Published in final edited form as:

Nat Struct Mol Biol. 2010 July ; 17(7): 808–814. doi:10.1038/nsmb.1860.

Structural Analysis of Rev-erba Bound to NCoR Reveals a Unique Mechanism of Nuclear Receptor-Corepressor Interaction

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Abstract

Repression by the nuclear receptor (NR) Rev-erba plays an integral role in the core molecular circadian clock. We report the crystal structure of a Nuclear Receptor Corepressor (NCoR) Interaction Domain 1 (ID1) peptide bound to truncated human Rev-erba ligand binding domain (LBD). The ID1 peptide forms an unprecedented anti-parallel β -sheet with Rev-erba, as well as an α -helix similar to that seen in NR/ID2 crystal structures, but out of register by four residues. Comparison with the structure of Rev-erb β bound to heme indicates that ID1 peptide and heme induce significantly different conformational changes in the LBD. Although heme is involved in Rev-erb repression, the structure suggests that Rev-erba could also mediate repression via ID1 binding in the absence of heme. The novel secondary structure induced by ID1 peptide binding advances our understanding of NR-corepressor interactions.

INTRODUCTION

Nuclear receptors (NRs) comprise a large family of ligand-sensitive transcription factors that regulate growth, development, metabolism, circadian rhythm, and other physiological processes^{1–3}. These receptors bind coactivator molecules in the presence of ligand and, to different degrees, corepressors in the absence of ligand, which facilitates local chromatin remodeling and target gene transcriptional activation or repression^{4–7}. NRs contain two

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ACCESSION CODE

Atomic coordinates and structure factors have been deposited with the Protein Data Bank under accession code 3N00.

AUTHOR CONTRIBUTIONS

D.J.P. performed the fluorescence energy transfer assays, M.H.L. and S.P.W. performed structure based construct design, T.M.B. grew the cells, J.B. purified the protein, V.M. crystallized the protein complex, R.T.G. and R.T.N. solved the structure, R.T.G. refined and analyzed the structures, C.A.P. performed the mammalian two-hybrid assay, X.H. performed the TR analysis, S.P.W. and R.T.N. provided critical review and C.A.P., R.T.G., M.H.L., and M.A.L. wrote and edited the manuscript.

modules that contribute to their activation function: a ligand-insensitive activation domain known as Activation Function-1 (AF-1) is located in the NR N-terminus; and a ligand-dependent activation domain, known as AF-2, is contained within the C-terminal ligand binding domain (LBD)⁸.

In the absence of ligand, receptor LBDs differentially associate with the corepressors NCoR (Nuclear Co-Repressor) and SMRT (Silencing Mediator of Retinoid and Thyroid Receptors), which share significant amino acid sequence homology. Two regions within the aligned NCoR/SMRT sequence bind to NR LBDs^{9–11}. The first region, called ID1^{9–11}, near NCoR residues 2062–2084 and SMRT residues 2128–2150, contains the motif IxxI/VI. The second region, called ID2^{9–11}, near NCoR 2270–2292 and SMRT 2235–2357, contains the motif LxxII. Subsequently, a third interaction domain was identified in NCoR but not SMRT¹². In two of the studies, the motifs were combined into a single I/LxxI/VI motif^{9,10}, implying that the sequences should be aligned to preserve this motif as shown in Fig. 1a. Perissi et al.¹¹ used 3D modeling to anticipate that the corepressor α -helix should extend into volume normally occupied by the AF2 α 12 helix, and based on corepressor sequence alignments they proposed an extended motif, LxxI/HIxxxI/L (Fig. 1b). The subsequent crystal structure of a SMRT ID2 peptide bound to ligand antagonized PPAR α ¹³ confirmed the displacement of the AF2 α 12 helix, the extension of the corepressor α -helix into volume normally occupied by α 12, and the 3D alignment of the ID2 LxxxIxxxL motif with the LxxLL coactivator motif, which was subsequently confirmed in other crystal structures of NRs bound to SMRT ID2^{14,15}. However, these data did not directly confirm the motif or alignment with respect to the ID1 sequences. Furthermore, there was evidence suggesting that the LxxxIxxxI/L motif might not apply to ID1 sequences^{10,16}. Thus, the motif and alignment necessary for ID1 binding was largely unclear.

Rev-erb α (*NR1D1*) is a heme receptor^{17,18} that represses the circadian clock transcription factor Bmal1, to influence the circadian period length and amplitude^{19,20}. In addition, Rev-erb α directly regulates a number of genes^{21–28} including the transcriptional coactivator PGC1 α ²⁹, to alter glucose, lipid and bile acid metabolism. Rev-erb lacks the AF2 domain α 12-helix^{30,31} and represses gene activity by associating with NCoR^{20,32,33} via selective binding to the ID1 CoRNR motif contained in NCoR amino acids 2024–2065. The structure of the apo-Rev-erb β receptor³⁴ has been reported as well as the heme-bound Rev-erb β receptor³⁵, but the nature of corepressor association with the Rev-erb receptors is unclear. Binding of heme to the Rev-erb α is principally coordinated by a single histidine residue within α 11 of the C-terminus, reveals dramatic restructuring of the receptor but provides little insight into the mode of NCoR ID1 association. Thus, elucidating the molecular basis of Rev-erb α repression function is critical to further our understanding of nuclear hormone receptor-mediated repression as well as to provide insight into the potential pharmacological manipulation of this receptor in disease.

