Interaction of Transcriptional Intermediary Factor 2 Nuclear Receptor Box Peptides with the Coactivator Binding Site of Estrogen Receptor $\alpha$

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The activation function 2 ligand-dependent interaction between nuclear receptors and their coregulators is mediated by a short consensus motif, the so-called nuclear receptor (NR) box. Nuclear receptors exhibit distinct preferences for such motifs depending both on the bound ligand and on the NR box sequence. To better understand the structural basis of motif recognition, we characterized the interaction between estrogen receptor $\alpha$ and the NR box regions of the p160 coactivator TIF2. We have determined the crystal structures of complexes between the ligand-binding domain of estrogen receptor $\alpha$ and 12-mer peptides from the Box 2 and Box 3 regions of TIF2. Surprisingly, the Box 3 module displays an unexpected binding mode that is distinct from the canonical LXXLL interaction observed in other ligand-binding domain/NR box crystal structures. The peptide is shifted along the coactivator binding site in such a way that the interaction motif becomes LXXYL rather than the classical LXXLL. However, analysis of the binding properties of wild-type NR box peptides, as well as mutant peptides designed to probe the Box 3 orientation, suggests that the Box 3 peptide primarily adopts the “classical” LXXLL orientation in solution. These results highlight the potential difficulties in interpretation of protein-protein interactions based on co-crystal structures using short peptide motifs.

The estrogen receptor $\alpha$ (ER$\alpha$) is a ligand-activated transcription factor that mediates the biological effects of the steroid hormone estrogen. Like other nuclear receptors (NRs), ER$\alpha$ exhibits a characteristic modular domain organization that includes two autonomous transcriptional activation functions (AF1 and AF2) that regulate transcription through interactions with NR coregulators (1–3). AF1, which resides in the N-terminal region of ER$\alpha$, is constitutively active and regulated by growth factors (4–6). In contrast, AF2, which is located in the C-terminal ligand-binding domain (LBD) of ER, is entirely dependent on ligand for its activity.

In recent years, structural and functional studies of both the AF2 domain of ER$\alpha$ and associated coregulators have greatly enhanced our knowledge of ligand-dependent, ER-mediated transcriptional activation. A large number of coactivators have been isolated that primarily target the LBD of the receptor in a ligand- and AF2-dependent manner (7, 8). The most widely studied group of AF2 coactivators includes the p160 family of proteins (steroid receptor coactivator 1, TIF2/glucoorticoid receptor-interacting protein 1, and steroid receptor coactivator 3/AB1) (9–13) and the p300/cAMP-responsive element-binding protein-binding protein (14, 15). These factors possess intrinsic histone acetyltransferase activity and/or function in complexes with other acetyltransferases such as p300/cAMP-responsive element-binding protein-binding protein-associated factor (16, 17). They act to remodel chromatin through the regulation of histone acetylation status (18) and are therefore believed to influence promoter accessibility. The critical importance of p160 coactivators in ER signaling has been highlighted by recent knockout studies and the discovery of p160 gene amplification in ER-positive breast cancer (19, 20).

A common feature of all AF2 coactivators is that they contain one or more copies of a leucine-rich signature motif, referred to as the LXXLL motif (where X denotes any amino acid) or the NR box (7). p160 factors, such as TIF2, contain a central NR-interacting domain with three evenly spaced LXXLL motifs. Differences in the spacing of the LXXLL modules in combination with variable flanking sequences has been shown to account for the overall receptor affinity and in particular NR specificity (21, 22). p160 recruitment to ER is also influenced by the bound receptor ligand (23). Full-length ER$\alpha$ and the isolated ER$\alpha$LBD are able to interact with all three LXXLL motifs of TIF2 but have a distinct preference for the second motif (13).

