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Protein-protein interactions in the regulation of RAR–RXR heterodimers transcriptional activity

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Abstract

The three retinoic acid receptor subtypes (RAR α , RAR β and RAR γ) act as ligand-inducible transcription factors binding to DNA regulatory elements in the promoter regions of target genes by forming heterodimers with the retinoid X receptors (RXR α , RXR β and RXR γ). They act as ligand-dependent transcription factors that regulate a large variety of genes involved in cell growth, differentiation, survival and death. The (patho)physiological functions of RAR–RXR heterodimers rely on a dynamic sequence of protein-protein interactions, many of which being modulated by natural (retinoic acid) or synthetic ligands. Direct protein-protein interactions include heterodimerization between RARs and RXRs, recruitment (and release) of transcriptional coactivators and corepressors, cross-talk with other transcription factors, including nuclear receptors, or transient association with many enzymes involved in post-translational modifications to cite the most prominent ones. This chapter describes structural, biochemical, biophysical and cell-based assays to monitor protein-protein interactions relevant to the retinoic acid signaling pathways with a focus on those for which a structural description has been provided.

Keywords: Retinoid receptors, RAR, RXR, coactivators, corepressors, protein-protein interactions, ligands, transcription, dimerization

1. Introduction

All-*trans*-retinoic acid (RA) plays crucial roles in a wide variety of biological processes including embryonic morphogenesis and organogenesis, cell proliferation, differentiation and apoptosis, homeostasis, as well as in their disorders. These pleiotropic effects are mediated through retinoic acid receptors (RARs) consisting of three subtypes, α (NR1B1), β (NR1B2), and γ (NR1B3) belonging to the nuclear receptor (NR) superfamily (Germain et al., 2006a). RARs form functional heterodimers with retinoid X receptors (RXR α , β and γ , NR2B1-3) which bind to specific RA-responsive elements (RAREs) located in target gene promoters and regulate gene expression in a retinoid dependent manner (Germain et al., 2006b; Gilardi & Desvergne, 2014). Like other members of the NR family, RARs and RXRs display a modular structure with a variable N-terminal activation function (AF-1), a central conserved DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Fig. 1A). The multifunctional LBD is responsible for ligand binding or dimerization and contains a ligand-dependent activation function (AF-2), which corresponds to coregulator interaction surfaces that can be modulated by natural or pharmacological ligands (Gronemeyer, Gustafsson, & Laudet, 2004). The N-terminal domain is essentially unstructured, yet it contains phosphorylation sites which are known to modulate the interaction with Src-homology-3 (SH3) and tryptophan-tryptophan (WW) domains.

[Insert Figure 1 here]

In contrast both DBD and LBD display prototypical folds. The DBD, whose core is approximately 66 residues, comprises two zinc-binding motifs with one N-terminal recognition helix inserting into the major groove of the DNA and a second perpendicular helix stabilizing the core of the DBD and the interaction (Fig. 2). The LBDs of RARs and RXRs are organized in a primarily helical scaffold, termed “antiparallel α -helical sandwich”, of 12 helices (H1–H12) and a short β -turn (S1–S2) arranged in three layers. Helices H1–H3 form one face, H4, H5, S1-S2, H8, and H9 comprise the central layer, and H6, H7, H10 and H11 constitute the second face (Fig. 2). The C-terminal helix H12 is flexible so that its position and dynamics vary according to the nature of the bound ligand, thereby modulating the binding and release of specific transcriptional coregulators. The helical arrangement of the LBD generates a mostly hydrophobic ligand-binding pocket (LBP), a dimerization surface (H7, H9 and H10) and interaction surfaces involved in the binding of multiple coregulators (H3, H4 and H12).

Historically, most of the structural information on RARs and RXRs DBDs and LBDs has been gained from studies of isolated domains. It is only recently that the first structures of nearly full-length receptors bound to their cognate response elements have been reported based on crystallographic and solution studies, thereby revealing their overall topologies and the role of DNA in domain organization and communication (Chandra et al., 2017; Rochel et al., 2011) (Fig. 2). Interestingly, the structures of the isolated DBDs and LBDs or within the entire receptors appear very similar, indicating that no major internal domain distortion is required upon establishment of the DNA-bound multidomain heterodimer. Nevertheless, physical interconnections between the various receptor subunit domains may allow the transmission of signals between distant domains in an allosteric manner, making ligand-, DNA-, and coregulator-binding interdependent events (Khorasanizadeh & Rastinejad, 2016).

[Insert Figure 2 here]

Retinoid receptors mainly function as ligand-regulated transcription factors whose activity depends on multiple interacting proteins including a diverse group of transcriptional coregulators, other transcription factors or enzymes involved in post-translational modifications, resulting in the cross-talk between different signaling pathways. Note also that a small fraction of RARs is present at the plasma membrane and exert some extra-nuclear and non-transcriptional effects through the activation of kinase signaling pathways (for a review see (Piskunov, Al Tanoury, & Rochette-Egly, 2014)). RXR–RAR heterodimers may act either as repressors or activators of gene transcription depending on their ligation status that in turn determines the ability of DNA-bound receptors to recruit so-called “corepressors” or “coactivators”. Coactivators and corepressors correspond to large groups of proteins with a varied range of protein activities and enzymatic functions that contribute to transcription regulation by many transcription factors. Coactivator recruitment is usually ligand-dependent, whereas corepressors interact in most cases with unliganded receptors. Coactivators can be subdivided into two groups. Primary coactivators, such as those of the TIF-2/SRC-1/RAC3 (p160) family, mediate the interaction of coactivator complexes with NRs. CBP, p300, P/CAF, and some p160 coactivators themselves are reported to act as histone acetyltransferases (HATs) (Glass & Rosenfeld, 2000; Lonard & O'Malley B, 2007). They are capable of acetylating specific residues in the N-terminal tails of

different histones, a process that is believed to play an important role in the opening of chromatin during transcription activation (Chen, Lin, Xie, Wilpitz, & Evans, 1999; Imhof et al., 1997).

Secondary coactivators represent a subgroup of molecules that are constituents of multisubunit coactivator complexes and that also contribute to the enhancement of NR-mediated transcription, but that do not directly contact the NRs. Corepressors N-CoR and SMRT have been shown to reside in, or recruit, high molecular weight complexes that display the opposite activity of coactivator complexes. While coactivator complexes acetylate histones, thereby weakening the interaction of the N-terminal histone tails with the nucleosomal DNA, corepressors recruit histone deacetylases (HDACs) that reverse this process (Heinzel et al., 1997; Nagy et al., 1997). Deacetylated histones are associated with silent regions of the genome, and it is generally accepted that histone acetylation and deacetylation shuffle nucleosomal targets between a condensed and relaxed chromatin configuration, the latter being requisite for transcriptional activation. Some histone-modifying enzymes other than HATs and HDACs, such as chromatin remodelers and histone chaperones, have been documented to serve as NR coregulators (Kato, Yokoyama, & Fujiki, 2011). Following ligand-induced nucleosome remodeling, the recruitment to the promoter region of the transcription machinery (RNA Polymerase II, General Transcription Factors and the Mediator complex) can occur, leading to the transcription of target genes. Transcription termination could be achieved by RAR ubiquitinylation and degradation by the proteasome.

Progress in NR structure research has revolutionized our view on how NRs in general and retinoid receptors in particular act as essential regulators of fundamental cellular processes. The structural principles of the interaction of receptors with their DNA response elements, ligands and coregulators have been decoded through crystallographic and other biophysical approaches revealing the link between NR conformation, ligand-induced allosteric changes and their resulting abilities to communicate with the intracellular components.

