-4α-E in the presence of 50% TFE differed significantly as compared with 0% TFE in L&C buffer (Fig. 10, A, C, and E). The highest changes in response to TFE were observed with HNF-4α-E-F (Fig. 10B), since its molar ellipticity absolute values at 208 and 222 nm were higher in 50% TFE than in the absence of TFE. The full-length HNF-4α and the HNF-4α-E mutant showed no change in molar ellipticities at 208 nm and only small changes at 222 nm (Fig. 10, A and E). Quantitative and statistical analysis of the CD spectra demonstrated that TFE induced (i) 30% more helix, no change in β-strand content, and a decrease in turns by 90% and in random coil by 2.2-fold in full-length HNF-4α (Fig. 10B); (ii) 70% more helix, a total disappearance of β-strands from 4% to 0, and significant reductions of turns by 8-fold and random coil by 6.8-fold in HNF-4α-E-F (Fig. 10D); (iii) 30% increase in helix structure, no change in β-strand, and a hardly significant (p < 0.02) decrease in turns (by 2-fold) and random coil (by 3-fold) in HNF-4α-E (Fig. 11F). These data demonstrated that TFE was most effective in inducing ordered helical structures in HNF-4α-E-F, in which the F-domain was present and the ratio of F-domain/whole molecule length was higher than in full-length HNF-4α. TFE was the least effective on HNF-4α-E, which did not contain the F-domain. This conclusion may suggest that the F-domain was the most sensitive part of HNF-4α to molecular environment and adopted either β-strand structure under a polar environment or helical structure under a hydrophobic environment. Because TFE precipitated PBS out, the effect of TFE on HNF-4α proteins when in PBS could not be studied.

**Effects of Ligands on Secondary Structure of HNF-4α Truncation Mutants: Circular Dichroism**—In order to assess the effect of LCFA-CoA and LCFA on the secondary structures of HNF-4α-E-F (aa 132–455) and HNF-4α-E (aa 132–370), the recombinant proteins were incubated with palmitoyl-CoA
(C16:0-CoA) or palmitic acid (C16:0), and far UV CD spectra were measured and analyzed (Fig. 11). Palmitoyl-CoA altered the CD spectra of HNF-4α-E-F (Fig. 11A), thereby significantly (p < 0.0001) increasing α-helix structure (by 70%), with a simultaneous decrease of β-strand (by 3.6-fold) and turns (by 60%) (Fig. 11B). In contrast, palmitic acid had only a small significant influence on the secondary structure of HNF-4α-E-F as compared with HNF-4α-E-F in the absence of ligand (Fig. 11, A and B) (i.e. a 40% decrease of β-strand percentage in the presence of C16:0). CD spectra of HNF-4α-E were not significantly altered by C16:0-CoA (Fig. 11, C and D). Free palmitic acid induced only small changes in secondary structures of HNF-4α-E: a 20% increase in helical structure and 40% decrease in β-strand structure of HNF-4α-E (Fig. 11, C and D; p < 0.01). These results indicated that conformational changes resulting in different secondary structure compositions of HNF-4α were induced by palmitoyl-CoA only when the F-domain was present. Free fatty acids induced smaller changes than CoA-thioesters in HNF-4α independently of the F-domain, and these changes were more evident when the F-domain was missing. Since ligand-induced conformational changes are a hallmark of ligand-responsive nuclear receptors, these data were consistent with LCFA-CoAs potentially being a physiologically significant ligand. Circular dichroism experiments with full-length HNF-4α indicated that palmitoyl-CoA but not palmitic acid elicited small alterations in the circular dichroic spectrum, suggesting increased helical content (data not shown). When the spectra were resolved into individual components, palmitoyl-CoA trended to increase the amount of helical structures in full-length HNF-4α, but this increase did not achieve statistical significance. This may be explained by the fact that full-length HNF-4α contains 131 additional amino acids and exhibits a much higher amount of helical component in its secondary structure as compared with HNF-4α-E truncation mutant, so that a small increase of its helix content upon acyl-CoA binding was hardly detectable by our circular dichroism assay.