Hormone binding to NRs induces structural rearrangements in the LBD/AF2 domain, resulting in the formation of a specific binding site for coactivators and other regulatory modulators (24). Structural studies with peptides corresponding to the LXXLL interaction motifs of p160 coactivators have demonstrated that the binding site, which maps to the AF2 region of the receptor, comprises a shallow groove on the surface of the LBD (25). The structure of a complex between the LBD of peroxisome proliferator-activated receptor $\gamma$ and a fragment of the NR-interacting domain of steroid receptor coactivator 1 containing two LXXLL motifs revealed that each module binds along the AF2 site as a helix so that each docking site on the receptor homodimer is occupied (26). A similar mode of NR box...
interaction has been observed with agonist-ligated thyroid and estrogen receptors (25, 27). AF2 antagonists sterically prevent the correct assembly of the AF2/NR box-binding region, thereby blocking receptor/coactivator interaction (25, 28).

In this study, we have taken a structural and biochemical approach to further investigate the interactions between ERα and the LXXLL motifs of TIF2.

**EXPERIMENTAL PROCEDURES**

**Proteins and Peptides**—Recombinant human ERα (1–595) (>95% purity) was obtained from PanVera, and recombinant human ERβ (residues 301–553) (>95% purity) was provided by Karo Bio AB (Huddinge, Sweden), respectively. N-terminally biotinylated peptides (95% purity) were purchased from Interactiva (Ulm, Germany). The human TIF2 LXXLL peptide sequences were as follows: Box 1 (residues 95–110), KGQTKLLQLLTTKS; Box 2 (residues 685–698), EKHKILHRLLQD; Box 3 (residues 740–753), KEKALLRYLLDKDD. The mutant peptide sequences were as follows: Box 2/4Y, EKHKILHRLLQD; Box 3/7K, KEKALLRYLLDKDD; Box 3/5Q, KENALLRYLQDKDD; Box 3/3K/1H, KENALRHYLQDD; Box 3/3K/1H/5Q, KENALRHYLQDKDD; Box 3/3H, KENALRHYLQDKDD; and Box 3/3Q, KENALRHYLQDKDD. The 12-mer peptides used for co-crystallization had the following sequences: Box 2, EKHKILHRLLQD; Box 3, KEKALLRYLLDKDD and had unmodified N and C termini.

**Structure Determination**—Human ERα LBD (residues Ser301-Thr553) was expressed, purified, and carboxymethylated as described previously (30). The RALcore and E2-ligated LBDs were prepared by the inclusion of 75 μM of the respective ligand in the E2-Sepharose column elution buffer. Prior to crystallization, peptide complexes were prepared by the addition of peptides at a peptide to LBD ratio of either 1:1 (Box 2/RALcore) or 5:1 (Box 3/E2) followed by incubation at 18°C for 12 h. The crystals were grown using the hanging drop vapor diffusion technique at 18°C.

**Data Collection, Phasing, and Refinement**—Diffraction data were collected in-house (Box 2) and at the European Synchrotron Radiation Facility (Box 3) to a maximum resolution of 2.45 and 2.4 Å, respectively. RALcore/Box 2 crystals were cryoprotected by sequential transfer to 25% to 5% ethylene glycol in 5% steps. E2/Box 3 crystals were transferred to 30% (v/v) glycerol prior to freezing. The data were recorded using either a MAR345 image plate (Box 2) or ADSC Quantum4 CCD detector (Box 3) and indexed, reduced, and scaled using the HKL suite of programs (31).

The structures of the hERα-RALcore/Box 2 and hERα-E2/Box 3 were solved using molecular replacement. In both cases, a single LBD dimer was located within the crystallographic asymmetric unit using the coordinates of a hERα-LBD-E3 dimer (Protein Data Bank code 1ERE) (28) as a search model in AMoRe (32). Initial electron density maps, calculated after rigid body refinement, clearly indicated the position of the bound NR box peptides. Both complexes were refined with REFMAC, version 4.0 (33), using all available data with no sigma cut-offs. Bulk solvent contributions, calculated in XPLOR, version 3.843 (34), were incorporated in the form of partial structure factors. All model building was carried out in the molecular graphics package QUANTA (Accelrys, San Diego, CA). Water molecules that made at least one hydrogen bond to the protein were included as long as their B values remained below 70 Å². The details of crystallization conditions, data collection, and refinement statistics are given in Table I.