Here we present the 2.6Å cocrystal structure of an NCoR-derived ID1 CoRNR peptide bound to the human Rev-erb α LBD. The N-terminal NCoR ID1 residues mediate a change in the receptor's C-terminal secondary structure and together form an unprecedented anti-parallel β -sheet. Subsequently, NCoR ID1 adopts a well defined α -helical structure with the CoRNR motif lying in the coactivator groove but not in register with either previously predicted corepressor sequence alignments¹¹ or one based on the ID2 SMRT peptide bound antagonized PPAR α crystal structure¹³. Structure based sequence alignment corrects and suggests a new extended motif that better defines the binding requirements and differences for ID1 and ID2 sequences. Contrast of the NCoR ID1 Rev-erb α and heme bound Rev-erb β crystal structure reveals NCoR ID1 would be displaced by significant steric hindrance between the β -sheet and the heme dependent restructuring of the α -helix 3, receptor core residues and the porphyrin ring.

METHODS

Crystallization and Structure Determination

Dilute solutions of purified human Rev-erba (281–614Δ324–422) complexed with 2–3 molar excess of NCoR CoRNR1 peptide (2045 NH2- THRLITLADHICQITQDFAR -OH 2065) concentrated to 5–6 mg per mL were used to obtain diffraction grade crystals. Vapor diffused hanging drops at 22°C with a 1:1 (v/v) ratio of the Rev-erba NCoR complex and the precipitant solution produced 100–200 μM crystals within a few days. Precipitant solutions contained 6–9% PEG 3350, 8% glycerol, 200 mM proline, and 80 mM HEPES at pH 7.5. Prior to flash freezing in liquid N₂, the crystals were transiently mixed with a cryoprotectant solvent consisting of the precipitant solution amended with 20% glycerol. X-ray diffraction data at 100°K from a single crystal data were collected at a 1 Å wavelength using a MAR165 detector at the IMCA-CAT, sector 17ID at the Advanced Photon Source synchrotron. HKL2000 was used to index, integrate and scale the data⁴³. The structure was solved using a proprietary liganded Rev-erba NCoR structure solved to 1.9 Å with heavy atom phases (to be published). A search model derived from hVDR (1db1) lacking helix 12 also yielded a valid solution by molecular replacement with AMORE or MOLREP^{44–46}. The resulting structure contains one molecule in the asymmetric unit that forms a symmetric dimer along helix α10 with an adjacent molecule. The initial model was iteratively built with Quanta (Accelrys) and refined with CNX⁴⁷ and the final structure built with COOT⁴⁸ and refined with Refmac^{45,49} using all hydrogens and maximum likelihood restraints. The crystallographic data and final refinement statistics are summarized in Table 1. Reported interatomic distances are between heavy atoms unless specified and were measured with COOT or PyMol. The COOT SSM utility was employed to superimpose previously published structures upon the Rev-erba monomeric LBD. The deposited coordinates have a MolProbity⁵⁰ structure quality score of 1.89 (98%) and Ramachandran analysis within COOT reported that 191 residues are in the preferred, 4 in the allowed and 2 in the outlier regions. Structure figures were generated with PyMol from Delano Scientific (www.pymol.org).

Time-Resolved Fluorescence Energy Transfer

From a collection of 70 different biotinylated coactivator or corepressor peptides, NCoR (2040–2065) and SMRT (2124–2149) with the CoRNR1 motif were identified to bind selectively to Rev-erba. A volume of 12.5 μL at 20 nM of streptavidin-conjugated europium chelate in assay buffer (50 mM MOPS, 50 mM NaF, 0.05 mM CHAPS, 5 mM DTT) was added to 1 μL of each peptide at 500 μM concentration. After 1 hour incubation, 12.5 μL of 20 nM recombinant Rev-erba LBD labeled with 20 nM streptavidin-conjugated allophycocyanin in assay buffer was added to the plate and allowed to equilibrate 2 hrs before reading on a Perkin Elmer ViewLux Microplate Imager. The data from 4 experiments was averaged and plotted versus the peptide sequence. An increase in signal over background was considered a potential interacting peptide with Rev-erba.

For the alanine mutagenesis study, a 40 nM NCoR peptide (2040–2065) labeled with 20 nM streptavidin-conjugated europium chelate was bound to 10 nM recombinant Rev-erba LBD labeled with 10 nM streptavidin-conjugated allophycocyanin in the assay buffer. Volumes of 10 μL of the Rev-erba NCoR solution were added to 384 well plate containing 0.1 μL with increasing concentrations of the NCoR (2040–2065) site mutated alanine peptide dissolved in DMSO. Plates were allowed to equilibrate for 1 hour then binding was monitored by time resolved fluorescence on a Perkin Elmer ViewLux Microplate Imager. Signal values were normalized as %inhibition then fit to a 4 parameter logistical fit to determine the IC₅₀. Data were reported as the average and standard error of the means for three experiments and compared to the control NCoR (2040–2065) value.

Plasmids

Human VP16-Rev-erba, Human VP16-TRα, Gal-DBD, 5xUAS-SV40-Luciferase and Gal-NCOR-ID1 have been described previously^{20,32,51}. Site-directed mutagenesis was performed using Stratagene site-directed mutagenesis II XL according to manufacturer's instructions and commercially available primer design program (Stratagene). All point mutants were verified by sequencing.