**DISCUSSION**

Since the discovery of HNF-4α over a decade ago (1), there has been considerable controversy regarding whether this orphan nuclear transcription factor is (8, 11, 14, 19) or is not (12, 16, 18) ligand-activated. Due to problems with crystallizing the full-length HNF-4α (aa 1–455) or HNF-4α mutants containing an intact C-terminal F-domain (17, 18), almost nothing is known regarding the ligand specificity, structure, or conformational dependence of the full-length HNF-4α (aa 1–455). Furthermore, whereas studies using truncated mutants assume that N-terminal deletion of the DNA binding domain or C-terminal deletion of the F-domain have no effect on ligand binding to HNF-4α, the validity of these assumptions has never been demonstrated. The latter is especially surprising, since the C-terminal F-domain is a well recognized negative regula-
tor of HNF-4α transactivation and is thought to directly interact with the ligand binding domain E (8, 16, 22, 38). Since high affinity ligand binding and ligand-induced conformational changes are hallmarks of ligand-dependent nuclear receptors, fluorescence binding assays and circular dichroism were used in the present study to directly address these issues by examining ligand specificity and conformational responsiveness to ligand. Furthermore, the effects on these parameters of deleting the N-terminal DNA binding domain (i.e. HNF-4α-E-F (aa 132–455)) as well as truncation of both the N-terminal DNA binding domain and C-terminal F-domain in HNF-4α-E (aa 132–370) were individually examined.

First, it was shown that full-length HNF-4α (aa 1–455) preferentially binds LCFA-CoAs with high affinity, namely $K_d$ values in the very low nanomolar range, as indicated by several direct fluorescence binding assays. The high affinity for LCFA-CoAs was further confirmed by a displacement assay wherein LCFA-CoA displaced a bound fluorescent ligand with nanomolar $K_d$ values similar to nanomolar $K_d$ values exhibited in direct binding assays for LCFA-CoA. In addition, FRET revealed that the bound LCFA-CoA was in close molecular proximity (i.e. a few Å) to the Trp (especially Trp 340) of full-length HNF-4α. It is important to note that the affinities of full-length HNF-4α reported herein for LCFA-CoAs are in the same range as the concentration of LCFA-CoAs present in purified nuclei (reviewed in Refs. 37 and 43), nuclei of living cells (44) and nucleoplasm of living cells (44) (namely $<10$ nM, $23$ nM, and $3$ nM, respectively). Furthermore, the functional significance of LCFA-CoA interaction with HNF-4α is established in a variety of studies in cultured cells and in vitro. Thus, LCFA-CoAs that can be converted to inhibitory (C22:6-CoA) or activating (C14:0-CoA) with H9251 with HNF-4α transactivation of full-length HNF-4α (aa 132–455) as well as truncation of both the N-terminal DNA binding domain and C-terminal F-domain in HNF-4α-E (aa 132–370) were individually examined.

Second, it was shown here that full-length HNF-4α (aa 1–455) only weakly bound LCFA-CoAs as exhibited by $K_d$ values that were 140–440-fold lower as compared with those for LCFA-CoAs. The lower affinity for LCFA-CoAs than LCFA-CoAs was confirmed by a displacement assay by which LCFA-CoA displaced a bound fluorescent ligand with nanomolar values exhibited in direct binding assays for LCFA-CoA (8, 11). In contrast, LCFA-CoAs that are not metabolized to LCFA-CoA (i.e. a,α'-tetrachloro-tetradecane dioic acid) do not affect HNF-4α-mediated transactivation (14). LCFA-CoA interaction with HNF-4α is also confirmed by the effect of cotransfections with constructs overexpressing LCFA-CoA synthase or LCFA-CoA-thioesterase (8, 11, 14) as well as by in vitro binding assays and protease sensitivity assays with HNF-4α. Furthermore, missense mutations of HNF-4α (aa 132–455) that result in maturity onset diabetes of the young dramatically reduce affinities for LCFA-CoA and transactivation in cultured cells (8).

Third, HNF-4α transactivation (i.e. HNF-4α-E-F (aa 132–455)) had little effect on the high affinity for LCFA-CoA and ligand specificities (LCFA-CoA $\gg$ LCFA) as compared with those exhibited by the full-length HNF-4α (aa 1–455). Consistent with this, N-terminal deletion of the DNA binding domain, as in HNF-4α-E-F (aa 96–455) (11) and HNF-4α E-F (aa 128–455) (22), does not change the transactivation rate in intact cells. Thus, the ligand binding characteristics of N-terminal truncated mutants of HNF-4α reflect those of the full-length protein. This was in contrast to the progesterone receptor (PR), wherein N-terminal deletion of the DNA binding domain reduces ligand affinity by 6-fold (45).