The atomic coordinates and structure factors for the hERα-RALcore/Box 2 peptide and hERα-E2/Box 3 peptide complexes have been deposited in the Protein Data Bank (www.rcsb.org; accession codes 1GWR (hERα-RALcore/Box 2) and 1GWR (hERα/E2/Box 3)).

**Surface Plasmon Resonance (SPR) Analyses**—The measurements were performed using a BiAcore 2000 (BIAcore AB, Uppsala, Sweden). All of the experiments were performed at 25°C in a binding buffer comprising 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20. Research grade streptavidin sensor chips were obtained from BIAcore AB. The streptavidin chips were first treated with three 1-min pulses of 50 mM NaN3 and 1 mM NaCl at a flow rate of 5 μl/min. Biotinylated peptides were immobilized on individual surfaces to variable responses (20–150 RU). Samples of full-length ERα or ERβ-ligated with 17β-E2 were then injected over each surface and a control surface with no peptide bound. The chip surfaces were regenerated down to the peptide level by applying two or three 1-min pulses of 10 mM NaN3. Kinetic and affinity determinations were performed using the BiAevaluation software 3.0 (BIAcore AB). Different binding models (different rate equations) were tested in the global curve fitting procedure, and the model best describing the experimental data was a conformational change model (21).

**RESULTS**

**Structures of ERαLBD/LXXLL Peptide Complexes**—To examine the structural basis of ER AF2/LXXLL recognition, we co-crystallized ERαLBD with two 12-amino acid peptide derived from the second (Box 2) and third (Box 3) NR box regions of TIF2. To facilitate crystal growth we screened a range of liganded LBDs and found that a different agonist/ER combination yielded crystals depending on the peptide used. The resultant NR Box 2 and NR Box 3 co-crystals diffract to around
Estrogen Receptor-TIF2 LXXLL Interactions

FIG. 1. Interaction of NR box peptides with ERαLBD. A, close view of the TIF2 Box 2 peptide bound to ERαLBD. The molecular surface of the coactivator binding groove is shown and colored according to electrostatic potential. Red, negative charge; blue, positive charge. The Cα trace of the TIF2 peptide is represented by the purple coil. For clarity, only the side chains of the three leucines of the LXXLL module (Leu690, Leu693, and Leu694) along with the preceding isoleucine (Ile689) are shown. The peptide residues are numbered based on their sequence position relative to the LXXLL motif. B, schematic representation of the interactions made by the Box 2 peptide. The residues that form the LXXLL binding site on the surface of ERαLBD are shown in their approximate positions. The hydrogen bonds are depicted as dotted lines. The van der Waals’ contacts are shown as radial arcs around the relevant residues of the peptide, and spokes point toward the LBD residues with which they interact. C, close view of the TIF2 Box 3 bound to ERαLBD (see A for details). The side chains of Ala1143, Leu1146, Tyr1147, and Leu1148 of the Box 3 motif are depicted. D, panel i, structural alignment of the Box 2 and Box 3 sequences based on their respective binding modes. Amino acids shown as lowercase letters represent residues that are disordered in the two co-crystal structures. The leucine residues of the LXXLL motifs are circled. The residues that interact with ERαLBD are boxed. Both sequences are numbered according to standard conventions (see text for details). Panel ii, Sequences of wild type (WT) and mutant Box 3 peptides used for BIACore binding studies.

2.4 Å resolution, and their structures were determined by molecular replacement (Table I).