Cell Culture and Transfection

293T cells were maintained in DMEM plus 10% fetal bovine serum in 0.5% penicillin/streptomycin at 37°C, 5% CO₂. 24 hours prior to transfection, cells were seeded into 24-well plates. Transfections were performed in fresh media lacking antibiotics. For mammalian two-hybrid assays, each well was transfected with 0.25 µg Gal-ID1 or Gal-DBD, 0.25 µg of VP16 or VP16-Rev-erba, 0.2 µg of 5xUAS-SV40-Luciferase, and 0.1 µg of β-galactosidase and 2 µL per well of Lipofectamine 2000 (Invitrogen) in 100 µL Opti-MEM. For repression assays, cells were co-transfected with 0.2 µg DR-Luciferase or Bmal1-Luciferase, 0.6 µg of pcDNA or FLAG-Rev-erba and 0.1 µg of β-galactosidase.

Luciferase Assay

Cells were lysed in 100 µL of Passive Lysis Buffer (Promega) followed by freeze-thaw at -80° C. 5 µL of lysate was used for luciferase assay (Promega) or β-galactosidase assay. Relative light units for luciferase were normalized to β-gal activity.

Protein Clones, Expression and Purification

This information is provided as Supplemental Material.

RESULTS

Rev-erba-LBD NCoR ID1 cocrystal structure

Poor expression and solubility of full length Rev-erba LBD (281–614) required use of a soluble deletion mutant, (281–614 Δ324–422), which lacks the proline rich region between α-helix1 (denoted as α1) and α3 containing the putative α2 and X-domain (406–418)³⁶, but has the C-terminal Y-domain (602–614). The mutated Rev-erba was cocrystallized with an NCoR (2045–2065) peptide, based on TR-FRET data that confirmed preferential, high affinity binding of CoRNR ID1 peptides by Rev-erba (Supplementary Fig. 1). Despite the deleted region and the naturally absent AF2 α12 helix, the global structure has ten α-helices and is mostly consistent with the canonical 3-layer α-helical sandwich seen in many NR LBD structures³⁷ (Fig. 2a,b). The Rev-erba α10 mediates dimer formation with α10 to form a crystallographic symmetry related monomer. Helix α1 is constructed of residues Pro284–Phe300 that are built into well ordered electron density, however, subsequent residues Thr301–Pro323 and Tyr423–Thr431 are not defined by the data. Surface helix α3 is continuous and spans Val432–Leu457, followed by α3' from Pro458–Ser464 with the Arg461 side-chain hydrogen-bonding to Gln465 and Gln468 followed by α4 from Gln465–Gly475. Thereafter, core α5 runs from Thr476–Phe484 where the backbone then meanders and disappears at residue Asp492. For residues 493–506, the anticipated canonical s1 and s2 β-strands could not be built into weak, disordered electron density. A remnant of α6 is defined by Glu507–Gly512 and is followed by a long stretch of well defined helices that spans α7 – α10 and into α11. Just after α10 Pro593 the C-terminal helix winds through His602 and is capped by Ser603. Subsequently, Lys605, whose side-chain hydrogen-bonds to the Gly512 CO, marks the transition into a new β-strand within the Y-domain, denoted sY, that spans from Leu607 before terminating at Val611 (Fig. 2c,d). The exposed CO of Leu606 and NH of Ser608 hydrogen-bond to the α3 Trp436 and Ser440 side-chains further

stabilizing this region. As an unprecedented NR structural feature, the sY β -strand establishes a new molecular framework that enables Rev-erba to form an anti-parallel β -sheet with the N-terminus of the NCoR corepressor peptide.

The NCoR Thr2045 CO hydrogen-bonds with the amide of Rev-erba Val611 to initiate β -strand β 1N, whose classic β -sheet hydrogen-bonding pattern terminates with the Ile2049 CO and Leu607 amide (Fig. 1c,d). Side-chains from Phe609 and Leu607 provide hydrophobic packing on the top side of the β -sheet between Arg2047 and α 3. Underpinning the receptor sY β -strand residues are a 2.7Å hydrogen-bond from the His2046 imidazole side-chain to the Glu437 carboxylate and the Trp436 indole in α 3 (Fig. 1d). As sY Leu606 and Lys605 pass under NCoR -5T2050, a change to α -helix, termed α 1N, is made at Leu2051, whose amide hydrogen-bonds to Lys605 CO (2.7Å) and side-chain is directed towards the receptor core. Denoted as ID1 corepressor motif residue -4, Leu2051 marks the first of a 15 residue amphipathic α -helix, where -4Leu2051, +1Ile2055, +4Ile2058, +5Ile2059 and +9Phe2063 travel up the entire coregulator binding cleft with hydrophobic side-chains pointing towards the mostly hydrophobic Rev-erba binding surface (Supplementary Fig. 2). Prior to +1Ile2055, the -2Asp2052 carboxylate hydrogen-bonds to the -5Thr2050 OH and the -1His2054 imidazole hydrogen-bonds to the conserved α 3 Thr444 hydroxyl side-chain (2.8Å) (Fig 2c,d). Further stabilizing α 1N on the C-terminal flank are water mediated hydrogen-bonds from the side-chain of the highly conserved Lys455 to the +8Asp2062 backbone CO (2.9Å, 3.3Å) and side-chain (3.0Å), a hydrogen-bond from Lys473 to the +6Thr2060 side-chain OH (3.0Å), the terminal +11Arg2065 side-chain to the +8Asp2062 side-chain (3.0Å) and the enclosure of the +9Phe2063 phenyl by Val451, Val469, Val472 and Arg461 and Gln465. The high degree of stability achieved by the bound NCoR peptide is reflected by the fact that the entire 21-residue NCoR peptide structure is well defined by good quality electron density except for the +7Gln2061 side-chain (Supplementary Fig. 3, Table 1).