2. Structural basis of RXR–RXR and RXR–RAR interactions

Dimerization is a general mechanism to increase binding site affinity, specificity, and diversity. In this regard, RXRs play a central role in various signal transduction pathways since they can both

homodimerize and act as promiscuous heterodimerization partner for almost fifteen NRs. Crystal structures of DBD and LBD homo- and heterodimers have defined the surfaces involved in dimerization. As exemplified with the RAR–RXR heterodimer (Fig. 2), two types of dimerization functions mediate homo- and heterodimerization. One involves several surface residues in the DBD that establish weak response element-specific interfaces with corresponding surfaces in the partner DBD. The second is a strong dimerization function in the LBDs of both partners that differs between homo- and heterodimers and to some extent between the partners of RXR. The structures of RXR LBD heterodimers with RAR (Bourguet, Vivat et al., 2000; Pogenberg et al., 2005; Sato et al., 2010), PPAR (Gampe et al., 2000), TR (Putcha, Wright, Brunzelle, & Fernandez, 2012), LXR (Svensson et al., 2003), FXR (N. Wang, Zou, Xu, Zhang, & Liu, 2018), PXR (Wallace et al., 2013) and CAR (Suino et al., 2004; R. X. Xu et al., 2004) or full-length RAR β –RXR α (Chandra et al., 2017), PPAR γ –RXR α (Chandra et al., 2008) and LXR β –RXR α (Lou et al., 2014) heterodimers demonstrate a topologically conserved dimerization surface with identical structural elements generating the interface (Fig. 2). Residues from helices H7, H9, H10, as well as loops L8–9 and L9–10 of each protomer form an interface comprising a network of complementary hydrophobic and charged residues and further stabilized by neutralized basic and acidic surfaces. Amino acid variations at the surface of the various NRs determine their specific dimerization characteristics.

3. Molecular paradigms governing interactions between RXR–RAR and coregulators

3.1. Coactivators

Coactivators (Fig. 1B) interact directly with the LBD of both RXR and RAR in an agonist-dependent manner by means of short signature sequences located in their nuclear receptor interacting domains (NRIDs). These domains are typically composed of three to four repeats of LxxLL motifs, in which x is any amino acids and L is leucine, embedded in an α -helical peptide (NR box) and connected by disordered linker regions. It is now well established that these motifs are necessary and sufficient to mediate the interaction of coactivators with a cognate surface in the nuclear receptor LBD. A number of crystal structures of RAR bound to both an agonist and a coactivator fragment containing the LxxLL motif have been reported (le Maire et al., 2010; Pogenberg et al., 2005). As with other

receptors, the C-terminal helix H12 seals the LBP and together with helices H3, H4, and the loop L3-4 generates a hydrophobic surface which accommodates the short LxxLL-containing helical motif. The helix is held in place *via* interactions of its leucine residues with the hydrophobic receptor groove but also through hydrogen bonds that involve two conserved residues. These amino acids are a lysine at the C-terminus of H3 and a glutamate in H12 (Fig. 3A). Both are hydrogen-bonded to a main-chain peptide bond of the LxxLL motif and together form a “charge clamp” that stabilizes the interaction and defines the precise length of the helical motif that can be accommodated by the cleft.

[Insert Figure 3 here]

The fact that coactivators often contain multiple NR boxes, most of which appear to be functional in terms of NR binding *in vitro*, brought up the question of whether this multiplicity reflects redundancy or may confer some specificity to the interface. Structural studies using the entire NRID of several coactivators and various RXR heterodimers have been conducted by Small Angle X-ray Scattering (SAXS), analytical ultracentrifugation, Nuclear Magnetic Resonance (NMR), Hydrogen-Deuterium exchange coupled to mass-spectrometry (HDX-MS) or Förster Resonance Energy Transfer (FRET). The data reveal that ligand and DNA binding cooperatively enhance coactivator interaction, thereby suggesting that allosteric communication integrating signals from ligand, DNA and coactivator play a role in DNA recognition, promoter specificity and gene regulation (de Vera et al., 2017; J. Zhang et al., 2011). However the details of the interaction, including stoichiometry or the involvement of each protomers of the heterodimers and of the LxxLL motifs of the coactivators, remain poorly understood. Whereas ligand binding to RXR was shown to be the primary driver of coactivator recruitment to the PPAR γ -RXR α heterodimer (de Vera et al., 2017), another study proposed that the coactivator only binds to the liganded partner receptor within the context of RAR-RXR (Rochel et al., 2011). In the same line, the presence of the ligands of both RXR and the partner receptor (*e.g.* PPAR, RAR) contribute to enhance the interaction, but whether this ligand combination stabilizes the interaction of one LxxLL motif on each receptor (deck model) is not known.

3.2. Corepressors

In their unliganded form or in the presence of so-called inverse agonists, a number of NRs, including RARs, act as repressors of transcription by recruiting corepressors to their target genes. Analyses of biochemical data and protein sequences provided evidence that coactivator and corepressor recruitments share similar molecular features and involve topologically related NR surfaces, with the important difference that the helix H12 is not involved in the interaction with corepressors. Similarly to coactivators, the NRID of SMRT and N-CoR is an intrinsically disordered domain with three functional LxxI/HIxxxI/L corepressor/nuclear receptor box (CoRNR box 1-3) motifs (Fig. 1C). CoRNR boxes are not equivalent as, for example, RAR interacts preferentially with CoRNR1, and further biochemical studies revealed that residues flanking the core helical sequence determine nuclear receptor specificity (Hu, Li, & Lazar, 2001; Perissi et al., 1999; H. E. Xu et al., 2002). The structural basis of the repression function of RAR has been revealed through crystallographic studies of RAR α LBD in complex with a peptide containing the CoRNR1 sequence of N-CoR (le Maire et al., 2010). The structure reveals that the interaction of RAR α with corepressors utilizes the bipartite motif CoRNR1 that contains a four-turn helical motif LxxxIxxI/VIxxxF/Y docking to a hydrophobic surface formed by residues from helices H3 and H4 of the receptor and a N-terminal extended β -strand (β 1) forming a specific antiparallel β -sheet with specific RAR residues (Fig. 3B). Interestingly, the RAR residues which adopt the β -strand conformation S3 in the corepressor-bound structure belong to helix H11 in the agonist-bound structure, and mutations affecting specifically the integrity of either β -strands (β 1 or S3) abrogated corepressor interaction and transcriptional repression by RAR (le Maire et al., 2010). The secondary structure change from α -helix to β -strand has also been observed in the structure of the heme receptor RevErb bound to CoRNR1 of N-CoR (Phelan et al., 2010) but not with other NRs that have been shown to interact rather weakly with the shorter CoRNR2 motif of corepressors (Madauss et al., 2007; L. Wang et al., 2006; H. E. Xu et al., 2002). Thus, the basal repressive activity of RAR is conferred by an extended β -strand that forms an antiparallel β -sheet with specific corepressor residues. Agonist binding induces a β -strand to α -helix transition that allows helix H11 formation which in turn provokes corepressor release, stabilization of the mobile helix H12 in the

so-called “active conformation”, and coactivator recruitment. More recently, combination of a large set of biophysical and computational methods revealed that while the NRID of corepressors is mainly disordered, it presents transient but robust intramolecular contacts upon interaction with the heterodimer, indicating that disorder-to-order transitions of both receptors and corepressors are key events in the regulation of RXR–RAR heterodimers (Cordeiro et al., 2019). The study also showed that the NRID forms a dynamic complex with RAR–RXR with singly and doubly bound states.