In both complexes, the asymmetric unit contains a single noncrystallographic LBD dimer that is identical in structure to other ERαLBD agonist complexes (25, 28). Each LBD displays the canonical NR fold composed of 12 α-helices (H1–12) (35). The coactivator binding site of each LBD monomer, formed by residues from helices H3, H4, H5, and H12, is occupied by peptide. Although the electron density for the NR Box 2 and Box 3 peptides is clear and continuous, not all of the peptide motif was visible. In each case, only nine residues of 12 could be defined, and the remaining residues were disordered. The ordered residues form two turns of α-helix that encompasses the core hydrophobic motif and two flanking residues at either end (peptide numbering −2 to +7). By convention, the residues in the coactivator peptide are numbered so that the first leucine of the LXXLL motif represents the +1 position. Residues prior to the core consensus sequence are designated by negative numbers (−1 to −5), with the amino acid immediately preceding the LXXLL motif as the −1 position. Interestingly, the region of both LXXLL motifs that is well ordered in the crystal structures corresponds closely to the minimal core sequence capable of binding to NRs (36).

The binding mode of the Box 2 peptide is identical to that observed in analogous complexes between related coactivator LXXLL motifs and NR LBDs (25–27). As Shiau and co-workers (25) have previously described, the interactions made by an identical peptide bound to hERαLBD complexed with diethylstilbestrol; this structure will only be described in brief. Here, hERαLBD was liganded with the arylbenzothiophene core of raloxifene (RALcore). In contrast to raloxifene, which is an ER AF2 antagonist, RALcore (raloxifene minus its basic amine-containing side chain) acts as a potent receptor agonist. Despite the difference in bound ligand, the two TIF2 Box 2 co-crystal structures are essentially identical. In the Box 2 complex presented here, the ligand, RALcore, adopts a similar orientation within the binding cavity to that observed with other ER agonists (e.g. E2 and diethylstilbestrol). However, the binding mode of RALcore is reversed when compared with raloxifene so that its phenolic moiety is directed toward Glu543 and Arg394.

The 12-mer Box 2 peptide adopts a helical conformation with its N-terminal end interacting with Glu542 (H12) through hydrogen bonds between the carboxylate group and the main chain amides of Ile689 and Leu692 (Fig. 1, A and B). The C-terminal end of the peptide helix is capped by the ε-amino group of Lys362 through hydrogen bonds with the main chain carbonyls of Leu693 and Leu694. All three leucines of the LXXLL peptide are in contact with the LBD. The leucines at the +1 and +5 positions of the motif are buried at the LBD-peptide interface and project into two shallow pockets in the binding groove. The isoleucine at −1 and the +4 Leu lie on the periphery of the binding site (Fig. 1A).
Unexpectedly, examination of the initial ERαLBD-E2/TIF2 Box 3 complex electron density maps reveals that the Box 3 LXXLL motif peptide adopted a novel binding orientation that was distinct from all NR box-NR LBD complexes determined to date (Fig. 1C). At first glance, the position adopted by the peptide appears identical to that observed in the Box 2 structure. Both hydrophobic pockets along the binding groove are filled by leucine side chains from the Box 3 peptide. However, on closer examination, it is apparent that the peptide has “corkscrewed” along the coactivator cleft by one residue in the direction of Lys362. Consequently, the completely buried leucines are contributed by the −1 and + 4 residues and the amino acids at the −2 (Ala) and + 3 (Tyr) positions lying on the edge of the groove (Fig. 1D). Therefore, in the ErαLBD-Box 3 peptide complex, the interacting motif comprises the sequence LXXYL rather than the consensus LXXLL. Despite this fundamental difference, the van der Waals’ contacts between the peptide and LBD are virtually identical to those observed for Box 2.

The side chain of Leu744 (−1) is completely buried and interacts with Ile358 (H3), Val376 (H5), Leu379 (H5), Glu380 (H5), and Met543 (H12). Leu748 (+4) is also sandwiched between the peptide and the LBD and sits in an indentation formed by Ile358, Leu372, and Val376. Ala743 (−2) and Tyr747 (+3) rest on the edge of the groove and make nonpolar contacts with the side chains of Val355 (H3), Ile358 (H3), and Leu539 (H12), respectively (Fig. 1C). A minor variation in the positioning of the Box 3 peptide is observed between each monomer within the noncrystallographic ErαLBD homodimer and gives rise to slightly different N and C capping interactions made by Lys362 and Glu542.