Structural basis for corepressor alignment revision

Both ID1 NCoR bound to Rev-erba and ID2 SMRT bound to ligand antagonized PPAR α adopt α -helical structures that bind coaxially in the space traditional reserved for a coactivator and AF2 α 12 in activated NRs (Fig. 2a). In striking contrast, the longer and better defined ID1 NCoR peptide extends more than one turn further towards the bottom of the binding groove and terminates at -4Leu2051, whose side-chain makes hydrophobic contacts with Phe443 and Phe477. The -4Leu2051 amide donates a hydrogen-bond to the Lys605 CO at the position where the anti-parallel β -sheet is forged from the unwound α 11 by analogy to apo Rev-erb β . Noteworthy is that NCoR -4Leu2051, occupies the same space as the GW6471 antagonist ethyl amide in the PPAR α SMRT structure (Fig. 3a) and the conserved α 12 tyrosine in activated PPAR structures³⁸. Most remarkably, our structure reveals that -4Leu2051 is not the first residue in the three turn LxxI/HIxxxL/I motif as predicted from the ID2 SMRT PPAR α crystal structure¹³. We show in Fig. 3b that the ID1 NCoR ICQII CoRNR sequence actually aligns with the ID2 SMRT LEAII CoRNR, and not the LADHI sequence as originally proposed^{11,13}. Therefore, this structure based comparison requires a four position N-terminal shift of the ID1 NCoR and SMRT peptide sequences in order to correctly align the motif alignment relative to the ID2 SMRT PPAR α GW6471 structure. Moreover, the realignment poses a recognition and specificity role for the +9Phe2063 that aligns with tyrosine in ID1 SMRT and leucine in ID2 SMRT and NCoR.

Structure guided analysis of repression by NCoR ID1

The novel mode of CoRNR peptide interaction was challenged functionally with NCoR ID1 mutations that were tested for Rev-erba LBD binding affinity with an *in vitro* fluorescence resonance energy transfer assay (Fig. 3a, Supplementary Table 1). With the single solvent

exposed –9His2046 to Glu437 hydrogen-bond and limited side-chain contact of –7Leu2048 to Trp436, it may not be too surprising that alanine mutations report minimal effects, since alanine sustains the β -sheet backbone interactions. On the other hand, it was rather surprising that –4Leu2051A did not produce more of a loss, since the geometry and chemistry of the side-chain appears well suited for the extremely hydrophobic environment provided by Phe443, Phe477, Leu607 and +1Ile2055. Evidently, the loss of three carbons makes little difference in this extremely hydrophobic region, nor does loss of a hydrogen-bond in the case of –1H2054A. By contrast, mutation of residues at the +1, +4, +5 positions (Fig. 3a) which comprise the core ICQII CoRNR motif, dramatically reduced binding affinity for Rev-erb α LBD. Furthermore, a near nine fold reduced affinity for ID1 +9F2063A on Rev-erb α is in accord with the similarly weakened binding affinity for the L693A mutation at the equivalent position in the ID2 SMRT motif when assayed against PPAR α and TR β ³⁸. This and the structure based alignment in Fig. 2b lends credence to the far right flanking playing a greater role and suggests extending the core CoRNR motif to I/LxxI/VIxxxY/L.

The structural predictions were also tested in living cells using a mammalian two-hybrid assay. Co-transfection of the NCoR-CoRNR peptide fused to the DBD of the Gal4 transcription factor, with Rev-erb α fused to the VP16 transcriptional activation domain led to the potent activation of a luciferase reporter gene containing Gal4 binding sites, indicative of an interaction between the NCoR peptide and Rev-erb α (Fig. 3b). Mutation in the CoRNR residues at the +1, +4, and +5 positions (Fig. 3b) abolished the interaction, whereas those in the extended N-terminal α -helix, residues –4 and –1, had little effect. Thus, the core ICQII motif was required for interaction with Rev-erb α , whereas the LADH extension was not.

The corrected CoRNR motif alignment applies to the thyroid hormone receptor

To further explore the mode of core ICQII motif binding to Rev-erb α , and to determine whether this binding mode might occur with other NRs, we tested two chimeric CoRNR-derived peptide constructs for their ability to interact with thyroid receptor (TR). TR preferentially binds NCoR³⁹, where the ID1 and ID2 CoRNR sequences are ICQII and LEDII, respectively. Hu and Lazar⁹ and Nagy et al.¹⁰ originally aligned the ICQII with the LEDII (Fig. 1a) to derive the CoRNR motif I/LxxI/VI. This alignment suggests chimeric peptides such as ID1/2 in Fig. 3c, which combines the LADHICQII from the ID1 sequence with the RKAL downstream from the aligned LEDII in the ID2 sequence. By contrast, Perissi et al. and Xu et al. aligned the ICQII directly with the downstream IRKAL sequence (Fig. 1b). This alignment suggests that the ICQII sequence be replaced with the IRKAL sequence, as in peptide ID1/2-RKAL in Fig. 3c. Note that both peptides fit the original LxxxIxxxI/L N-terminally extended motif, but only the first peptide fits the I/LxxI/VI CoRNR motif. In a mammalian two-hybrid assay testing the interaction of TR and the described CoRNR peptides in cells, Gal-ID1/2 demonstrated robust interaction whereas the alignment utilizing the downstream RKAL sequence did not (Fig. 3d). This result agrees with the structural work summarized in Fig. 2 in supporting revision of the core CoRNR motif alignment.