4. Protocols for the study of protein-protein interactions

4.1. Two-hybrid analysis

The mammalian two-hybrid assay can be recommended to investigate interactions between nuclear receptors and transcriptional co-regulators in a cellular environment. This cell-based reporter gene assay has been adapted from the principle of yeast two-hybrid system to mammalian cells. This method was originally conceived to reveal protein-protein interaction using the GAL4 transcriptional activator of the yeast *Saccharomyces cerevisiae* (Fields & Song, 1989). The assay is based on the fact that most eukaryotic transcription factors consist of distinct functional and physical domains. The DNA binding domain (DBD) mediates binding of these proteins to the gene promoter by sequence specific DNA recognition and the activation domain coordinates the assembly of the factors required for transcription allowing RNA polymerase II to transcribe the reporter gene downstream of the DBD. Both domains are required for the induction of gene expression, but they do not have to be present within the same protein. Thus, if the DBD of a transcription factor is fused to a bait protein and a second prey protein is fused to the activation domain of the transcription factor, efficient gene induction will be seen if the two proteins bind to each other, thereby bringing the two modules into spatial association. However, no gene expression will be observed if the two transcription factor modules are not brought in proximity to each other. Reporter genes encode for proteins that are distinguishable and easily quantified in the background of endogenous proteins. The choice of a reporter gene takes into account various parameters such as detection sensitivity, rapidity, convenience in quantitation, and the possibility of measuring gene expression in living cells. These criteria have led to the preferential use of firefly luciferase as the reporter gene in an increasing number of studies.

Quantification relies on enzymatic assays of this protein. In addition to the reporter used to measure the transcriptional response, a second reporter, generally the bacterial β -galactosidase or the Renilla luciferase, serves as an internal control to normalize the data obtained from the experimental reporter. Transfection corresponds to the process of inserting foreign genetic material into a eukaryotic living cell without killing it. Virtually all mammalian cell lines can be considered. However, the chosen cell line should be relatively cheap, easy to culture, and robust enough to withstand application of the investigate methods and reagents. In this respect, COS, HeLa, and HEK293T cell lines are widely used. The following is an example of the use of a mammalian two-hybrid system to study NCoR-RAR α interactions (Fig. 4).

Required materials

- COS cell line
- Luciferase-based reporter gene ((17m)5x- β Gal-Luc), cytomegalovirus- β -galactosidase (CMV- β Gal) vector, and BSK plasmid
- pSG5-based Gal-NCoR and RAR α -VP16 chimera expression vectors
- JetPei transfectant (Ozyme, France)
- Lysis buffer: 25 mM Tris phosphate (pH 7.8), 2 mM EDTA, 1 mM DTT, 10% glycerol, and 1% Triton X-100
- Luciferin buffer: 20 mM Tris phosphate (pH 7.8), 1.07 mM MgCl₂, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT
- Luciferase buffer (2x): 40 mM Tris-phosphate, 2.14 mM MgCl₂, 5.4 MgSO₄, 0.2 mM EDTA, 66.6 mM DTT, pH 7.8 (store at -20 °C)
- Luciferase assay reagent: 500 μ l 2 x luciferase buffer, 47 μ l luciferine 10 mM, 53 μ l ATP 10 mM, 27 μ l Coenzyme A (lithium salt) 10 mM 430 μ l distilled water
- β Gal buffer: 60 mM Na₂HPO₄ (12H₂O), 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgCl₂ (6H₂O), 50 mM β -mercaptoethanol
- ONPG (4 mg/ml)
- 24-well plates for cell culture
- Opaque white Optiplate-96-well plates (Perkin Elmer)

- Luminometer (for instance MicroLumat LB96P, Berthold)

Protocol

1. Maintain COS cells in Dulbecco's Modified Eagle Medium (DMEM) with Glutamax and 10% (v/v) fetal calf serum (FCS)
2. Seed 10^5 cells per well in DMEM with Glutamax and 10% (v/v) FCS
3. For each well, prepare the mix of plasmids in 50 μ l of 150 mM NaCl buffer (80 ng Gal-NCoR + 50 ng VP16-RAR α + 150 ng (17m)5x- β Gal-Luc + 50 ng CMV- β Gal + 670 ng BSK plasmid; vortex gently and spin down briefly
4. For each well, prepare the JetPei solution (2 μ l JetPei in 50 μ l of 150 mM NaCl buffer); vortex gently
5. Add the JetPei solution to the DNA solution all at once; vortex gently and spin down briefly
6. Incubate for 15-30 min at room temperature
7. Add 100 μ l of the mix drop-wise to the medium in each well and incubate the cells for 24 h at 37 °C in a 5% CO₂ incubator; transfect 3 wells per condition to have triplicate measurements
8. Replace the culture medium with fresh medium containing the ligand at active concentration and incubate again the cells for 24 h at 37 °C in a 5% CO₂ incubator
9. Remove the medium from the well and gently apply phosphate saline buffer (PBS) to wash the surface of the well
10. Dispense into each well 100 μ l of 1 x lysis buffer and place the plate on an orbital shaker with gentle rocking for 15 min
11. Distribute 30 μ l of lysate and 50 μ l of luciferase assay reagent per well of a 96-well white plate (luciferase assay reagent can be automatically added if a luminometer with injector is used)
12. Perform the measurement of the luciferase activity using a luminometer
13. Distribute 10 μ l of lysate, 150 μ l of β -Gal assay buffer and 30 μ l of ONPG (4 mg/ml) in a 96-well plate
14. Incubate the plate at 37 °C and measure the time until the yellow color has appeared
15. Stop the reaction by adding 75 μ l of 1 M Na₂CO₃

16. Read the absorbance immediately at 420 nm in a plate reader to measure the β -Gal activity (β -Gal unit = 100 x total volume x absorbance/(assay volume x time (h))

17. Normalize the luciferase values with the β -Gal values.

[Insert Figure 4 here]

4.2. Electrophoretic mobility shift assays

The ability of RXR-RAR heterodimers bound to specific DNA sequences to associate with transcriptional co-regulators can be assessed by electrophoretic mobility shift assays (EMSAs). EMSA is also referred as gel retardation assay, gel shift assay, or band shift assay (Hellman & Fried, 2007). In this method, solutions of proteins and nucleic acid are combined and subjected to electrophoresis under native conditions (non-denaturing TBE-polyacrylamide gels or TAE-agarose gels) to resolve protein-DNA complexes from free DNA. The adequate gel percentage is depended on the size of the used linear DNA probe as well as the size, number and charge of the interacting proteins. After electrophoresis, the distribution of species containing nucleic acid is revealed, usually by autoradiography of radiolabeled DNA target with ^{32}P -labeled by 5' end labeling using an $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase. Typically, protein-DNA complexes migrate more slowly than the corresponding free nucleic acid. This method is also recommended for investigating higher-order complexes containing several proteins, observed as a “supershift” assay. Interestingly, proteins of interest may be obtained from a crude nuclear or whole cell extract, *in vitro* transcription product or a purified preparation from various expression systems. Here a protocol is given as example of EMSA to determine the effect of various retinoid combinations on the interaction of both co-repressors (SMRT) and co-activators (TIF2) with the RXR α -RAR α heterodimer bound to a specific DR5 DNA sequence (le Maire, Teyssier, Balaguer, Bourguet, & Germain, 2019) (Fig. 5).