**SPR Analysis of ERαLBD/LXXLL Peptide Interactions**—SPR analyses were performed to investigate the interactions between ERα AF2 and the LXXLL motifs of TIF2 in a more quantitative manner. Biotinylated 14-mer peptides containing the LXXLL motifs of wild type TIF2 (NR Boxes 1–3) were immobilized on a streptavidin-coated sensor chip surface. Fig. 2A demonstrates the steady state response at different concentrations of injected E2-ligated ERαLBD over a surface immobilized with NR Box 3. The plot was fitted to a steady state model, and the “steady state affinity” was determined from the following relationship between the level of binding and the concentration.

$$R_{\text{eq}} = \frac{K_{Cp} \Gamma_{max}}{1 + K_{Cp} \Gamma}$$  \hspace{1cm} (Eq. 1)

where $\Gamma$ specifies how many binding sites on the immobilized molecule that are on average blocked by binding one analyte molecule (n = 1). $R_{\text{max}}$ is the theoretical binding capacity, and $R_{\text{eq}}$ is the steady state binding level.

The steady state dissociation constant ($K_D$) was determined to be 260 nM. We then pursued kinetic measurements for this interaction and calculated the affinity from the rate constants. Fig. 2B shows the concentration-dependent association of E2-ligated ErαLBD with NR Box 3 along with the calculated line of best fit using a conformational change model (see “Experimental Procedures” and Ref. 21). This model assumes that the receptor first forms an unstable complex with peptide and then undergoes a conformational change that leads to a more stable complex. However, in reality the actual binding model could be more complicated because liganded ER is typically dimeric and therefore capable of binding two peptide motifs (one for each LBD in an ER homodimer). Based on the reported equilibrium constants for ER dimerization (37, 38), we have assumed that, at the concentrations of ER and ErαLBD used in this study (≥ 16 nM), the receptor exists as a homodimer. To further simplify the system, we also immobilized limited amounts of each peptide to the chip surfaces so as to make it impossible for two peptides to bind simultaneously to a single ER homodimer. The resulting apparent dissociation constant calculated for NR Box 3 was around 250 nM, which agrees very well with the dissociation constant calculated from steady state data and supports the choice of binding model used in data interpretation. Kinetic measurements were also performed for TIF2 NR Box 1 and NR Box 2 peptides. Table II summarizes the rate constants and affinities for all of the LXXLL motifs of TIF2. As shown previously using the full-length ER (21), the NR Box 2 motif binds to ER with the highest affinity, followed by Box 1, which binds slightly better than the Box 3 motif. In a control study, no ER binding over background was observed using a non-LXXLL peptide with a randomized sequence (data not shown).

To validate the unusual binding orientation observed in the ErαLBD/Box 3 crystal structure, we designed a series of mutant Box 3 peptides (Fig. 1D, panel ii). We supposed that replacement of certain residues within the motif would preclude a classical LXXLL/receptor interaction (as in the NR Box 2 complex) while maintaining the possibility of the novel binding mode observed in the Box 3 co-crystal structure. Accordingly, the first and/or third Leu in the Box 3 sequence (Leu745 and Leu749) were replaced with the corresponding residue from Box 2 based on the structural alignment shown in Fig. 1D. Previous structural studies have demonstrated that the lysine residue in the −2 position of the Box 2 motif makes a salt bridge with Glu380 (H5) (25). In addition, the basic sequence that precedes NR Box 2 has been implicated in the high affinity of this motif for ErαLBD (39). We therefore replaced Asn742 with Lys742. 

