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Solution NMR Studies of the Ligand-Binding Domain of an Orphan Nuclear Receptor Reveals a Dynamic Helix in the Ligand-Binding Pocket

Nicolas Daffern¹, Zhonglei Chen¹, Yongbo Zhang², Leslie Pick³, and Ishwar Radhakrishnan^{1,*}

¹Department of Molecular Biosciences, Northwestern University, Evanston, IL 60208

²Department of Chemistry, Northwestern University, Evanston, IL 60208

³Department of Entomology, University of Maryland, College Park, MD 20742

Abstract

The ligand-binding domains (LBD) of the NR5A subfamily of nuclear receptors activate transcription via ligand-dependent and ligand-independent mechanisms. The *Drosophila* Ftz-F1 receptor (NR5A3) belongs to the latter category and its ligand-independence is attributed to a short helical segment ($\alpha 6$) within the protein that resides in the canonical ligand-binding pocket (LBP) in the crystalline state. Here, we show that the $\alpha 6$ helix is dynamic in solution when Ftz-F1 is bound to the LxxLL motif of its cofactor Ftz, undergoing motions on the fast (picosecond-nanosecond) as well as slow (microsecond-millisecond) timescales. Motions on the slow timescale (ca. 10^{-3} s) appear to pervade through the domain, most prominently in the LBP and residues at or near the cofactor binding site. We ascribe the fast timescale motions to a solvent-accessible conformation for the $\alpha 6$ helix akin to those described for its orthologs in higher organisms. We assign this conformation where the LBP is ‘open’ to a lowly-populated species while the major conformer bears the properties of the crystal structure where the LBP is ‘closed’. We propose that these conformational transitions could allow binding to small molecule ligands and/or play a role in cofactor dissociation from the binding site. Indeed, we show that Ftz-F1 LBD can bind phospholipids, not unlike its orthologs. Our studies provide the first detailed insights into intrinsic motions occurring on a variety of timescales in a nuclear receptor LBD and reveal that potentially functionally significant motions pervade the domain in solution, despite evidence to the contrary implied by the crystal structure.

Introduction

Nuclear receptors (NRs) constitute one of the largest families of transcription factors in metazoans that play key roles in regulating a broad range of biological processes that impact on growth, development, reproduction, and homeostasis.^{1–5} A distinctive feature of the NR family of transcription factors is that many receptors are inducible factors that upon binding

*To whom correspondence should be addressed: Department of Molecular Biosciences, Northwestern University, 2205 Tech Drive, Evanston, IL 60208-3500; Tel: +1 847-467-1173; Fax: +1 847-467-6489; i-radhakrishnan@northwestern.edu.

Supplementary Information. Five supplementary figures and figure captions are available in this section.

to chemical ligands activate or repress transcription through an allosteric mechanism that involves changes in the conformation of a C-terminal helix in the ligand-binding domain (LBD).^{1, 6}

The *Drosophila* Ftz-F1 receptor belongs to the NR5A subfamily whose members are found in diverse eumetazoans.⁷ Subfamily members bind DNA as monomers and function as strong transcriptional activators in a broad range of cell types.^{8–18} *Drosophila* Ftz-F1 plays a critical role in establishing the segmented body plan in the embryo, as *ftz-f1* mutations cause pair-rule segmentation defects, similar to those described for *ftz* mutants.^{19–21} Although Ftz-F1 is expressed in all somatic cells, its effects on transcription are confined to alternating ‘stripes’ of cells where the pair-rule homeodomain-containing gene product Ftz is also expressed. The proteins activate transcription in a synergistic manner through a mechanism involving cooperative assembly on the DNA and direct protein-protein interactions.^{19, 22}

Early studies suggested that members of the NR5A subfamily were *bona fide* orphan receptors, activating transcription in a ligand-independent manner. The idea was reinforced by the crystal structure of murine liver receptor homologue 1 (LRH1) LBD that featured an empty ligand-binding pocket (LBP) and the C-terminal helix locked in an active conformation, ready to engage with coactivators.²³ Unexpectedly, the crystal structures of the murine and human versions of steroidogenic factor 1 (SF1) and human version of LRH1 revealed serendipitously co-purified phospholipid ligands in their large LBPs with the lipophilic moieties filling the pocket and the charged head groups at the periphery.^{24–28} Unlike in the case of murine LRH1, pocket mutations in these receptors adversely impacted transcriptional activity and recent evidence suggests that lipids such as phosphoinositides (PIP₃) and phosphatidylcholines (DLPC) serve as cognate ligands for these receptors, underscoring the diversification in the mechanisms of activation by members of this subfamily.^{29–32}