NCoR ID1 association potentially induces significant secondary structural change

The foremost structural difference between apo Rev-erb β ³⁴ and NCoR Rev-erb α is the difference in secondary structure for equivalent Y-domain residues from α 11 (β residues His568–Pro578) and the sY β -strand (α residues His602–Gln614) (Fig. 4a, b). Rev-erb β α 11 residues Leu572, Leu573 and Phe575 (LLxF) (Supplementary Fig. 4) provide intramolecular hydrophobic packing with Leu482, Val413 and Trp402 (Fig. 4b, c). The α 3 break at Pro411³⁴ (Fig. 4c) packs the Phe405 side-chain deeper into the cognate ligand

pocket and Trp402 fills space between $\alpha 3$ and $\alpha 7$ and the Glu403 side-chain is directed to the solvent front. Contrasted with the highly homologous Rev-erba receptor bound with NCoR ID1, the reconfigured Y-domain secondary structure creates an extended binding site that appears specific for CoRNR ID1 motifs. This places the α Leu606 below $\beta 1N$ and α Leu607 and α Phe609 (LLxF) on top of the sY β -strand that forms the anti-parallel β -sheet with NCoR (Fig. 4b, d). Interestingly, this also positions the NCoR -4Leu2051 directly where the equivalent $\alpha 11$ β Leu572 resided and assumed contact between -4Leu2051 and α Leu606, which may encourage the structure change and help to register the NCoR peptide on the coregulator groove (Fig. 4b–d). Furthermore, unlike $\alpha 3$ in apo Rev-erb β , corepressed Rev-erba $\alpha 3$ is continuous and its N-terminus to α The444 ensemble pivots toward sY, thus freeing α Trp436 to rotate under sY and α Glu473 to hydrogen-bond with NCoR -9His2046. Assuming the described structural differences between the apo and ID1 bound states are shared by both the Rev-erba and Rev-erb β receptors, the NCoR ID1 bound Rev-erba structure reveals a potential molecular mechanism for a repression mode that occurs in the absence of heme. Also, despite that the Rev-erba $\alpha 3$ moved as much as 4.3Å between α -carbons for α Ile435 / β Ile401 and 2.7Å for α Phe439 / β Phe405, the ligand binding pocket remained occluded by many conserved hydrophobic side-chains as previously described³⁴. Therefore, in order to accommodate a ligand such as heme, a substantial change in tertiary structure and side-chain orientation of the Y-domain histidine would have to occur³⁵.

Contrasting the apo-Rev-erba/NCoR and heme-bound Rev-erb β crystal structures

Although heme binding stabilizes the interaction of full-length Rev-erba to NCoR, heme binding to the Rev-erba LBD paradoxically destabilizes the interaction of Rev-erba with the ID1 peptide¹⁸. This suggests that heme binding alters the conformation of Rev-erba LBD in a manner that prevents interaction of the NCoR-derived peptide in the absence of additional contacts with other regions of one or both proteins. Comparison of the apo-Rev-erba/NCoR and heme-bound Rev-erb β ³⁵ crystal structures shows that heme binding induces changes for $\alpha 5$, $\alpha 7$ and more interestingly has a Y-domain (β His568-Pro578) conformed to $\alpha 11$ that is accompanied by a large scale structural change in the bottom half of $\alpha 3$ (Fig. 5). The conserved β Trp402 α -carbon located in $\alpha 3$ below β Pro411 swings almost 8Å left and away from the continuous $\alpha 3$ of the NCoR structure and 10Å from that in the apo Rev-erb β structure and positions the $\alpha 3$ N-terminus directly in the space occupied by the NCoR Rev-erba β -sheet (Fig. 5b). The trajectory of the conserved tryptophan demonstrates the versatility of the receptor and how it adapts in response to different binding partners. Specifically, in apo-Rev-erb β , β Trp402 contributes hydrophobic packing at the base of the cognate ligand pocket, while with heme bound, it contributes direct hydrophobic packing along with β Leu572 and β Phe409 and in apo-Rev-erba bound to NCoR ID1, α Trp436 hydrogen-bonds to α Leu606 and underpins sY. Heme binding also remodels core residues, such as β Phe409 that would produce steric clash with NCoR -1His2054 (1.7Å) and α Phe609 (2.8Å) and directly mediated contacts from the porphyrin ring to -4Ile2051 and α Leu607 (both <2.1Å). Assuming heme bound Rev-erba would yield a similarly remodeled structure, these and other potential steric clashes are likely to interfere with ID1 binding. In all probability this explains why we have been unable to obtain crystals of the Rev-erba LBD bound to NCoR ID1 in the presence of heme (R. Gampe, unpublished).

