Structural Basis for HNF-4α Activation by Ligand and Coactivator Binding*

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In addition to suggesting that fatty acids are endogenous ligands, our recent crystal structure of HNF-4α showed an unusual degree of structural flexibility in the AF-2 domain (helix 12). Although every molecule contained a fatty acid within its ligand binding domain, one molecule in each homodimer was in an open inactive conformation with α12 fully extended and colinear with α10. By contrast, the second molecule in each homodimer was in a closed conformation with α12 folded against the body of the domain in what is widely considered to be the active state. This indicates that although ligand binding is necessary, it is not sufficient to induce an activating structural transition in HNF-4α as is commonly suggested to occur for nuclear receptors. To further assess potential mechanisms of activation, we have solved a structure of human HNF-4α bound to both fatty acid ligand and a coactivator sequence derived from SRC-1. The mode of coactivator binding is similar to that observed for other nuclear receptors, and in this case, all of the molecules adopt the closed active conformation. We conclude that for HNF-4α, coactivator rather than ligand binding locks the active conformation.

As a member of the nuclear receptor family of transcription factors, HNF-4α can be separated into DNA and ligand binding domains (LBD).1 The HNF-4α LBD (residues 140–368) contains distinctive features such as a hydrophobic ligand binding pocket, a dimerization interface, and an AF-2 transactivation domain (residues 360–368) (1). HNF-4α expression is highest in the liver, kidney, and the small and large intestines and is also considered to be the inactive form. In the second conformation, helix 12 folds back onto the LBD to seal off the ligand binding pocket. Because this “closed” form interacts with coactivators, it is also considered to be the inactive form. In the second conformation, helix α12 folds back onto the LBD to seal off the ligand binding pocket. Because this “closed” form interacts with coactivators, it is also considered to be the inactive form of the NR. Structural comparisons between unliganded (apo-domain) and ligand-bound and coactivator-bound forms of various NRs have led to the supposition that it is ligand binding that activates NRs by converting molecules in the open inactive conformation to an all closed active state (16, 17).

Our previously reported crystal structure of HNF-4α challenges this hypothesis (1). One major finding from both the HNF-4α and HNF-4γ structures was the serendipitous presence of fatty acids in their LBDs, suggesting that these might be endogenous ligands (1, 18). However, only half of the fatty acid-bound molecules in the HNF-4α structure adopted the closed conformation expected for the activated state. The second molecule in each homodimer was in a conformation expected for the inactive state with helix α12 fully extended and colinear with helix α10. This finding suggested that fatty acid binding might be necessary but not sufficient for activating

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§ The abbreviations used are: LBD, ligand binding domain; PDB, Protein Data Bank; PEPCK, phosphoenolpyruvate carboxykinase; NR, nuclear receptor; FFA, free fatty acid; RXR, retinoid X receptor.

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The LBD of human HNF-4α was crystallized with both fatty acid ligand and a coactivator peptide sequence derived from SRC-1.

**MATERIALS AND METHODS**

**Protein Production and Crystallization**—The protein boundaries for expression of the human HNF-4α LBD were defined by our previous structure of the rat apodomain (1) (the LBDs of rat and human HNF-4α are 97% identical). DNA encoding the LBD of human HNF-4α (residues 140–382) was subcloned into the pET 28a vector (Novagen) by PCR.

Protein was expressed in *Escherichia coli* BL21(DE3) (Invitrogen) cells and isolated from lysates using Talon cobalt affinity resin (Clontech). The His₆ affinity tag was cleaved using bovine thrombin (10 units/ml), and the protein was further purified by ion-exchange chromatography (Mono Q fast protein liquid chromatography). A 3-fold molar excess of SRC-1 peptide (SSLTERHKILHRLLQEGSPS, residues 681–700) was used for crystallization. Fatty acids are once again present in all of the crystals with one protein molecule in each asymmetric unit. The density map was well defined in the electron density map with the exception of side chains from residues of the α1-α3 loop.

| Data and refinement statistics |  |
|-------------------------------|---|
| **Native Data Statistics**     |  |
| Unit cell                     |  |
| a = b = 81.49 Å, c = 104.71 Å |  |
| Wavelength (Å)                | 0.970 |
| Resolution (Å)                | 29.9–2.1 |
| Unique reflections            | 20,187 |
| Redundancy                    | 12.10 |
| Completeness (%)              | 85.1 (76.7) |
| |  |
| **Refinement Statistics**     |  |
| Resolution (Å)                | 20–2.1 |
| R-factor (%)                  | 19.974 |
| R<sub>sym</sub>, 5% of data (%)| 22.6 |
| Non-H atoms per asymmetric unit | 1936 |
| Protein/peptide               | 16 |
| Water                         | 137 |
| (B)                           | 42.82 Å² |
| R.m.s. deviation,d             | 0.006 Å |
| Bond angles                   | 1.08° |
| Dihedral angles               | 18.92° |
| Improper angles               | 0.74° |