Crystallographic analyses of *Drosophila* Ftz-F1 LBD bound to the LxxLL peptide of its co-factor Ftz further revealed the extent of diversification.³³ The Ftz-F1 LBP is occupied by a helical segment within the domain, thereby precluding the binding of a small molecule ligand to the pocket. It also features the C-terminal helix locked in an active conformation and thus meets all the criteria for a *bona fide* orphan receptor. Also, non-conservative substitutions in this helix ($\alpha 6$) adversely affected transactivation, implying that an occupied LBP is a requisite for normal Ftz-F1 function. Unlike in *Drosophila* Ftz-F1, the $\alpha 6$ helix normally resides on the surface of the protein on one side of the LBP in both SF1 and LRH1 in higher organisms. Removal of the Ftz-F1 $\alpha 6$ helix *in silico* creates sufficient space to accommodate phospholipids in the same conformation as the one found in LRH1/SF1 complexes,^{24–32} implying that changes within the helix played a vital role in the functional diversification of this subfamily during evolution.

Few detailed studies of NR LBDs in solution by NMR have been reported, with almost all previous studies focusing on LBD interactions with other NRs or with small molecule ligands.^{34–40} Here, we describe the conformation and dynamics of *Drosophila* Ftz-F1 LBD in solution. We show that the $\alpha 6$ helix exhibits motions on a wide range of timescales, belying the static nature of this segment implied by the crystal structure.

Experimental Details

Production of the wild-type and mutant Ftz-F1 LBDs, Ftz LxxLL peptide and mouse SF1 LBD

The coding sequence for *Drosophila melanogaster* Ftz-F1 LBD (residues 785-1027) was amplified by PCR, cloned into the pMCSG7 expression vector,⁴¹ sequenced to verify identity, and expressed in *E. coli* BL21(DE3) cells at 16 °C. After 20 h, the cells were harvested, resuspended in lysis buffer (50 mM Tris, pH 8.5, 200 mM NaCl, 1 mM BME, 0.5 mM TCEP, 5 mM Imidazole, 0.1% Triton X-100) and lysed by sonication for 30 min. The lysate was clarified by centrifugation at 12,000 rpm for 25 min and the soluble supernatant was incubated with a Ni²⁺-NTA resin (Qiagen), washed with buffer (50 mM Tris, pH 8.5, 200 mM NaCl, 1 mM BME, 0.5 mM TCEP, 5 mM imidazole) and eluted with the same buffer containing 250 mM imidazole. Eluted fractions of the protein were dialyzed overnight at 4 °C against the lysis buffer lacking detergent and imidazole and incubated in the presence of 1:20 (w/w) TEV protease:protein to simultaneously remove the His₆-tag. The dialysate was either concentrated and subjected to size exclusion chromatography or was incubated with Ni²⁺ resin and the flow-through collected. The sample was then dialyzed into the appropriate buffers and concentrated for further studies.

The G913Y Ftz-F1 LBD mutant was produced by mutating the expression vector encoding the wild-type protein using the QuikChange site-directed mutagenesis protocol (Agilent Genomics). Following verification of the DNA sequence, the mutant protein was expressed and purified using a similar protocol as the wild-type protein.

The *Drosophila melanogaster* Ftz LxxLL peptide corresponding to residues 103-119 (EERPSTLRALLTNPVKKY) was synthesized using automated methods (University of Utah Peptide Facility) and purified by reversed phase HPLC. The sequence contains a non-native tyrosine residue at the C-terminus to facilitate concentration measurements. The identity of the peptide was verified by mass spectrometry.

The mouse SF1 LBD (residues 221-462) for phospholipid binding assays was produced using the same approach as described above for the *Drosophila* Ftz-F1 LBD.

Fluorescence Thermal Shift (FTS) assays

FTS assays with the wild-type and the G913Y LBD mutant were performed on a Bio-Rad IQ5 Real-Time PCR machine at a protein concentration of 1.8 μM in the absence or presence of 50 μM Ftz LxxLL peptide in 20 mM sodium phosphate buffer (pH 7.2) containing 50 mM NaCl, 1mM TCEP, and 5x SYPRO Orange dye. Fluorescence data were acquired by varying the temperature from 25 °C to 95 °C at the rate of 0.5 °C/s. Data were processed using scripts written in R developed in-house.