Fourth, for the first time it was shown that the C-terminal regulatory F-domain was critical to determining ligand affinity and specificity of HNF-4α. C-terminal deletion of the F-domain not only enhances transactivation in intact cells (12, 16, 22) but correlates with marked alteration in ligand affinities and specificities. Deletion of the C-terminal F-domain as in HNF-4α-E (aa 132–370) differentially altered the ligand affinities (i.e. binding affinities for LCFA-CoAs were reduced 52–243-fold, whereas those for LCFA were increased 4–52-fold). The net effect of these differential influences of the F-domain on HNF-4α affinity for LCFA-CoA versus LCFA was that deletion of the C-terminal F-domain completely reversed the ligand specificity of the HNF-4α. Consistent with this finding, almost all of the F-domain of the androgen receptor (AR) participates in ligand binding (46). Similarly, deletion of the C-terminal F-domain in the PR greatly reduces affinity for or abrogates binding of progesterone but minimally affects affinity for antagonist RU486 (reviewed in Ref. 45) (46). Also, mutations in the 4th from last residue of the glucocorticoid receptor and mineralocorticoid receptor significantly decrease binding of both agonists and antagonists (reviewed in Ref. 46). In contrast, deletion of the C-terminal F-domain in the estrogen receptor increases affinity for estradiol by 11-fold but oppositely affects the activity of 4-hydroxytamoxifen, a partial agonist/antagonist (47). Thus, the exact nature of the ligand-dependent nuclear receptor determines whether the F-domain inhibits or enhances ligand binding.

Fifth, the C-terminal negative regulatory F-domain contributed significantly to the secondary structure of HNF-4α-LBD. Circular dichroism revealed that truncation of the C-terminal F-domain significantly decreased the proportion of $\beta$-strand, turn, and unordered content. This was consistent with the overall secondary structure of the F-domain being poorer in helix and higher in $\beta$-strand structure than the E-domain. Based on the amino acid sequence of HNF-4α F-domain, which is rich in proline (23%) (22), computer modeling predicts that an 428–441 may form a $\beta$-strand, and the only two additional organized regions within the F-domain may be very short, consisting of aa 383–389 (α-helix) and aa 392–396 ($\beta$-strand) (12, 13). Mutagenesis studies indicate that two regions within the F-domain (i.e. aa 428–441 and 371–414) are most highly associated with its repressor effect on transactivation in intact cells (reviewed in Ref. 38) (12). Quantitative analysis of the CD structures of HNF-4α-E and HNF-4α-E-F (Fig. 7) and comparison with x-ray crystal data of HNF-4α (aa 132–382) (18) and HNF-4γ (aa 103–408) (17) suggested that the F-domain interacted with the E-domain to disorganize helical components and favor the formation of $\beta$-strand secondary structure in HNF-4α-E-F. As a result, C-terminal truncation of the F-domain may have significantly reduced the size of the ligand binding pocket as evidenced by x-ray crystallography and also abolished cooperative interactions between HNF-4α subunits. Indeed, Hill plots of constant ligand titrated with increasing HNF-4α protein indicate that C-terminal deletion of the F-domain abolishes the cooperativity between HNF-4α-E (aa 132–370) subunits similarly to that observed upon disruption of the helix present in the F-domain of the estrogen receptor (47). Since the data presented herein and earlier showed that the F-domain significantly increased the $\beta$-strand content of the protein (shown herein) and altered the cooperativity between HNF-4α-E (aa 132–370) subunits, this would suggest that the F-domain may significantly interact with the ligand binding E-domain to alter its structure. Indeed, the C-terminal F-domain region of HNF-4α is apparently mobile, as evidenced by the inability of x-ray data to assign structures to the remaining portions of the C-terminal F-domain in HNF-4α (aa 132–382) (18), HNF-4γ (aa 103–408) (17), or HNF-4α-D-E-F (aa 103–465) (17). The high flexibility and inability to assign structure to the C-terminal F-domain residues is also evident in the closely related RXR and retinoic acid receptor (revised in Ref. 46). By modulating the structure of the ligand binding E-domain, the
F-domain Determines HNF4α Ligand Specificity

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