**RESULTS AND DISCUSSION**

**Domain Architecture**—The LBD of human HNF-4α is dimeric in the crystals with one protein molecule in each asymmetric unit. It adopts the canonical nuclear receptor LBD fold containing nine α helices and two β strands (Fig. 1A). The helices were numbered according to conventional NR nomenclature as we previously numbered the helices in the rodent protein (1). Helix α2, which is variably present in NR LBDs, is not present in HNF-4α. α6 consists of only one helical turn, and because α10 and α11 are contiguous, this helix is referred to simply as α10. The nine α helices are organized into three layers within the helical sandwich. Helices α4, α5, α8, and α9 form the central layer. Helices α1 and α3 comprise the outer layer at one side of the central layer, and helix α10 forms the opposite outer layer. Helix α12 is separate from α10, consistent with the HNF-4α LBD being in the active state and bound to coactivator. The dimerization interface, comprising residues from α9 and α10, lies along a plane of 2-fold crystallographic symmetry. At 2.1-Å resolution, the amino acid side chains are well defined in the electron density map with the exception of side chains from residues of the α1-α3 loop.

There are two obvious differences between the structures of the binary complex of the FFA-bound rat domain (Fig. 1B) reported earlier (1) and the ternary complex of the human domain reported here (Fig. 1A). The first is the presence of the SRC-1 peptide in one structure and not the other, which provides the opportunity to determine the effects of coactivator binding both on global structure and more specifically on the orientation of α12 (the AF-2 domain). The second difference, linked to the first, addresses the issue of open and closed configurations and what they mean. The LBD adopts two distinct conformations in the binary complex, despite both molecules being bound to fatty acid ligand (Fig. 1, B and C) (1). The first molecule is in an open conformation in which α10 and α12 are contiguous and colinear. This long helix is fully extended. The second molecule of the homodimer adopts a closed conformation with α12 situated against the body of the LBD. In the closed state, a hydrophobic patch on α12 (Leu-360, Leu-361, Met-364, and Leu-365) affixes it via hydrophobic interactions to the body of the domain. Residues from α3 (Met-182, Ala-183, Leu-186, Leu-187, Leu-189, and Val-190) and α4 (Leu-211, Ala-215, Gly-216, and Leu-219) form a complementary patch on the body of the domain. Both molecules in the ternary LBD complex (HNF-4α/FFA/SRC-1) adopt identical closed conformations (Fig. 1C). This structure is indistinguishable from that of the molecule in the closed conformation of the binary complex (Fig. 1C), yielding an root mean square deviation value of 0.57 Å when all of the Ca atoms of the two structures are superimposed. We have concluded from these comparisons that ligand (FFA) binding is conformationally permissive, because it facilitates the formation of the activated closed state but does not lock it. Coactivator binding, on the other hand, is restrictive because it apparently locks FFA-bound HNF-4α into the activated closed state.

**HNF-4α/Fatty Acid Interactions**—X-ray crystal structures of rat HNF-4α and human HNF-4γ indicated that both of these proteins bind fatty acids as natural ligands (1, 18). Because fatty acids incorporated spontaneously into the ligand binding pockets of the proteins during bacterial expression and because it was not possible to remove the fatty acids without at least partially denaturing the proteins, we had hypothesized that ligands, presumably fatty acids, might be critical to the stably folded domains (1, 18). Our new structure shows that human HNF-4α similarly sequesters bacterial fatty acids during protein production. Fatty acids are once again present in all of the molecules of the structure, despite not having been added intentionally.

The ligand binding pocket forms a narrow channel that is lined almost exclusively with side chains of hydrophobic resi-
dues (Fig. 2). The side chain of Arg-226 at the base of the binding pocket is the exception. Considerably smaller than the ligand binding pockets of other reported NR LBDs, HNF-4/H9251 has a cavity area of 370 Å³ as calculated by the program Voidoo (23). This is the approximate molecular volume of a long chain fatty acid. The fatty acids bound to our structure are anchored in the ligand binding pocket via an interaction between the fatty acid headgroup and the side chain of Arg-226. Both of the oxygen molecules in the fatty acid headgroup interact with

**FIG. 1. Structures of HNF-4α.** A, ribbon diagram of the ternary complex of HNF-4α bound to fatty acid ligand and SRC-1 coactivator peptide. Helices α1-α9 are colored turquoise, α10 is yellow, and α12 is in red. Bound fatty acid ligand is colored magenta, and the SRC-1 peptide is dark blue. B, ribbon diagram of the binary complex of HNF-4α bound to fatty acid ligand. Elements of secondary structure and the fatty acid ligand are colored as in A (1). C, structure so that the HNF-4α LBD homodimers are superimposed with the binary HNF-4α-FFA structure colored turquoise and the ternary HNF-4αFFA-SRC-1 structure in magenta.