NMR sample preparation and NMR spectroscopy

Uniformly ¹⁵N and/or ¹³C-labeled samples of wild-type and mutant Ftz-F1 LBD were prepared as described above except cells were grown in media with U-¹⁵N-ammonium sulfate and/or U-¹³C-glucose (Cambridge Isotopes) as the sole nitrogen and carbon sources;

isotope incorporation was verified by mass spectrometry. The sample concentration for NMR studies were in the 0.4-0.7 mM range and the samples were prepared in 20 mM sodium phosphate buffer (pH 6.8) containing 50 mM NaCl, 1 mM TCEP, and 0.5 mM EDTA. NMR data were acquired principally on an Agilent DirectDrive 600 MHz spectrometer equipped with a cold probe at 29 °C. NMR data were processed using Felix 98.0 and analyzed using NMRFAM-Sparky. Backbone assignments were accomplished manually using TROSY versions of 2D ^1H - ^{15}N HSQC, 3D HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HNCO, and ^{15}N -edited NOESY-HSQC (all part of the Agilent BioPack library).

CPMG relaxation dispersion measurements⁴²⁻⁴⁴ were performed for wild-type Ftz-F1 LBD at field strengths of 14.1 and 16.45 T at 29 °C; a separate measurement was conducted at 24 °C at 14.1 T. In addition to a reference spectrum, spectra with a constant time CPMG delay (T_{CP}) of 40 ms were recorded with the following pulsing rates: 50, 100 (x2), 150, 200, 300, 400 (x2), 600, 800, 1000 Hz (at 14.1 T) and 50 (x2), 150, 250, 350, 450, 550, 650, 750, 850 Hz (16.45 T). Spectra were acquired with a recycle delay of 2.5 s to mitigate the effects of heating from radiofrequency pulses; compensatory pulses were applied prior to the recycle delay to maintain similar levels of heating in all the spectra. Experiments with different pulsing rates were acquired in an interleaved mode in randomized order to minimize variation in experimental conditions. Peak heights were measured in NMRFAM-SPARKY⁴⁵ and reformatted for analysis using the relax software suite.^{46, 47}

The $R_{2,\text{eff}}$ dispersion curves were evaluated for consistency across all three data sets. The curves were fitted using the relax software suite assuming no exchange, 2-site fast exchange,⁴⁸ and 2-site slow exchange analytic models;⁴⁹ the best model was chosen based on the Akaike Information Criterion.^{46, 47} The curves were fit after clustering spatially proximal residues (local clustering) followed by global clustering of all exchanging residues. The goodness-of-fits between the various clustering schemes were evaluated by comparing χ^2 values. Monte Carlo simulations were used to model uncertainties in the fitted values.

$\{^1\text{H}\}$ - ^{15}N heteronuclear Overhauser effect (NOE) experiments were conducted at 14.1 T following the approach of Ferrage et al.⁵⁰ Experiments were performed in triplicate with a recycle delay of 6.5 s (either greater than or ca. $5 \times ^{15}\text{N}$ T_1). To minimize variation in experimental conditions, the reference and steady-state experiments were performed in an interleaved mode. ^1H pulses in the steady-state experiment were centered on the $^1\text{H}^{\text{N}}$ region (8.2 ppm) and applied at a lower power ($\gamma B_1/2 \pi = 9$ KHz) over 4 s of the recycle delay; water magnetization was carefully preserved through flip-back pulses in the reference experiment. Peak heights were measured in NMRFAM-SPARKY⁴⁵ and uncertainties in the measurements were propagated to the calculated NOE values.

Phospholipid binding assays

PIP Strips™ (Echelon Biosciences) were incubated for 1 h at room temperature with 20 mM Tris buffer (pH 7.6), 150 mM NaCl, and 0.1% (v/v) Tween 20 (TBST buffer) containing 1% (w/v) casein hydrolysate (Sigma). Purified His₆-tagged Ftz-F1 and SF-1 LBD proteins were then incubated with the strips in the same buffer at room temperature for 1 h. Bound protein was detected following incubation for 1 h each with anti-His₆ and anti-mouse IgG

antibodies. All incubation steps were followed by three washes with TBST buffer. The strips were imaged using a SuperSignal™ chemiluminescence kit (Thermo Fisher) and detected with a Syngene PXi imager.