DISCUSSION

We present the first crystal structure of an ID1 corepressor peptide in complex with an apo-NR LBD. Bound to a deletion mutant of Rev-erba LBD, the NCoR ID1 peptide adopts an amphipathic α -helical structure that binds the coregulator groove and overlaps the α -helix of ID2 CoRNR peptides bound to PPAR α , ERR γ and PR. Consistent with earlier predictions¹¹ the longer $\alpha 1N$ extends further into receptor volume normally occupied by the

AF2 helix of activated NR structures. However, NCoR Leu2051, originally predicted to be at the +1 position in the LxxxIxxxI/L motif actually maps to the -4 position. Upon correction, the ID1 ICQII and the ID2 LEAII sequences are in register, consistent with the I/L xx I/V L motif for positions +1 through +5^{9,10}. Supporting this finding are cell-based and *in vitro* assays of NCoR alanine mutants indicating the +1Ile2055, +4Ile2058, +5Ile2059 play major roles in ID1 CoRNR binding. Also in agreement with the corrected alignment is that proper registry and presence of the CoRNR ID1 ICQII motif is required for binding to TR. Furthermore, the corrected alignment suggests extending the motif to include the +9Phe2063 and equivalent SMRT tyrosine. The crystal structures also show the NCoR +9Phe2063 does superimpose reasonably well onto the +9 leucine in the ID2 corepressor sequences and the final leucine of the LxxLL motif of coactivators. These observations and the near 9-fold loss of binding for +9F2063A support extension of the corrected CoRNR motif to I/LxxI/VIxxxL/F/Y. Although there is some quantitative variation between the different peptides and receptors, the mutagenesis work presented here with ID1 peptides bound to Rev-erba generally agrees with earlier mutagenesis work on ID2 peptides bound to PPARα and TR^{13,40-42} showing that the +1, +4, +5 and +9 side-chains tend to be the most important.

The formation of the anti-parallel β-sheet between Rev-erba and the ID1 NCoR corepressor peptide was unanticipated, especially when considering that β-structure has never been observed for the corresponding residues in all other NR structures. Nor did features in the Rev-erbβ structures or Rev-erba sequences suggest that α11 could unwind to a β-strand. The presence of β-strand enhancing residues prior to -4Leu2051 in ID1 strongly suggests that this elegant secondary structure switch is specifically tailored for the CoRNR ID1 motif. The remodeled Y-domain extends the ID1 binding surface by 5 residues, which in the case of apo and heme bound Rev-erbβ has recoiled to α11 and packed against the ligand binding pocket. Insensitivity of -9His2046A or -7Leu2048A established their side-chain interactions contribute little to forming the β-sheet and indicate that insertion of β-strand breaking residues from ID2 motifs or simultaneous deletion is probably required to dissect the key interactions. Thus our data and crystal structure help to better explain the molecular recognition and determinants for ID1 corepressor binding to apo-Rev-erba LBD *in vitro*.

It is important to consider whether the intermolecular β-sheet could form in other NR corepressor complexes aside from the NCoR ID1 sequence bound to Rev-erba. In the region where NCoR pairs with the sY β-strand, positions -9 through -5, and in the extended part of the α-helix, positions -4 through -1, the NCoR and SMRT ID1 sequences are similar to each other, but quite different from the ID2 sequences. The ID1 sequences have amino acids that tend to promote α-helical and β-sheet structure, whereas the ID2 sequences tend to break these secondary structures. The NCoR ID2 sequence has a proline that might interfere with the hydrogen-bonding of the β-sheet (Supplementary Fig. 4b). This suggests that CoRNR ID2 sequences probably would not induce the intermolecular β-sheet as presented here for NCoR ID1 bound to Rev-erba.

Rev-erba exhibits several structural features that probably contribute to β-sheet formation and ID1 selection. The Ser603 side-chain helps cap α10 where it unwinds into the sY β-strand. An extensive hydrogen-bond network involving the α3 W436 and S440 side-chains effectively caps the edge of the sY β-strand, while the Trp436 indole also contributes to the lipophilic cluster below the sheet. The N-terminal α3 residues, Glu437 and Gln433, both hydrogen-bond to -9His2046. Further along in α3, Thr444 hydrogen-bonds to -1His2054, while in α4, Lys473 hydrogen-bonds to +6Thr2060, noting that the -1His2054 and +6Thr2060 are both present in ID1 but not ID2 sequences. These receptor residues are all conserved in Rev-erbβ, except for Gln433, which is replaced by histidine. Among nuclear receptors, Trp436 is conserved only in RAR and ROR. While the Ser440 serine is conserved

only in RAR, a threonine is at this position in TR, LXR, FXR and ER. The Thr444 is conserved in TR, RAR, ROR, and others. The $\alpha 3$ Gln433 glutamine and Glu437 glutamate are probably less important, because their interactions are exposed to solvent, but they are conserved, respectively, in LXR and in ROR γ , COUP and TLX (Supplementary Fig. 4). Overall, the conservation of Trp436, Ser440, Thr444 suggests that in addition to Rev-erb β both RAR and ROR might bind ID1 corepressor sequences via the β -sheet interaction seen here, provided any interference from the AF2 $\alpha 12$ helix could be overcome in the case of RAR or ROR.