Required materials

- The TNT® Quick Coupled Transcription/Translation System (Promega, Madison, WI 53711, USA)
- pSG5-based RXR α Δ AB and RAR α Δ AB (receptors deleted of their AB domain, harboring both DBD and LBD) expression vectors
- Purified TIF2 (TIF2.42 residues 624–828 containing the NRID) and SMRT (SMRTct residues 982 to end containing the NRID)

- ³²P-DR5 (5'-TCGAGGGGTAGGGGTCACCGAAAGGTCACCTCG-3'; direct repeat underlined) oligonucleotide; specific radiolabeled DNA sequence recognizing RXR-RAR heterodimers
- Binding buffer: 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.4 mM DTT, 5% glycerol
- TBE (10 x): 108 g Tris base, 55 g boric acid, 4 ml 0.5 M EDTA (pH 8.0)
- Dye (10 x): 0.1 % bromophenol blue, 0.1 % xylene cyanol, 60 % glycerol
- Vertical electrophoresis apparatus and electrophoresis power supply
- Autoradiography film cassette
- Kodak XAR-5 film (with film developer) or cassette and storage phosphor screen (with a phosphorimager instrument)
- Vacuum gel drier

Protocol

1. Produce RXR α Δ AB and RAR α Δ AB receptors by *in vitro* transcription–translation using the TNT rabbit reticulocyte lysate system programmed with pSG5-based expression vectors according to the instructions provided by the manufacturer
2. Mix lysates containing RXR α Δ AB and RAR α Δ AB on ice for 30 min (volumes of TNT reaction products containing the receptors are indicated in the legend of Fig. 5)
3. Add ligands (stock solution in ethanol) on ice at a final concentration of 1 μ M and incubate for 10 min
4. Add both purified transcriptional co-regulators TIF2 and SMRT on ice for 10 min (amounts of co-regulators are indicated in the legend of Fig. 5)
5. Mix Proteins (volumes of TNT reaction products containing the receptors and concentrations of co-regulators are indicated in the legend of the figure 5) and pre-annealed DNA probe that is labeled with ³²P (approximately 25 000 c.p.m.)
6. Incubate for 15 min on ice the mix proteins–DNA in a final volume of 20 μ L binding buffer, containing 2 μ g poly(dI-dC), as well as 150 mM KCl and 20 mg/mL BSA
7. Add 10 % of 10 x dye and carefully mix by pipetting; immediately load onto the gel (use Hamilton syringe)

8. Resolve protein-DNA complexes through 6% polyacrylamide gels in 0.5 x TBE buffer for 2 h at 100 V/4°C (pre-run the gel for 3 hours at 200 V/4°C)

9. Dry the gel for at least 45 min and expose to x-ray film for desired period of time

[Insert Figure 5 here]

4.3. Protein expression and purification

The availability of homogeneous NR monomers and dimers is a prerequisite for biochemical and biophysical studies addressing interactions of NRs with partners (other NRs, coregulators, DNA). A number of protocols have been optimized for such purifications (Bourguet, Andry et al., 2000; Iyer et al., 1999). These protocols apply to both isolated LBDs and full-length receptors. The following protocol is an example given for a rapid two-step copurification procedure yielding nonaggregated and functionally homogeneous RAR α -RXR α LBD heterodimers in large quantities suitable for structural and other *in vitro* studies.

Required materials

- pET-15b and pET-3a expression vectors (Novagen)
- Competent E. coli BL21(DE3) cells (Novagen)
- Shaker/incubator set at 37°C
- Sonicator
- Centrifuge
- Liquid chromatography system (AKTA purifier, GE Healthcare)
- Lysis buffer: 20 mM Tris-HCl pH 8.0, 500 mM NaCl,
- Elution buffer: 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 500 mM imidazole
- Gel filtration buffer: 20 mM Tris-HCl pH7.5, 150 mM NaCl, 2 mM DTT
- HisTrap chelating columns (GE Healthcare)
- HiLoad 26/60 Superdex 75 column (GE Healthcare)
- UV-Vis spectrophotometer (NanoDrop, Thermo Scientific)

Protocol

1. Express the RAR α LBD and the RXR α LBD cloned, respectively, into the pET-15b vector

(as a histidine-tagged protein) or into the pET-3a vector in *E. coli* BL21 (DE3) cells. Cells are grown at 37°C in LB supplemented with 50 µg/ml of ampicillin to an OD₆₀₀ of 0.6–0.8, and induce T7 RNA polymerase expression by the addition of 1 mM IPTG to 1 mM. After an additional incubation overnight at 25°C, harvest the cells by centrifugation (5000g during 15 min).

2. Resuspend the pellets together (usually 3 L of RARα LBD and 2 L of RXRα LBD) in 100 ml of ice-cold lysis buffer. Sonicate the suspension three times (60% amplitude) for 2 min and centrifuge (18 000 rpm, 30 min) to obtain the crude extract.

3. Pass the crude extract over a 5 mL HisTrap chelating column previously equilibrated with 98% of lysis buffer + 2% of elution buffer, at a flow rate of 5 mL/min. Wash the column with 20 volumes of , 98% of lysis buffer + 2% of elution buffer. Remove unspecific bounded protein and potential excess of RXRα LBD by passing 10 volumes of 90% of lysis buffer + 20% of elution buffer. Elute the heterodimer with 20 volumes of 50% of lysis buffer + 50% of elution buffer. Analyze the elution fractions by SDS–polyacrylamide gel electrophoresis, pooled, and concentrated until 5 ml.

4. Optionally, cleave the His tag from RARα LBD by adding thrombin (1 unit per mg of protein), overnight in ice.

5. Further purify the heterodimer by gel filtration using a HiLoad 26/60 Superdex 75 column preequilibrated with the gel filtration buffer. Analyze the 2 ml by SDS–polyacrylamide gel electrophoresis, pooled, and concentrated to 5 mg/mL using Centriprep 30 for subsequent in vitro analysis.

6. At this step, the structural homogeneity of the preparations can be evaluated by electrophoretic analysis using nondenaturing conditions (native gels), dynamic light scattering (DLS), or circular dichroism (CD).

4.4. Polarization of fluorescence

Steady-state fluorescence anisotropy relies on the differences in rotational mobility of bound vs free molecule. It is a widely used method for investigating protein-protein, protein-DNA and protein-ligand interactions as polarization measurements are done at equilibrium and can be easily and rapidly monitored. The recruitment of coregulators by RXR and RAR LBDs has been characterized by measuring the affinities (Kd) of fluorescently-labeled peptides derives from CoAs and CoRs, in the

absence or presence of different ligands (Cordeiro et al., 2019; le Maire et al., 2010; Pogenberg et al., 2005). The following protocol is an example given for the determination of the binding affinities of a peptide of PGC-1 α corresponding to the NR box 2 (EEPSLLKKLLLAPA) for RXR α LBD in its unliganded form and in the presence of the full RXR α agonist CD3254 that reinforces the interaction (Fig. 6) (le Maire et al., 2009).