Results and Discussion

Solution conformation of the Ftz-F1 LBD in the apo- and Ftz-bound states

The ^1H - ^{15}N correlated spectra of the apo- and Ftz LxxLL peptide-bound Ftz-F1 LBD were characterized by generally well-dispersed resonances and narrow resonance linewidths, although TROSY spectra yielded much higher sensitivity than the HSQC counterpart, consistent with the ~28-30 KDa size of the protein/protein-peptide complex (Figure 1A). The apo-Ftz-F1 LBD was not soluble for extended periods of time, precipitating over a ~24 h period, and hence our analyses were largely confined to the peptide-loaded form of the LBD (but see below). Out of 236 expected backbone correlations, 219 were detected. Of these, 209 were assigned sequence-specifically via standard triple-resonance approaches, 7 could not be assigned (due to poor magnetization transfers caused by resonance broadening effects), while 3 belonged to residues in a minor conformation (due to *cis-trans* proline isomerization) near the N-terminus of the protein. Most of the undetectable and unassignable resonances mapped to the C-terminal region of $\alpha 3$, the loop connecting $\alpha 3$ and $\alpha 4$, the C-terminus of $\alpha 5$, and the N-terminus of $\alpha 6$.

Analysis of the $^{13}\text{C}^\alpha$ secondary chemical shifts hinted at a backbone conformation for the LBD that is consistent with the crystal structure (Figure 1B). To quantitatively assess the backbone conformation of Ftz-F1 LBD, the $^1\text{H}^\text{N}$, amide ^{15}N , $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$ and $^{13}\text{C}'$ chemical shifts were analyzed using TALOS+ and predictions of ϕ and ψ torsion angles that were deemed reliable, were compared against those found in the crystal structure (Supplementary Figure S1). Except for a few residues located between regular secondary structural elements, the ϕ/ψ predictions were consistent with the crystal structure, including residues in helix $\alpha 6$, implying that the conformation at the backbone level in solution resembles the one in the crystal.

Since no high-resolution structure of the apo-form of Ftz-F1 LBD has yet been described, we asked whether binding to the Ftz LxxLL peptide is accompanied by any major conformational changes in the domain. Although apo-Ftz-F1 LBD is not soluble for extended periods as noted above, the vast majority of resonances could be assigned sequence-specifically using sparse triple-resonance data in conjunction with the assignments that were obtained for the holo-protein. A comparison of the backbone amide chemical shifts of the two states revealed that the most significant chemical shift perturbations involved a contiguous surface formed by residues within helices $\alpha 3$, $\alpha 4$, and $\alpha 12$ (the C-terminal helix) that together constituted the LxxLL peptide-binding surface (Figure 2). Relatively smaller perturbations could be detected for residues in helices $\alpha 5$, $\alpha 8$, $\alpha 9$ and $\alpha 12$ that form the 'secondary shell' by making contacts with residues in helices $\alpha 3$, $\alpha 4$, and $\alpha 12$. By contrast, residues in the loop connecting helices $\alpha 11$ and $\alpha 12$ as well as those in direct contact with these residues at the N-terminus of $\alpha 3$, showed little or no changes. Collectively, these results imply that the C-terminal helix ($\alpha 12$) does not undergo any

significant conformational changes upon LxxLL peptide binding and that it adopts the 'active' conformation in the apo-state, poised to bind to its cofactor.

Intrinsic motions in the LBD on the microsecond-millisecond (μ s-ms) timescale

Close inspection of the ^1H - ^{15}N correlated spectrum of the peptide-bound Ftz-F1 LBD revealed several broadened resonances with diminished intensities compared to other correlations (Supplementary Figure S2). Taken in conjunction with the absence of or severe resonance broadening for several correlations, we asked whether slow timescale motions were prevalent within the domain. Towards this end, backbone ^{15}N spin CPMG relaxation dispersion experiments^{42–44} were performed at 14.1 and 16.45 T at 29 °C. Whereas most residues showed no readily discernible, systematic variation in the effective R_2 rates as a function of CPMG pulse repetition rate, a subset of residues showed evidence of dispersion (Figure 3). These residues map to several different regions in the domain.

A visual analysis reveals an unusual concentration of residues affected by exchange in and around the $\alpha 6$ helix (Figure 4), including many that contribute to the canonical LBP. These include residues in the $\alpha 2$, $\alpha 3$, and $\alpha 5$ helices as well as the loop connecting $\alpha 5$ with the β -hairpin. The affected residues extend to the β -hairpin and the C-terminus of the $\alpha 2$ helix as well as the loop that follows $\alpha 2$. A similar clustering of residues can be seen for the segment spanning the C-terminus of $\alpha 3$ and the succeeding loop segment including the short $\alpha 3'$ helix and up to the beginning of $\alpha 4$; residues in close proximity to this segment at the C- and N-termini of the $\alpha 8$ and $\alpha 9$ helices, respectively complete the cluster (Figure 4). Two comparatively smaller clusters, one comprising residues near the N-terminus of the LBD and the C-terminus of $\alpha 9$, and the other made up of residues at the C-terminus of $\alpha 1$ and the N-terminus of $\alpha 8$, round out all the residues affected by exchange.