Fig. 2. Quantitative evaluation of the interaction between ERαLBD and wild type NR Box 3 peptide. A, several different concentrations of liganded ERαLBD were injected over a surface immobilized with NR Box 3 wild type (WT) peptide. After 1.25 h of injection, the binding had reached its steady state level, and the response was measured and plotted against concentration (squares). The solid line shows the fit of the data to a steady state model. A flow rate of 4 μl/min was used. B, overlayed sensograms showing injections of liganded ERαLBD at dimeric-protein concentrations of 8, 16, 32, 64 128, and 256 nM over a surface immobilized with NR Box 3 WT peptide (dotted lines) and the best calculated fit using a conformational change model (solid lines). A flow rate of 25 μl/min was used. The data shown have been corrected for bulk and background effects using a control surface.
with a lysine (−3K) to test whether this substitution would improve the affinity of Box 3 for ERαLBD.

Equal amounts of mutant peptides were immobilized to separate surfaces and probed for binding to both full-length ERα (Fig. 3) and to the isolated LBD (Fig. 4). Interestingly, the single (1H and 5Q), double (1H/5Q) and triple mutations (−3K/1H/5Q) completely abolished the interaction between the immobilized peptide and both forms of the receptor (Figs. 3B and 4B). As a negative control, we used a NR Box 2 peptide containing alanine substitutions in the core motif (AXXLAL). Previous structural and mutagenesis studies have shown the essential role of leucine residues in NR box-dependent interaction with NR LBDs (22). As expected, the AXXLAL peptide showed no significant binding to either the full-length receptor or the isolated LBD (Figs. 3A and 4A). Only the −3K mutant exhibits some binding activity, but this is clearly reduced compared with the wild type motif.

To directly probe the importance of the tyrosine side chain (+3) in the Box 3 sequence for NR box interaction, we evaluated the binding of mutant peptides in which this position was replaced with either a histidine (3H) or a glutamine (3Q) (Fig. 1D, panel ii). Both peptides exhibited diminished binding to both the isolated LBD (Fig. 4C) and full-length ERα (data not shown) compared with the wild type. Finally, a mutant Box 2 peptide, in which the second leucine (+4) in the motif was replaced by a tyrosine, was tested to evaluate the binding of the alternate LXXYL core sequence seen in the Box 3 crystal structure. This 4Y mutant bound to both the full-length ERα and the LBD (Figs. 3A and 4A) but had slightly lower binding capacity than the wild type motif.

**DISCUSSION**

The surprising finding of the crystallographic studies was that peptides derived from the NR Box 2 and Box 3 regions of TIF2 appear to interact with ERαLBD in a distinct manner. The NR Box 2 motif adopts the classical LXXLL binding mode that has been observed in a number of NR/coactivator peptide complexes (25–27, 40). In contrast, the Box 3 peptide displays a novel mode of interaction. Such a difference in binding orientation may represent a quirk of the sequence that flanks the NR Box 3 core consensus LXXLL module of TIF2. The NR Box 3 sequence is much more hydrophobic than the corresponding Box 2 region of TIF2. Furthermore, the pattern of hydrophobic residues of the Box 3 motif (residues 2 to +5) permits two possible peptide orientations that fulfill the general requirements for LBD/LXXLL interaction, namely LKRYLL and LLRYYL. The major difference between the alternate orientations of the core motif is the residue that occupies the peripheral +4 position.

The significance of the altered binding mode, LLRYYL, is not clear but illustrates the general principle that the binding groove of ERα can accommodate other sequence motifs apart from the characteristic LXXLL module. Further evidence of such a nonstandard binding within the coactivator groove can be seen in complexes of ERLBD with AF2 antagonists such as raloxifene (28, 29) where the LXXML motif of H12 (residues 540–544) mimics the interactions made by coactivator peptides (25). In such structures, the side chain of Met indicates that the coactivator binding groove can accommodate several different large hydrophobic groups at the +4 position of the consensus motif.