Required materials

- Safire2 microplate reader (TECAN) or CLARIOstar (BMG Labtech)
- Corning NBS 384 well low volume microplates (Corning Incorporated)
- Anisotropy buffer : 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 10% glycerol
- Fluorescein-labeled PGC-1 α (FITC-EEPSLLKKLLLAPA) at 20 mM in DMSO
- Ligand CD3254 at 10mM in DMSO
- Purified RXR α LBD at 5–10 mg/ml

Protocol

1. Prepare 60 μ L of protein stock solution diluted at two times the maximal concentration in anisotropy buffer (C_{max}) and add the ligand at 3 molar excess ($3 \cdot C_{max}$). In fact, the protein will be diluted 1:1 with the fluorescent target (see step 5), so the final protein concentration will be half of the stock concentration. In order to saturate the binding event, the maximum protein concentration should be 10 to 20 fold above the expected K_d .
3. Dispense 30 μ l in the first well. Add 30 μ L of anisotropy buffer into the remaining 30 μ L of the protein solution prepared in step 1, mix gently. Transfer 30 μ L of this mix in the second well.
4. Repeat step 3 until the last well.
5. Prepare 500 μ L of peptide solution containing 8 nM of fluorescent peptide in anisotropy buffer and dispense 30 μ L in each well. Of anisotropy buffer in an additional well as a reference measurement and to ensure that the total intensity of wells containing the fluorescent peptide is at least ten times more than ones of this reference well.
6. Determine anisotropy values of the Safire 2 or ClarioStar microplate readers with the excitation wavelength set at 470 nm and emission measured at 530 nm, for a FITC fluorophore.

7. Fit binding data and determine K_d using a sigmoidal dose-response model in GraphPad Prism (GraphPad Software, San Diego).

[Insert Figure 6 here]

4.5. Electrospray ionization mass spectrometry

Since the nineties, electrospray ionization mass spectrometry (ESI-MS) has been regularly used to study noncovalent complexes and offers new possibilities for the study of such complexes, providing direct evidence for their formation and an accurate determination of their binding stoichiometry (Fenn, Mann, Meng, Wong, & Whitehouse, 1989; Loo, 1997; Rajabi, Ashcroft, & Radford, 2015). For instance ESI-MS was previously used to monitor the ligand-mediated interactions between RXR α -RAR α heterodimers and transcriptional coregulators (Sanglier et al., 2004). Hence supramolecular mass spectrometry is a powerful tool to rapidly and unambiguously NRs-protein interactions. The following protocol is an example given for the use of ESI-MS under nondenaturing conditions to monitor the interaction between the RXR–RAR LBD heterodimer and CoRNR1 of the corepressor NCoR (Fig. 7).

Required materials

- Electrospray time-of-flight mass spectrometer (LCT, Waters)
- Buffer A: 50 mM ammonium acetate pH 6.5
- Purified RXR α -RAR α LBD heterodimer at 5–10 mg/mL (see above for the description of a purification method)

Protocol

1. Calibrate the instrument using the multiply charged ions produced by an injection of horse heart myoglobin diluted to 2 pmol/mL in a water/acetonitrile mixture (1:1, v/v) acidified with 1% (v/v) formic acid.
2. Prior to ESI-MS analysis, desalt the samples on Centricon PM30 microconcentrators (Amicon, Millipore) in buffer A.
3. Verify purity and homogeneity of the samples in denaturing conditions by diluting the complex solution to 5 pmol/mL in a water/acetonitrile mixture (1:1, v/v) acidified with 1% (v/v) formic acid.

Record spectra in the positive ion mode on the mass range 500–2500 m/z. Verify that the measured molecular masses are in agreement with those calculated from the amino acid sequences.

4. Dilute samples to 10 pmol/mL in buffer A and infuse continuously into the ESI ion source at a flow rate of 6 mL/min through a Harvard syringe pump. The accelerating voltage (V_c) must be set to 50 V in order to preserve ternary complex formation and good mass accuracy. ESI-MS data are acquired in the positive ion mode on the mass range 1000–5000 m/z.

5. Measure the relative abundance of the different species present on ESI mass spectra from their respective peak intensities, assuming that the relative intensities displayed by the different species reflect the actual distribution of these species in solution.

[Insert Figure 7 here]

4.6. Microscale thermophoresis (MST)

MicroScale Thermophoresis is a method to quantify biomolecular interactions and its applications range from small-molecule binding events to protein-protein, protein-DNA interactions and interactions of multi-protein complexes (Jerabek-Willemsen, Wienken, Braun, Baaske, & Duhr, 2011; W. Zhang, Duhr, Baaske, & Laue, 2014). It measures the motion of molecules along microscopic temperature gradients and detects changes in their hydration shell, charge or size. By combining the fluorescence detections with thermophoresis, MST provides a way to accurately measure affinity constants (K_d). The following protocol is an example given for the determination of the binding affinities of the nuclear interaction domain (NRID) of the corepressor NCoR for the heterodimer RXR α -RAR α LBDs in the presence of various ligands (Cordeiro et al., 2019).

Required materials

- Monolith NT.115 (NanoTemper)
- Monolith NT.115 Premium Capillaries (NanoTemper)
- MST optimized buffer: 50 mM Tris-HCl pH7.4, 150 mM NaCl, 10 mM MgCl₂, 0.05 % Tween-20
- Purified N-CoR_{NID} fragment to which a fluorescent probe (Atto647N maleimide, Invitrogen) was attached to the thiol group of its sole cysteine, according to the manufacturer's instructions.
- Purified RXR-RAR LBDs heterodimer at 5-10 mg/ml
- Ligands (AM580 and BMS493 from Tocris) at 10mM in DMSO

Protocol

1. Prepare 16 small reaction tubes of a volume inferior to 200 μL (delivered by NanoTemper with the capillaries).
2. Prepare 20 μL of protein stock solution diluted at two times the maximal concentration in MST buffer. In fact, the protein will be diluted 1:1 with the fluorescent target (step 4), so the final protein concentration will be half of the stock concentration. In order to saturate the binding event, the maximum protein concentration should be 10 to 20 fold above the expected K_d .
3. Transfer 10 μL of this stock solution in tube 1. Add 10 μL of MST buffer in the 10 μL left of your protein stock solution and mix very well by carefully pipetting up and down several times. Transfer 10 μL of this mix to tube 2. Continue until you reach tube 16 to obtain a serial dilution.
4. In each tube, add 10 μL of fluorescently labelled molecule (at double the concentration of the final desired concentration, 80nM in our case) to the 10 μL of the serial protein dilution and mix well by pipetting up and down several times.
5. Incubate at conditions of your choice (room temperature or on ice) before loading into the capillaries, during 10 minutes.
6. Fill the capillaries with the mix by soaking each capillary in each tube.
7. Put the capillaries into the tray of the machine, and start an MST measurement using a LED power to obtain 200-1500 units and 40% MST power.
8. Load the results in the MO.Affinity Analysis software (NanoTemper). To derive a binding curve from the MST traces, the fluorescence ration before and after heating is calculated and plotted against the ligand concentration. A fitting by a sigmoidal dose-response curve allows determine the binding affinity of the interaction.

[Insert Figure 8 here]

4.7. Thermal shift assays (TSA)

Thermal shift assay is a powerful method for examining binding interactions in proteins (Boivin, Kozak, & Meijers, 2013). It is based on the fact that stabilities of proteins are changed (typically increased) upon addition of ligand (small molecule, protein partner...) and these changes are quantitatively linked to the affinity of the interaction. Thermal shift assay quantifies shifts in thermal

denaturation temperature of a protein under varying conditions, by monitoring the thermal unfolding of the protein of interest using fluorescent dyes such as sypro orange. Data collection is rapid and straightforward using readily available real-time polymerase chain reaction instrumentation. The following protocol is an example given for the study of the interaction of RAR α LBD with a CoA peptide corresponding to the NR2 motif of TIF2 (KHKILHRLDSS), in the presence of the agonist AM580 (Fig. 9).