To gain insights into the timescale of exchange, the dispersion data for individual residues were initially fit to well-established two-site fast exchange and two-site slow exchange models. Virtually all the exchanging residues were best fit by a two-site fast exchange model. Because of the large uncertainties in the fitted values stemming from these individual fits, residues were assigned to one of the five clusters above and data for the residues in each cluster were simultaneously fit to the two-site fast exchange model. This yielded good fits with fitted values for the exchange rates that ranged from $2080 \pm 310 \text{ s}^{-1}$ to $3750 \pm 730 \text{ s}^{-1}$. The fits were deemed to be better (from a comparison of the resulting χ^2 values) when all five clusters were merged into a single cluster which yielded an exchange rate of $3530 \pm 90 \text{ s}^{-1}$ (Figure 3). To enhance the confidence in the measured rate, we conducted CPMG relaxation dispersion experiments at a lower temperature (24 °C) at 14.1 T. Following clustering, the data for all residues deemed to undergo exchange were fit simultaneously which yielded a diminished exchange rate of $1720 \pm 60 \text{ s}^{-1}$ (Supplementary Figure S3), in line with expectation. Collectively, these results suggest conformational transitions occurring faster than the millisecond timescale within the LBD but most notably involving the $\alpha 6$ helix and segments lining the canonical LBP.

Intrinsic motions in the LBD on the picosecond-nanosecond timescale

We then sought to gain insights into motions occurring at the backbone level on the picosecond-nanosecond (ps-ns) timescale by recording ^1H - ^{15}N heteronuclear NOE experiments.^{50, 51} As expected, NOE values are low in unstructured segments at or near the N-terminus (-0.02 ± 0.55 ; reported values are the average and standard deviation for each segment), the C-terminus (0.26 ± 0.41), and the loops between secondary structural elements including the long loops connecting $\alpha 2$ with $\alpha 3$ (0.65 ± 0.07) and $\alpha 11$ with $\alpha 12$ (0.43 ± 0.15), consistent with enhanced flexibility in these regions (Figure 5A). Rather unexpectedly, residues within the $\alpha 6$ helix and the segment immediately following it, both of which are embedded within the LBP exhibit reduced NOE values (0.63 ± 0.11). By contrast, the other helices exhibit NOEs close to the limiting value (0.79 ± 0.10), consistent with the enhanced order expected in these regions. Interestingly, the temperature factors (B-factors) for $\alpha 6$ in the crystal structure are somewhat low ($46.5 \pm 3.6 \text{ \AA}^2$) but comparable to the other helices ($51.0 \pm 8.8 \text{ \AA}^2$), belying any evidence of significant disorder (Figure 5B).³³

We note that two other regions, including the polypeptide segments at/near the N-terminus as well as the loop linking $\alpha 11$ and $\alpha 12$ also exhibit low NOE values (-0.02 ± 0.55 and 0.43 ± 0.15 , respectively) but moderate values for the B-factors ($51.6 \pm 4.0 \text{ \AA}^2$ and $50.3 \pm 2.7 \text{ \AA}^2$, respectively; Figure 5B).³³ However, in both cases, the respective segments engage in contacts with crystallographically related molecules and thus the apparent discrepancy between the solution NMR and crystallographic data can be readily rationalized. In contrast, there are no such intermolecular crystal contacts in the vicinity of the $\alpha 6$ helix. However, since our studies show that the $\alpha 6$ likely exists in two different conformations, the results can still be reconciled by invoking the possibility of ‘selection’ of the major conformer by the crystallization process with the low NOEs emanating from enhanced flexibility in this region in the minor conformer (note that all the NOE measurements and trends were reproduced using a completely different sample of the Ftz-F1-Ftz LxxLL peptide complex, so that the lower values for the $\alpha 6$ helix cannot be explained simply by a ‘random’ measurement error).

Is $\alpha 6$ embedded in the LBP in the major conformer?

Because of resonance broadening effects observed for residues in and around the $\alpha 6$ helix, which precluded direct ^1H - ^1H NOE-based interrogations of the precise location of the helix, we sought to answer this question by introducing a mutation in the vicinity of the helix and analyzing the resulting pattern of chemical shift perturbations. Towards this end, we engineered a glycine to tyrosine mutation of a solvent-exposed residue in $\alpha 7$ that was predicted by FoldX to have little or no effect on the stability of the domain.⁵² Although the G913Y mutant appeared to exhibit a slightly lower melting temperature compared to wild-type (45.2 ± 0.4 vs. $46.5 \pm 0.3 \text{ }^\circ\text{C}$; Supplementary Figure 4A), it bound to the Ftz LxxLL peptide efficiently and exhibited a very similar melting temperature as the wild-type in its presence (49.9 ± 0.9 vs. $49.6 \pm 0.3 \text{ }^\circ\text{C}$). The NMR spectrum of the mutant was also very similar to the wild-type in the presence of an equivalent amount of Ftz peptide, with what appeared to be mostly localized perturbations (Supplementary Figure 4B), consistent with the mutant sharing similar conformational features as the wild-type protein. This was confirmed by the lack of significant changes to the $^{13}\text{C}^\alpha$ chemical shifts for residues in the