Comparison of the structure of Rev-erb α bound to the NCoR ID1 with the heme-bound Rev-erb β structure provides an explanation of the biochemical findings that heme titration displaces bound CoRNR ID1 peptide from the Rev-erb α LBD, as the inter-molecular Rev-erb α β -sheet would be significantly disrupted by the bound heme and the remodeled Rev-erb α LBD. It also presents the possibility that a unique molecular mechanism is employed by CoRNR ID1 to achieve repression in the absence of bound heme. Binding of NCoR ID1 to Rev-erb α or β in this manner could represent a previously undescribed basal repression state, which might be further subject to alterations in repression function by association with heme, or by other circadian and metabolic events. However, the structures do not provide insight into how heme recruits or stabilizes the interaction between full-length NCoR and the Rev-erb proteins. Future studies are likely to reveal other regions of NCoR and Rev-erb α that contribute to stabilizing the complex when bound with heme.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH DK45586 (MAL) and by the Penn-GSK Academic Drug Discovery Initiative. We thank Nan Wu for critically reading the manuscript and Thomas B. Stanley and Tim M. Willson from GSK for useful discussions.

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a 'CoRNR' alignment extended

Residue Position: -4321123456789

IXXIIXXXF

NCoR ID1: VPRTHRLITLADHICQIITQDFAR

SMRT ID1: PGVKGHQRVVTLAQHISEVITQDYTR

NCoR ID2: GHSFADPASNLGLEDIRKALMGSF

SMRT ID2: QAVQEHASTNLGLEAIRKALMGKYD

b 'LXXIIXXXI' alignment

Residue Position: 123456789

LXXIIXXXI

NCoR ID1: VPRTHRLITLADHICQIITQDFAR

SMRT ID1: PGVKGHQRVVTLAQHISEVITQDYTR

NCoR ID2: GHSFADPASNLGLEDIRKALMGSF

SMRT ID2: QAVQEHASTNLGLEAIRKALMGKYD

Figure 1. NCoR and SMRT sequence alignment templates

(a). CoRNR ID1 and ID2 alignment of NCoR and SMRT, which utilizes an IxxII motif. The corrected I/LxxI/VIxxxF/Y/L extended motif based on the ID1 NCoR Rev-erba crystal structure is indicated with red text and shifted -4Leu2051 in blue. (b) The previously predicted LxxI/HIxxxL/I corepressor sequence alignment based on ID2 SMRT bound to PPAR α with GW6471 is indicated with blue text. (c) **Cocrystal structure of Rev-erba Δ 323–423 LBD with NCoR CoRNR ID1 (2040–2065).** Global front view of the NCoR CoRNR ID1 peptide β -strand, β 1N and α -helix, α 1N (magenta) bound to the Rev-erba LBD (yellow) with a mostly canonical nuclear receptor fold. Backbone of contact residues

in helices α_3 , α_4 , α_5 and the new C-terminal Y-domain β -strand, sY, are shown in green with supporting α_3 residues in brown. The sequence deleted and unstructured region between α_1 and α_3 is connected by a red line and the unstructured region between α_5 and α_6 by a black line. **(d)** Zoomed top view showing the Rev-erba and NCoR anti-parallel β -sheet T2045HRLI (−10 to −6) and −4L2051 transition into the α -helix that encompasses the +1I2055-CQITQDF (+1 to +9) extended motif and terminates after the water mediated hydrogen-bonds between the +8D2062 CO and the side-chain of the conserved α_3 charge clamp residue, K455. Distances in Å and hydrogen-bonds depicted with solid orange lines.

Figure 2. NCoR ID1 bound to Rev-erba compared to SMRT and NCoR ID2 bound crystal structures

(a) Overlay of ID1 NCoR bound Rev-erba (magenta, yellow and green) and ID2 SMRT bound PPARα/GW6471 crystal structures (orange, cyan/orange sticks, 1KKQ). Both ID1 and ID2 corepressor peptides have a helical structure that occupies the classical coactivator cleft between helices α3 and α4. Only the ID1 NCoR αhelix extends further towards the bottom of Rev-erba and forges the novel β-sheet that overlaps with PPARα residues just prior to the displaced PPARα AF2 helix α12 with its conserved Y464 colored red. Striped blue residues indicate the highly conserved α3 helix lysine. (b) Decomposed superposition of ID1 NCoR peptide from Rev-erba compared to crystal structures of ID2 SMRT from

PPAR α (peptide length 22-mer, 1KKQ), PR (17-mer, 20VH), ERR γ (21-mer, 2GPV) and ID2 NCoR PR (25-mer, 20VM). The vertical lines clearly show the Rev-erb bound NCoR ID1 ICQII core motif is in register with all the ID2 LxxLL motifs of SMRT and NCoR from the ligand antagonized LBD structures and provides the structural basis for revision of previously reported corepressor alignments. Right flank extension of the ID1 NCoR motif to +9 F2063 is also supported by alanine mutagenesis. Revised and extended CoRNR motif sequences for both ID21 and ID2 of SMRT and NCoR are shown at the top.