Required materials

- 7500 Real Time PCR system (Applied Biosystem)
- 96-well PCR plates and optical sealing tapes (Bio-Rad)
- Thermalshift buffer: 20 mM Tris HCl (pH 7.5), 150 mM NaCl
- Sypro® Orange
- TIF2 NR2 peptide (KHKILHRLDSS)
- Ligand AM580 at 10mM in DMSO
- Purified RAR α LBD at 5–10 mg/ml

Protocol

1. Prepare 25 μ l of different solutions containing 5 μ M protein and eventually 10 μ M of TIF2 NR2 peptide, in the presence of DMSO (condition without ligand) or in the presence of 10 μ M of AM580, and 2X Sypro Orange in thermalshift buffer.
2. Transfer the solutions in the wells of a 96-well PCR plate and sealed the plates very firmly before
3. Heat the plate in a 7500 Real Time PCR system (Applied Biosystem) from 25 to 95°C at 1°C intervals. Fluorescence changes in the wells are monitored with a photomultiplier tube and the wavelengths for excitation and emission were 545 nm and 568 nm, respectively.
4. The melting temperatures (T_m) are obtained by fitting the fluorescence data with a Boltzmann model using the GraphPad Prism software.

[Insert Figure 9 here]

4.8 Isothermal Titration Calorimetry (ITC)

ITC is a label-free measurement of the binding affinity and thermodynamics of a wide variety of biomolecular interactions (Velazquez-Campoy, Leavitt, & Freire, 2004). It works by directly

measuring the heat that is either released or absorbed by the calorimeter when binding occurs during gradual titration of the ligand into the sample cell containing the biomolecule of interest. The following protocol is an example given for the formation of the RXR-THR (thyroid hormone receptor) heterodimer (Fig. 10).

Required materials

- MicroCal VP-ITC system (Malvern Panalytical)
- ITC buffer: 50 mM Hepes pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM TCEP
- 10 kDa molecular weight cut-off dialysis cassette (Slide-A-Lyzer 0.5 ml 10K MWCO, Thermo Scientific)
- Purified TR α LBD at 5–10 mg/ml
- Purified RXR α LBD at 5–10 mg/ml

Protocol

1. Dialyze the two proteins (TR α and RXR α LBD) for 2 X 2 h against the same ITC buffer using dialysis cassettes.
2. Fill the reference cell of the VP-ITC with pure water.
3. Dispose 2 ml of TR α LBD at 10 μ M in the sample cell.
4. Load 500 μ l of RXR α LBD at 120 μ M (concentration more or equal to 10 X concentration of the protein in the cell) in the syringe.
5. Set the temperature at 20°C and monitor heat exchanges throughout titrations consisting of 30 injections (one injection of 1 μ l in 2.5 s followed by 29 times 10 μ l in 7.1 s, spaced by 300 s) of RXR α LBD solution into the cell containing TR α LBD.
6. Analyze data with the MicroCal ITC-ORIGIN software (Malvern Panalytical) or using Affinimeter (<https://www.affinimeter.com/site/itc/>) for more complex interactions.

[Insert Figure 10 here]

4.8. Small Angle X-ray Scattering (SAXS)

Small angle X-ray scattering (SAXS) is a biophysical method to study the overall shape and structural transitions of biological macromolecules in solution (Petoukhov & Svergun, 2013; Prior, Davies, Bruce, & Pohl, 2020). It provides low resolution information on the shape, conformation and assembly

state of proteins, nucleic acids and macromolecular complexes. Contrary to crystallography, this technique offers powerful means for the quantitative analysis of flexible systems, including intrinsically disordered proteins (Kachala, Valentini, & Svergun, 2015). Recent developments in SEC-SAXS have proved powerful in isolating monodisperse species from polydisperse or aggregating samples, thereby yielding structural information on transient macromolecular conformations and complexes. The following protocol is an example given for the interaction study of the nuclear interaction domain of NCoR (NCoRNID) with the heterodimer RXR/RAR LBDs (Cordeiro et al., 2019).

Required materials

- X-ray synchrotron source (ESRF, Grenoble)
- SAXS buffer: 50mM Tris HCl pH7.5, 150mM NaCl, 2mM TCEP
- Purified complex between NCoRNID and RXR/RAR α LBD at 5–10 mg/ml

Protocol

1. Prepare the protein complex in SAXS buffer at different concentrations ranging from 0.5 to 10 mg/ml. Keep also the buffer solution for SAXS measurement.
2. Record SAXS data by exposing the samples to a synchrotron X-ray source. Do repetitive measurements to detect and to correct for radiation damage and collect data on buffer solution before and after the measurements of each protein sample.
3. Calculate the protein SAXS curves after subtraction of the averaged buffer scattering from the protein sample patterns using Primus (ref) and merge SAXS curves of different concentrations to avoid interparticle interactions.
4. Use the ATSAS suite (Franke et al., 2017) to derive the radius of gyration, the molecular weight, the maximal intraparticle distance (D_{max}), the pairwise distance distribution function of the studied protein or complex. Further structural modeling is also possible using the experimental data. For that, multiple approaches are available to model dynamic conformational ensembles encoded in the scattering curve (reviewed in Brosey, Tainer 2019).

[Insert Figure 11 here]

4.9. Structural analysis by X-ray crystallography

Protein crystallization is very efficient to characterize protein–protein interactions. It is based on the preparation of crystals of the complexes followed by their analysis using X-ray beams. The determination of the high-resolution three-dimensional (3D) structures that derive from this technique provides atomic-level information on the protein–protein interface. Several crystal structures of homo- and heterodimers of NR LBDs, in complex with CoA and CoR peptides have been reported (ref). Considering that the methods used for the crystallization of protein complexes are diverse and that crystallization conditions are unique to each protein (or protein complex), we provide here a very general procedure that may apply to all kinds of NR LBDs in complex or in isolation. The following protocol is an example given for the crystallization of the RAR α LBD bound to BMS493, a RAR α -selective inverse agonist and in complex with CoRNR1 of NCoR (le Maire et al., 2010).

Required materials

- Crystallization robot (Mosquito)
- Crystallization screens (Molecular Dimension, Hampton Research, Qiagen, etc.)
- Visualization robot (Formulatrix)
- 96-Well crystallization plates (Greiner Bio-One)
- 24-Well crystallization plates (Greiner Bio-One, Molecular Dimension)
- Siliconized glass coverslips (Greiner Bio-One)
- X-ray synchrotron source (ESRF, Grenoble)
- NCoR CoRNR1 peptide (EZBiolab)
- Ligand BMS493 at 10mM in DMSO
- Purified RAR α LBD at 5–10 mg/ml

Protocol

1. Mix the purified protein with a two-fold molar excess of ligand and a three-fold molar excess of the NCoR CoRNR1 peptide, concentrate it to 3.5 mg/ml and centrifuge for 20 min at 13,000 rpm to get rid of any aggregated material.
2. Perform an initial search for crystallization conditions by mixing the purified protein with sparse matrix screening conditions using crystallization robots, dedicated 96-well crystallization plates and

the sitting-drop vapor diffusion technique. The robot automatically performs each crystallization trial by mixing 100 nl of protein with 100 nl of precipitant condition in a well suspended over a reservoir containing 40 μ l of the corresponding condition.