vicinity (at the levels of primary and tertiary structure) of the mutation; the $^{13}\text{C}^\alpha$ shifts were deduced during the sequence-specific backbone resonance assignment process.

Backbone amide chemical shift deviations between the G913Y mutant and the wild-type revealed, as expected, significant changes for residues on either side of the site of mutation at the level of primary structure (Figure 6). However, significant chemical shift changes (>0.05 ppm), caused most likely by the aromatic side chain, are also noted for residues in $\alpha 6$ and, to a lesser extent, residues at the C-terminus of $\alpha 5$ and the $\alpha 5$ - $\beta 1$ linker region. This pattern would be consistent with and indeed expected if the major conformer resembled the conformation seen in the crystal. On the other hand, the pattern of chemical shift changes is inconsistent with the only other plausible conformation observed for $\alpha 6$ in the human and mouse NR5A receptors (Figure 6). Collectively, these results suggest that the major conformer in solution is most likely the conformation observed in the crystal with the $\alpha 6$ helix embedded in the LBP.

Implications of Protein Motions in Ftz-F1 LBD Function

A recurring theme from solution NMR studies of NR LBDs (all of which have focused exclusively on ligand-dependent receptors), is the localized or widespread resonance broadening in the apo protein, likely caused by slow timescale motions.^{35–40} Ligand binding leads to complete quenching of dynamics in some cases, as evidenced by the narrowing of linewidths and/or recovery of ‘missing’ resonances, but only partial quenching in others. Invariably, residues that comprise the LBP are affected by resonance broadening in the apo state, and it stands to reason that the slow timescale dynamics reflects the sampling of multiple conformational states to facilitate ligand binding.

Our studies of what is widely regarded as a *bona fide* orphan receptor in which the LBP is occupied by a helical segment ($\alpha 6$) within the protein reveals a surprisingly dynamic $\alpha 6$ helix with motions occurring on both the ps-ns and the μ s-ms timescales. The presence of slow motions was not expected because the receptor is not known to be regulated by small molecule ligands, which would require eviction of the helix and remodeling of the LBP. Therefore, what might be the purpose of such motions? One possibility is that these intrinsic motions could promote dissociation of the bound Ftz cofactor, not unlike the coupling noted previously between motional processes and the catalytic rate in enzymes.⁵³ Indeed, in our studies we find that the motions on the slow timescale are not just confined to $\alpha 6$ and segments in direct contact with the helix (*i.e.* residues that comprise the LBP); rather, they pervade through the domain, extending up to the cofactor binding surface, hinting at the presence of a communication network involving residue-level contacts that connects these sites, akin to those described previously for the orthologous LRH1 receptor.⁵⁴ Besides identifying regions affected by these motions, our studies yielded quantitative insights into the motional timescales with parsimonious models used for data fitting indicating exchange in the 10^{-3} s range.⁴⁸ This yields an association rate of ca. $10^9 \text{ M}^{-1} \text{ s}^{-1}$, since the equilibrium dissociation constant of the Ftz-F1 LBD-Ftz LxxLL peptide complex was previously measured to be ca. $1 \mu\text{M}$.³³ Because the inferred association rate is at the higher end of the spectrum,⁵⁵ we suggest that the μ s-ms timescale motions within the LBD may have a role in

the dissociation of the cofactor, although further studies are required to conclusively address this issue.