**FIG. 2. The ligand binding pocket of HNF-4α.** The structures of binary (HNF-4α-FFA) and ternary (HNF-4α-FFA-SRC-1) complexes of the LBD are superimposed. The internal contours of the ligand binding pockets are essentially identical and shown in gray with the carboxyl-terminal portions of α10 deleted for clearer viewing of the pocket. Residues that interact with the fatty acid ligand are depicted as sticks and colored blue or orange for the binary or ternary complexes, respectively. Fatty acids present in each structure are similarly colored green or pink for the binary or ternary complexes, respectively. Residues labeled in the figure arise from HNF-4α3 (Ile-175, Val-178, Cys-179, and Met-182), α5 (Leu-219, Leu-220, and Ala-223), β1 (Leu-236), α7 (Met-252, Val-255, and Ile-259), α10 (Met-342, Ile-346, and Ile-349), and the α5/β1 (Arg-226) and α10/α12 (Ile-357) loops.

**FIG. 3. SRC-1 coactivator peptide interactions.** For clarity, only those helices of HNF-4α (turquoise) and SRC-1 (green) that interact directly are depicted. Side chains (magenta) of residues from HNF-4α and the SRC-1 peptide (LXXLL) form a hydrophobic patch at the interaction interface. Charge clamp residues Glu-363 and Lys-194 from HNF-4α are similarly displayed.
the arginine guanidinium group, and one oxygen interacts as well with the backbone NH group of Gly-237. The ligand interaction is further stabilized by hydrophobic and Van der Waals interactions between the fatty acid carbon chain and residues lining the pocket. Residues that participate in these interactions include Ile-175, Val-178, Cys-179, Met-182, Leu-219, Leu-220, Arg-223, Leu-236, Met-252, Val-255, Ile-259, Met-342, Ile-346, Ile-349, and Ile-357 (Fig. 2).

Because our previous structure was of the binary FFA-bound LBD complex and our new structure is of the ternary HNF-4α-FFA-SRC-1 complex, we are in a position to compare ligand binding pockets in the absence and presence of bound coactivator. Improved electron density for the ternary complex allowed visualization of 14 carbons of the fatty acid, as opposed to the 12 carbons seen in the binary complex (1). Improved electron density similarly facilitated the identification of additional water molecules in the ligand binding pocket of the ternary complex. Otherwise, there appear to be no significant differences (Fig. 2). As noted previously, backbone Ca atoms are superimposable. Amino acid side chains that line the pockets are in similar positions in the two structures and maintain the same interactions, including the salt bridge between the side chain of Arg-226 and the fatty acid head. The trivial differences in fatty acid binding between the two structures can thus be accounted for by the enhanced resolution of the ternary structure, solved at a 2.1-Å resolution as opposed to a 2.8-Å resolution.

**HNF-4α/SRC-1 Interactions**—As a recurring structural theme for nuclear receptors, bound coactivators form α-helices with hydrophobic side chains of the LXXLL motif directed inward to interact with the LBDs (24). The HNF-4α-FFA-SRC-1 complex recapitulates this mode of interaction (Fig. 3). Of 20 residues in the crystallized SRC-1 peptide, 14 at the carboxyl terminus are well defined. The amino-terminal six residues are not visualized in the electron density map. Eight residues (ILHRLLQEG) encompassing the LXXLL motif form ≥2 full turns of an α-helix. The HNF-4α/SRC-1 interaction is dominated by hydrophobic interactions involving the leucine residues of the LXXLL motif and the canonical “charge clamp” created by hydrogen bonds between backbone atoms and the side chains of invariant residues Glu-363 of α12 and Lys-194 of α3. The interaction with Gly-363 requires that α12 be folded into place in the ligand binding pocket and thus mandates that coactivator binding occur only with the closed LBD conformation.

The hydrophobic face of the LXXLL helix is packed into a hydrophobic pocket created by residues in α3 (Leu-187, Val-190, and Lys-194), α3-α4 loop (Phe-199), α4 (Leu-211), and α12 (Glu-363). The charge clamp orients the LXXLL motif into place, which explains why modes of interactions are well conserved between NRs and coactivators. Subtler interactions are thought to govern specificity and relative affinity of a given NR for different coactivator LXXLL motifs. These may be mediated by flanking residues at the carboxyl-terminal end of the LXXLL helix (25, 26). In our structure, the amino-terminal flanking region of the SRC-1 peptide is disordered, whereas the carboxyl-terminal flanking region is well defined but lacks specific interactions with the LBD.