Figure 3. Rev-erba repression requires the IxxII CoRNR box motif of NCoR

(a) GST-Rev-erba LBD was incubated with increasing amounts of wild-type NCoR ID1 peptide or the indicated alanine mutants in a TR-FRET assay. Binding affinity and fold decrease for alanine mutants: -9His 0.54 / 2.5, -7Leu 0.57 / 2.5, -4Leu 0.60 / 2.7, -1His 0.25 / 1.2, +1Ile 50.3 / 229.0, +4Ile 8.1 / 36.8, +5Ile 19.4 / 88.2 and +9Phe 1.9 / 8.6 compared to 0.22 μ M / 0 for control wild type NCoR ID1 (2040–2065). (b). 293T cells were co-transfected with Gal-DBD or Gal-NCoR ID1 wild-type or mutants and either VP16 or VP16 fused to full-length Rev-erba, as well as a 5xUAS luciferase reporter and β -galactosidase (to measure transfection efficiency). Relative interaction was determined as luciferase units normalized to β -galactosidase activity. All data is mean of three independent experiments $\pm$ SEM. (c) **Nuclear hormone receptors utilize different modes of corepressor binding.** Gal ID1/2 construct is shown, which utilizes the two-turn “CoRNR alignment”. Gal ID1/2-RKAL construct is shown, which utilizes the N-terminally extended

α -helical motif sequence as in Fig 1b. **(d)** Mammalian two-hybrid assay using indicated Gal-fusions co-transfected in 293T cells along with VP16-thyroid receptor (TR) and Gal-5xUAS-luciferase reporter. Following normalization to β -gal control, relative interaction is shown normalized to Gal-DBD.

Figure 4. Structural changes for NCoR bound Rev-erb α versus ligand free Rev-erb β
 (a) Global superposition for NCoR Rev-erb α (yellow/green/brown) and the Rev-erb β (gray/orange) without corepressor peptide (2V0V) where the boxed region shows the respective differences between the $\alpha 3$ helix orientations and the secondary structure changes in the C-terminal Y-domain. (b) Expanded view of the boxed area showing the respective C-terminal Y-domain secondary structure differences. NCoR bound Rev-erb α adopts anti-parallel β -sheet structure where $\beta 1N$ is underpinned by L606 and sY benefits from hydrophobic packing by F609 and L607 on top and below by W436 and the W436 to Leu606 and E437 to -9H2046 hydrogen-bonds. This configuration draws the $\alpha 3$ helix away from the receptor

hydrophobic core by as much as 3.9Å based on the E437 or E403 α -carbon distance difference. The NCoR-free Rev-erb β Y-domain adopts an α -helical structure, α 11, that uses conserved equivalents F575 and L572 to provide packing between the α 3 and α 11 helices with L572 (cyan) lying very near the space occupied by NCoR –4L2051. **(c)** Decomposed view of **b** showing the C-terminal α 11, the H568 residue and α 3 residue conformational changes in the peptide free Rev-erb β crystal structure. **(d)** Decomposed view of **b** showing the C-terminal sY β -strand and the α 3 residue conformational changes in the NCoR peptide bound Rev-erb α crystal structure.

Figure 5. Structural changes for NCoR bound Rev-erbα versus heme bound Rev-erbβ
(a) Global superposition for NCoR Rev-erbα (yellow/green/brown) and heme bound Rev-erbβ (pink/violet/orange) (3CQV) with boxed region of interest. Relative to α3 with NCoR, the heme binding induces a large and clearly visible left shift whose magnitude can be gauged visually by comparing the positional difference between the conserved βW402/E403 (orange) and αW436/E437 (brown) residues. Less pronounced structure change is visible for α5. **(b)** Clockwise rotated view of expanded box region illustrating severe overlap of the NCoR mediated β-sheet and the heme shifted α3. Direct and close contacts are noted between the heme porphyrin to NCoR –4L2051 and from the restructured αF443/βF409 to

–1H2054 and α F609. Should similar changes occur in Rev-erb α , the secondary structure switch from β -sheet to α 11 helix would require that α L606 retract to a location similar to β L572 (cyan). **(c)** Decomposed view of **b** showing the Rev-erb β C-terminal α 11 helix and conserved α 3 helix residue conformational changes. Noted differences show the remodeled conformation for E403 and W402 and H568 that ligates heme and the retracted α 11 L572 and F575 with side-chains (cyan). **(d)** Decomposed view of **b** showing the Rev-erb α C-terminal sY β -strand, and the α 3 helix residue conformational changes. Noted differences show the orientations for E437 and W436 underpinning the sY β -strand and the alternate rotamer adopted by the H602 imidazole in the absence of heme.

Table 1

Data collection and refinement statistics

	Rev-erba (281–614, Δ324–422) NCoR ID1 (2045–2065)
Data collection	
Space group	R32
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	112.5 112.5 103.83
α, β, γ (°)	90 90 120
Resolution (Å)	50.0 -2.60 (2.69-2.60)
<i>R</i> _{sym} or <i>R</i> _{merge}	0.049 (29.6)
<i>I</i> / σI	50.5 (7.3)
Completeness (%)	99.9 (99.4)
Redundancy	10.9 (9.8)
Refinement	
Resolution (Å)	30.0 - 2.6Å
No. reflections	7693
<i>R</i> _{work} / <i>R</i> _{free}	19.9 / 26.6
No. atoms / No. residues	1612
Protein	1382
Ligand	168
Water	62
<i>B</i> -factors	
Protein	49.8
Ligand/ion	46.5
Water	53.0
R.m.s. deviations	
Bond lengths (Å)	0.010
Bond angles (°)	1.126

* Data was derived from one crystals

* Values in parentheses are for highest-resolution shell.