The detection of fast motions in the Ftz-F1 $\alpha 6$ helix is also unexpected. Although, the helix is short (5 residues long) and bears the hallmarks of both an α -helix and a 3_{10} -helix in terms of hydrogen bonding interactions,³³ how a secondary structural element deeply embedded in the hydrophobic core could undergo motions on the ps-ns timescale is confounding. However, since the LBD samples at least two distinct conformations, we surmise that the fast timescale dynamics and the resulting low values for the ^1H - ^{15}N heteronuclear NOEs for $\alpha 6$ most likely emanates from a second (minor) conformer (we note that the measured values for the NOEs are heavily skewed towards the major conformer, which we showed was consistent with the conformation observed in the crystal). The $\alpha 6$ helix in several NR5A orthologs is largely solvent-accessible and adopts a strikingly different conformation than the one described for *Drosophila* Ftz-F1.^{24–28} In crystal structures featuring NR5A receptors with an empty LBP, such as the apo human LRH1 LBD and the mouse LRH1 LBD, the $\alpha 6$ helix is characterized respectively by a lack of detectable density or by unusually high temperature factors, implying a dynamic helix.^{23, 54} We propose that the minor conformer for Ftz-F1 LBD in solution adopts an analogous conformation (Figure 7). In support of this model, the $\alpha 6$ helix and the segment leading to $\alpha 7$ has the sequence LMSLGLLGVP that features several residues that are poor helix formers, likely destabilizing the helix upon removal from the pocket, thereby contributing to the enhanced flexibility noted for this region in our studies.

To decisively test the aforementioned model, we conducted a well-established assay to assess whether Ftz-F1 LBD could bind to phospholipids (Supplementary Figure S5). If the $\alpha 6$ helix indeed spent time outside the LBP, this should allow ligands (such as phospholipids attributed to its orthologs in mouse and human) to enter and occupy the pocket. Consistent with our model, Ftz-F1 LBD bound to phospholipids previously shown to bind and function as *bona fide* ligands for its orthologs.^{29–32} Indeed, Ftz-F1 shows a strikingly similar preference for phospholipids as mouse SF1 LBD (Supplementary Figure S5). Given that the LBP has all the molecular determinants for binding phospholipids, we conclude that these lipids most likely bind to the pocket upon extrusion of the $\alpha 6$ helix.

Since the $\alpha 6$ helix in Ftz-F1 LBD samples two conformations as described above (Figure 7), a key question relates to how it might switch between the two states. Although additional studies are required to answer this question, we are intrigued by the high concentration of residues near/at the C-terminal region of $\alpha 2$, the $\alpha 5$ - $\beta 1$ loop and the β -hairpin that show evidence of exchange (Figure 4). We strongly suspect that these regions undergo correlated motions facilitating the switch from the major conformation to the minor conformation accompanied by the eviction of the helix from the LBP. Indeed, molecular dynamics simulations of liganded nuclear receptors have suggested that analogous regions of these receptors undergo localized changes in conformation for ligand entry and exit.^{57–59} Additionally, hydrogen-deuterium exchange data for the human LRH1 receptor also suggest conformational fluctuations on the seconds (or faster) timescale in these regions.⁶⁰

Ftz-F1 LBD shares a few striking parallels with that of another receptor. Nurr1 (NR4A2) was originally considered to be a *bona fide* orphan receptor based on the crystal structure that also showed an LBP occupied by $\alpha 6$.⁶¹ NMR studies later showed residues in $\alpha 6$ and adjacent regions to be affected by resonance broadening.³⁶ More recently, the receptor was shown to bind to unsaturated fatty acids, but unlike Ftz-F1, binding to ligand is necessary for efficient interactions with its coregulator.⁴⁰ Since our studies demonstrate that Ftz-F1 LBD can bind phospholipids, we surmise they may function to regulate the affinity for Ftz, as the two factors coordinately regulate transcription in *Drosophila*.^{7, 62} Importantly, these studies recommend caution while assigning the ligand-binding status of a receptor based solely on a crystal structure.

Nuclear receptors have evoked intense interest since their discovery, as these proteins bind to an extraordinarily diverse range of small molecules to control many aspects of development and cellular physiology, and have inspired many successful drug discovery programs. A key question in NR biology relates to their molecular evolution and functional diversification. In the prevailing view, many extant nuclear receptors are thought to have descended from an ancestral liganded receptor having undergone subtle changes to the LBP to accommodate diverse ligands while others evolved ligand-independence by acquiring mutations that stabilized the active conformation involving the C-terminal helix.⁶³ Members of the NR5A subfamily exhibit the full range of functional diversification described for the NR superfamily with most higher vertebrate NR5A receptors showing ligand dependence whereas arthropods were thought to be ligand-independent.^{23–33} Interestingly, some of the greatest variability within the subfamily at the sequence level lies in the vicinity of the $\alpha 6$ helix. The unexpected presence and extent of dynamics for the *Drosophila* receptor gleaned from our studies alludes to potential variation in the canonical mechanism of ligand binding and activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NR	nuclear receptor
LBD	ligand-binding domain
LBP	ligand-binding pocket

TROSY	transverse relaxation optimized spectroscopy
HSQC	heteronuclear single quantum coherence
NOE	nuclear Overhauser effect
NOESY	NOE spectroscopy
CPMG	Carr Purcell Meiboom Gill
LRH1	liver receptor homologue 1
SF1	steroidogenic factor 1

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Figure 1.