3. Once the 96 conditions have been dispensed, seal the plates with a transparent plastic film and incubate the plates at a fixed temperature (20°C) in the Formulatrix to take pictures of the drops at defined times. The crystals obtained at this step are generally small or not well shaped and need to be optimized through refinement of these preliminary crystallization conditions.

4. Perform the refinement by generating “home-made” grid screens where all the parameters of the initial hit are varied (pH, salt, concentration of precipitant, etc.). Use 24-well crystallization plates and mix 1 μ L of the concentrated protein with 1 μ L of each crystallization condition, the reservoir containing 500 μ L of the corresponding crystallization condition. The drops are suspended from siliconized glass coverslips (hanging drop method). This scale-up of the crystallization process allows to obtain large crystals suitable for further crystallographic studies in a crystallization solution consisting of 18% (w/v) PEG3350, 0.15M NH_4Cl .

5. Mount the crystal onto a cryoloop, transfer the crystal to a solution containing the mother liquor plus a cryoprotectant (25 % glycerol) for a few seconds and flash-frozen in liquid nitrogen to preserve it before and during the X-ray diffraction experiment.

6. Collect diffraction data at a synchrotron X-ray source and solve the crystal structure following all the steps of data processing, determination of the structure by molecular replacement, model building, and refinement (see le Maire 2010 for more details and programs used).

4.10. SEC-MALS

Coupling an analytical Size Exclusion Chromatography (SEC) column in-line with multi-angle light scattering (MALS), UV detector and refractometric detector provides a widespread approach and a useful tool for accurate analysis of molar mass, overall shape, aggregation, oligomerization, interactions and purity of proteins or macromolecular complexes in native solution, independent of the protein retention time analyzed by SEC (Some, Amartely, Tsadok, & Lebendiker, 2019). The following protocol is an example given for the biophysical characterization of RXR Δ AB-RAR Δ AB-DR5 in complex with NCOR_{NID} before more in-depth structural study (Fig. 12; unpublished results).

Required materials

- miniRAWN Treos – Multi-Angle Static Light Scattering + Optilab T-rex – Refractometer (Wyatt)
- 1260 Infinity II Liquid Chromatography system (Agilent)
- Superdex 200 Increase 10/300 GL (Life Sciences)
- MALS buffer: 50 mM Tris pH7.5, 150 mM NaCl, degassed and filtrated at 0.1 μm
- NCOR_{NID} purified at 2-10 mg/ml
- RXR Δ AB-RAR Δ AB-DR5 complex purified at 2-5 mg/ml
- RXR Δ AB-RAR Δ AB-DR5-NCOR_{NID} – BMS493 (RAR inverse agonist) complex purified at 2-5 mg/ml

Protocol

1. Equilibrate the system and the column in the MALS buffer. The chromatography system is controlled using the chromatography software from Agilent. Run this equilibration step until MALS detector is stable with drift lower than 10^{-4} .
2. Centrifuge the protein sample at 14 000 rpm during 20 minutes to remove precipitates and larger insoluble particles
3. Inject the protein sample (volume of 20-25 μl) on the column and continue the run at 0.5 ml/min (about 40 minutes for the 20 ml column)
4. Process the data using the ASTRA software. Set the baselines, select the peak(s) of interest, check the report, especially the Molecular Mass vs Time plot.

[Insert Figure 12 here]

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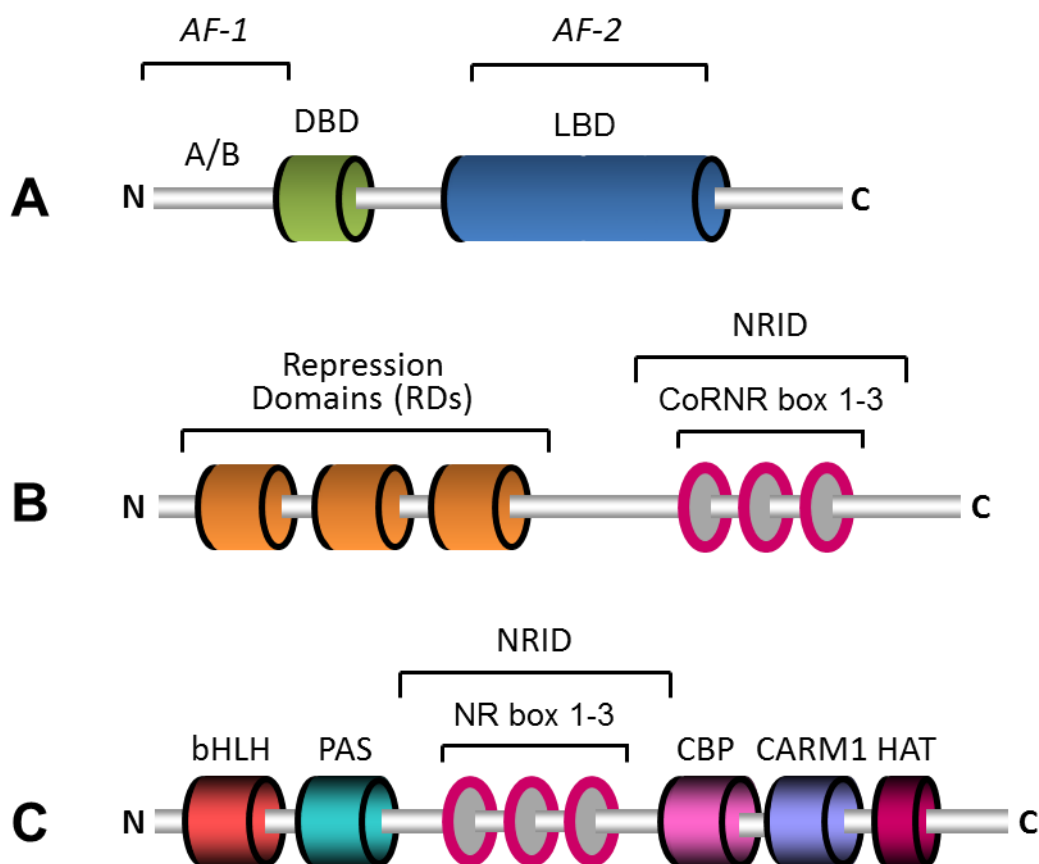


Figure 1: Schematic illustration of the structural and functional organization of retinoid receptors and coregulators. (A) Like other NRs, RARs and RXRs contain an evolutionary well conserved DNA-binding domain (DBD), a moderately conserved ligand-binding domain (LBD) and a highly divergent N-terminal A/B region. Two transcriptional activation functions have been described in these receptors: a constitutively active AF-1 in region A/B and a ligand-inducible AF-2 in the LBD. (B) The corepressors (N-CoR and SMRT) functional domains illustrated are the C-terminal NR interaction domain (NRID) and the repression domains (RDs). (C) The functional domains of the members of the SRC-1/p160 family of coactivators illustrated are the bHLH/PAS (basic helix-loop-helix-Per/Ah receptor nuclear translocation/Sim motif), the NRID comprising three NR boxes mediating NR interactions and the CREB-binding protein (CBP), the methyltransferase (CARM1), and the histone acetyltransferase (HAT) interaction domains at the C-terminus.