However, the data obtained from soluble ERα interacting with immobilized peptides casts some doubt on the physiological significance of the unusual NR Box 3 binding mode observed in the crystal structure. All of the mutant peptides that interfere with the core classical LXXLL motif Box 3 (1H, 5Q, and 1H/5Q) are unable to interact with either full-length ERα or the isolated LBD in solution. Only the −3K mutant, which alters the N-terminal flanking sequence but not the LXXLL motif, exhibits any detectable binding. In fact, any alteration of the LXXLL motif has serious consequences for the interaction of Box 3 with ER. This finding is somewhat surprising considering that the core sequence of the triple mutant peptide (−3K/1H/5Q) differs at only three positions (−3, −1 and +4) from the high affinity NR Box 2 motif. The complete lack of binding exhibited by this triple mutant therefore appears to result from disruption of the N-terminal flanking sequence of the motif. Previous studies have highlighted the importance of this region for NR box affinity (27, 39).

The role of the tyrosine residue (+3) in the interaction of Box 3 with ER in solution is somewhat ambiguous. Binding data from the tyrosine substitution mutants (3H and 3Q) demonstrate that this residue is not essential for receptor interaction. Nonetheless, the binding of both of these mutants is significantly weaker than the wild type motif. Clearly, a tyrosine is required for full binding, but it is not apparent whether this residue plays a direct role, as seen in the Box 3 crystal structure or contributes indirectly to the intrinsic stability of the helical conformation of the motif in complex with the receptor.

Data from the corresponding Box 2 4Y mutant, in which the leucine that occupies a position on the periphery (+4 position) of the binding groove is substituted by a tyrosine, indirectly demonstrate that changes at this site have a minor effect on receptor/NR box interaction. This result, combined with the observed Box 3 binding mode in the crystal structure, suggests that LXXYL motifs may be active in certain contexts.

The data summarized in Table II demonstrate that ERαLBD binds best to TIF2 Box 2 with a Kd of 76 nM and less well to Box 1 and 3 (Kd = 250 nM). The relative order of affinities of the NR box motifs of TIF2 determined here agree well with our previously study of the full-length receptor (21) and with those from competitive binding experiments (23, 41). However, the affinities reported for the full-length receptor are considerably higher than those for the isolated LBD. In the case of TIF2 Box 2, the dissociation constant was determined to be 1.4 nM for full-length protein compared with 76 nM for LBD. The higher observed affinity for full-length ER may be due to the involvement of other parts of the receptor in the recognition and binding to the LXXLL peptides. Such a difference could also arise if the initial interaction of NR box motifs with the receptor is stabilized, thereby reducing the dissociation rate. Unexpectedly, the lower affinity LXXLL motifs (Box 1 and 3) exhibit different binding kinetics compared with the Box 2 motif (Fig 4, A and B). The binding of either NR Box 1 or NR Box 3 is characterized by fast association and dissociation phases. In contrast, Box 2 does not have as fast an association rate and also exhibits a much lower dissociation rate (Table II). Although the origins of these binding differences are not clear, the observed kinetic and affinity parameters are consistent.
with a model in which each of the LXXLL motifs of TIF2 exhibit distinct NR preferences.

In summary, our SPR data from the wild type and mutant NR Box 3 peptides seem to favor a classical binding mode (LRYLLE) and suggests that the binding mode seen in the ERα-LBD/NR Box 3 crystal structure may not be a true representation of the predominant interaction that occurs under the conditions used for SPR. Is the mode of peptide interaction seen in the crystallographic study likely to result from packing constraints imposed by the crystalline lattice? The coactivator binding groove of ERLBD lies on the surface of the molecule, and the bound peptide makes minor contacts with a neighboring LBD molecule within the crystal. As noted above, the environments of the two NR Box 3 peptides bound to the ERLBD homodimer are different. In case of the one of the Box 3 motifs, the phenolic hydroxyl of Tyr747 makes an intermolecular hydrogen bond with the H2-H3 loop region of an adjacent molecule. However, because this interaction does not occur in the other coactivator peptide-binding site, it seems unlikely that this interaction is responsible for the unusual Box 3 orientation. Nonetheless, the possibility remains that the unusual binding mode of both peptides, which directly contact each other in neighboring homodimers within the lattice, is favored during crystal growth. The conflicting crystallographic and solution binding data highlight the potential pitfalls encountered when using short peptide motifs to mimic interactions between large macromolecules in structural studies.

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