Conformation of Ftz-F1 LBD and its interaction with the Ftz LxxLL peptide. (A) ^1H - ^{15}N correlated spectra of apo (blue) and Ftz LxxLL-peptide bound Ftz-F1 LBD (magenta). Select backbone correlations that exhibit significant chemical shift changes upon peptide binding are annotated. (B) $^{13}\text{C}^\alpha$ secondary chemical shifts mapped on to the backbone conformation of the crystal structure (PDB accession: 2XHS)³³ with residues showing large, positive secondary shifts ($>+2.5$ ppm) characteristic of helical conformations colored in teal and those showing large, negative shifts (<-2.5 ppm) in magenta and those at near random coil values (0 ppm) in white; colors are linearly interpolated to reflect the corresponding values. Residues that were unassigned are colored in yellow.

Figure 2.

The Ftz-F1 LBD C-terminal helix is in an active conformation in the apo-protein. Backbone chemical shift perturbations caused by Ftz LxxLL peptide binding mapped on to the surface of the LBD (A) and to the polypeptide backbone (B) of the crystal structure.³³ Perturbations were calculated using the formula: $\delta_{H,N} = (((\delta_H)^2 + 0.04 * (\delta_N)^2) / 2)$. The intermediate and high values in the color key $< \delta_{H,N} > + 1\sigma$ (yellow) and $< \delta_{H,N} > + 3\sigma$ (magenta), respectively, where $< \delta_{H,N} >$ is the average value and σ is the standard deviation. Colors are linearly interpolated to reflect the corresponding values. Prolines and residues with unassigned resonances are colored in light blue.

Figure 3.

Microsecond-millisecond timescale motions in the Ftz-F1 LBD in complex with Ftz LxxLL peptide. Representative $R_{2,\text{eff}}$ dispersion curves as a function of CPMG pulse repetition rate from different parts of the domain (residue annotations are color ramped from blue to red to reflect their position within the domain). The measurements were performed at 14.1 T (blue symbols and lines) and 16.45 T (green symbols and lines). The fitted values are drawn as continuous lines and are the result of a fitting process that assumed a shared exchange rate for all the residues deemed to undergo exchange.

Figure 4.

Sites undergoing conformational exchange in the Ftz peptide-bound Ftz-F1 LBD. Residues are colored based on whether exchange could be quantified via CPMG relaxation dispersion experiments (purple) or could not be quantified but are suspected to undergo exchange as implied by severe or substantial resonance broadening (gold). Prolines and residues with overlapped resonances are colored in light blue.

Figure 5.

Picosecond to nanosecond timescale motions in the Ftz-F1 LBD in complex with the Ftz LxxLL peptide. Views of the LBD with (A) $\{^1\text{H}\}$ - ^{15}N heteronuclear NOEs and (B) crystallographic B-factors³³ mapped on to the polypeptide backbone. Unassigned residues, prolines, and residues for which NOE data could not be reliably quantified are shown in yellow. Colors and the corresponding values are as depicted by the color keys; colors are linearly interpolated between the values displayed in the keys.

Figure 6.

The major conformer in solution resembles the conformation captured in the crystal for *Drosophila* Ftz-F1 LBD in complex with the Ftz peptide. Chemical shift deviations ($\delta_{H,N}$) between the Ftz peptide-bound forms of the wild-type and G913Y mutant of Ftz-F1 LBD. The deviations are mapped on to the polypeptide backbone of the crystal structure (PDB accession: 2XHS; *left*)³³ and a homology model for the same sequence based on the crystal structure of the mouse LRH1 ortholog (PDB accession: 1YOK; *right*)²⁴. The computationally predicted location of the tyrosine side chain in the mutant is depicted for reference. Chemical shift deviations were calculated using the same formula as in Figure 2. The intermediate and high values in the color key represent $\langle \delta_{H,N} \rangle + 1 \sigma$ (yellow) and $\langle \delta_{H,N} \rangle + 3 \sigma$ (magenta), respectively. Colors are linearly interpolated to reflect the corresponding values. Prolines and residues with unassigned resonances are colored in light blue.

Figure 7.

A structural model for exchange between two states for the Ftz-F1 LBD in complex with the Ftz cofactor. Since no high-resolution structure for the open complex of Ftz-F1 LBD has been described, the structure was generated via homology modeling using the structure of the mouse LRH1 ortholog (PDB accession: 1YOK)²⁴ as the template in the SWISS-MODEL webserver.⁵⁶