**Helix α12 Structural Changes**—Because ligand-bound HNF-4α adopts both closed (active) and open (inactive) conformations (1), whereas the SRC-1-bound LBD adopts only the closed conformation, α12 does not appear to be a ligand-dependent switch that upon ligand binding triggers a structural rearrangement, providing a surface for coactivator binding (27, 28). Moreover, α12 does not participate in direct interactions with the ligand as is seen in the interactions of other NRs with many synthetic ligands. Therefore, it appears that both ligand and co-activator binding are required to lock HNF-4α in the closed and active state.

**Comparisons between Unliganded NRs and Binary and Ternary Complexes**—The first apodomain structure to be determined was that of RXXR (29). Comparisons between the apodomain and structures of the binary ligand/RXXR and ternary ligand/coactivator/RXXR complexes formed the basis for developing a global theory regarding structural determinants in NR activation. The theory predicted that apodomains are open and that ligand binding induces a conformational transition that stabilizes a closed active state with α12 (AF-2) folded against the body of the domain to form a coactivator binding surface (16, 17). The theory further predicted that although coactivator binding might further stabilize the closed conformation, it would not produce additional conformational changes. Because HNF-4α does not fit this scheme, we have re-examined the many structures that have now been solved. Of the four published apodomain structures (29–32), RXXR is the only one with α12 in the open conformation (Fig 4A). This indicates that, in the absence of ligand, α12 is conformationally flexible and can adopt both open and closed configurations. The predominance of the closed form seen in structural studies, in addition to data from thermal melt studies, suggests that the closed form of the apodomain may be energetically favored (33).

Three structures of binary complexes of LBDs bound to naturally occurring ligands are shown in Fig. 4B. RXXR is bound to 9-cis-retinoic acid (PDB 1FBY) (16), retinoid acid-related orphan receptor α is bound to cholesterol (PDB 1N83) (34), and the androgen receptor is bound to dihydrotestosterone (PDB 1I37) (35). LBDs in previously reported structures of binary ligand-bound complexes adopt the active closed conformation, supporting the theory that ligand binding serves as the activating switch. However, the finding that binary HNF-4α-FFA complexes adopt both open and closed conformations in our recently solved structure runs counter to the theory. In reexamining the structural basis for activation, found that many endogenous ligands in published structures do not interact with α12 directly (16, 34, 35). We conclude that although natural ligands undoubtedly stabilize the closed configuration, they may not lock the LBD into the active form. By contrast, ligands with larger appendages that do interact with α12 (36–39) may well lock the LBD into an activated conformation.

Structures of ternary ligand/coactivator/LBD complexes are profiled in Fig. 4C. The LBDs in each of these structures and in the ternary HNF-4α structure presented in this paper all adopt the active closed conformation. The coactivator charge clamp located on either side of the LXXLL motif interacts directly with α12 in each of these structures. Thus, coactivator binding requires that the LBD be closed and, because the LBD must remain closed as long as the coactivator is bound, this essentially “locks” the LBD in an active conformation. We conclude for HNF-4α that although ligand binding and coactivator binding both stabilize the LBD, it is coactivator binding that locks it in the active state. As far as we can tell from available structures of other LBDs bound to endogenous ligands and coactivators, the same holds, i.e. the binding of natural ligands and coactivators stabilize the LBD but coactivator binding locks the active state. Synthetic ligands with bulky appendages that interact with α12 may have the added ability to lock LBDs in the active conformation.

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FIG. 4. Comparisons of nuclear receptor LBD conformations. A, crystal structures are of unliganded apodomains from hRXRα (PDB 1LBD) (29), hPXR (PDB 1ILG) (30), hPPARγ (PDB 3PRG) (31). B, crystal structures of binary LBD complexes containing endogenous ligands, including the RXR α/9-cis retinoic acid complex (PDB 1FBY) (16), androgen receptor (AR)/dihydrotestosterone (DHT) complex (PDB 1I37) (35), and the ROR α/cholesterol complex (PDB 1N83) (34). C, crystal structures of ternary LBD/ligand/coactivator complexes, including hRXR/retinoic acid/SRC-1 (PDB 1FM6) (41), hPXR/SR12813/SRC-1 (PDB 1NRL) (33), and PPARγ/GW409544/SRC-1 (PDB 1K7L) (40). The LBDs were aligned in roughly similar orientations.
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