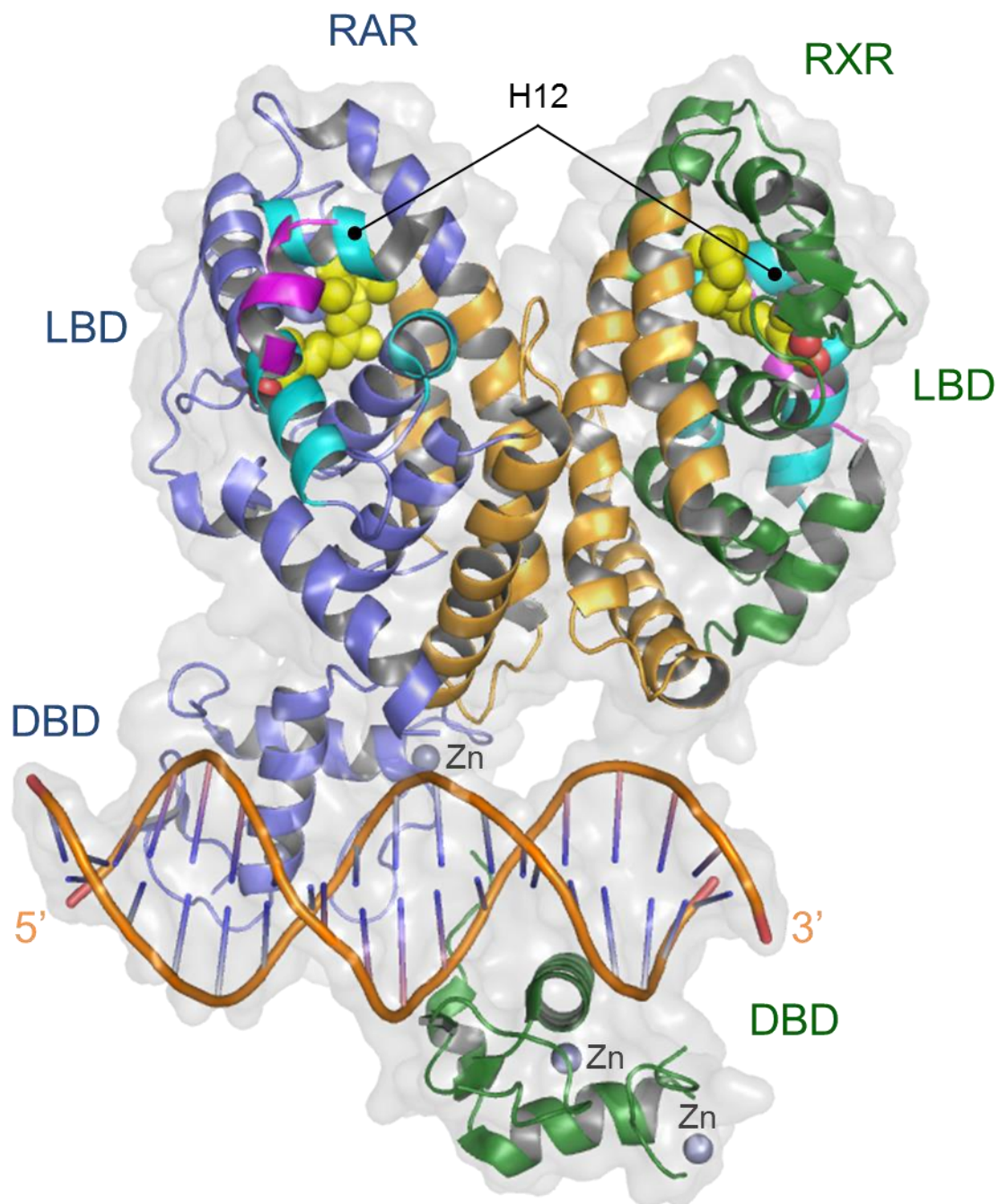


Figure 2: Crystal structure of RAR β -RXR α heterodimer bound to DNA, ligands and coactivator peptides. Ligands are shown as yellow van der Waals spheres, coactivator peptides in magenta, zinc ions in gray, the dimerization surfaces (H7, H9, H10) in orange and the coactivator binding surfaces on RAR and RXR (H3, H4, H12) in cyan. For color detail, please see color plate section.

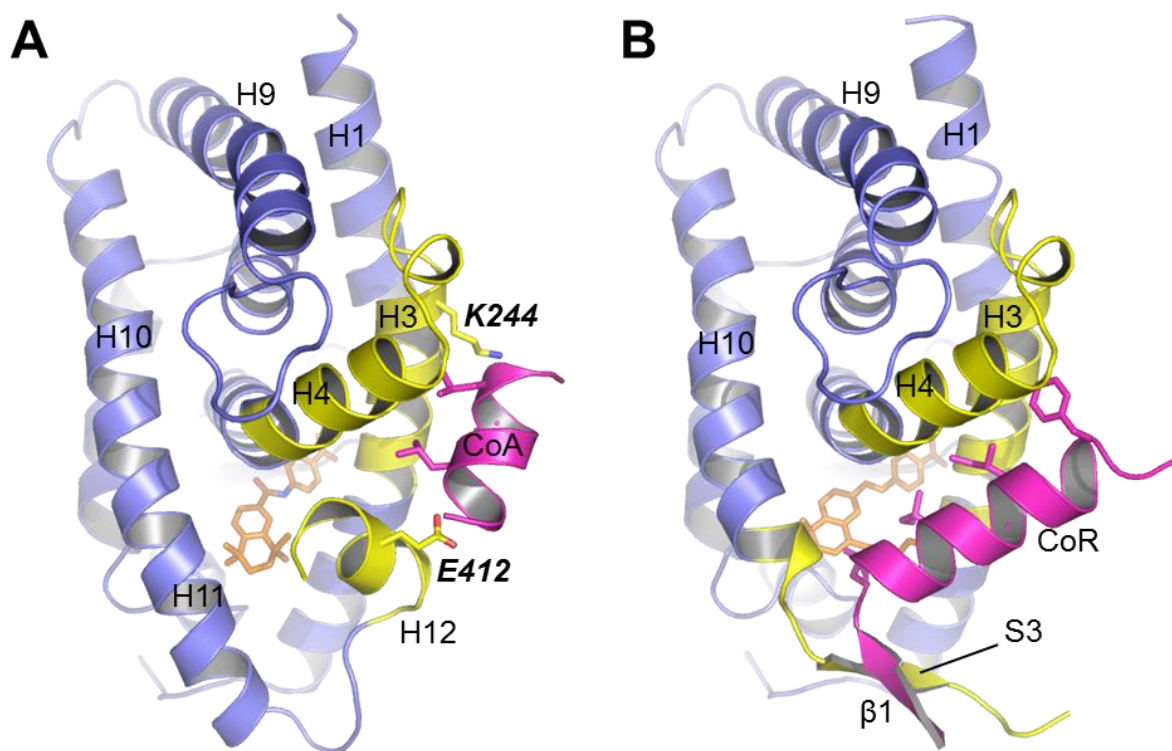


Figure 3: Structural basis of the interaction between RAR and coregulators. (A) Overall architecture of RAR LBD bound to a synthetic agonist (AM580 shown in orange) and a coactivator-derived FxxLF peptide (magenta ribbon). Helices H3, H4 and H12 constitutive of the AF-2 surface are colored in yellow. Conserved charge-clamp residues are labeled. (B) Overall architecture of RAR LBD bound to a synthetic inverse agonist (BMS493 shown in orange) and a peptide containing the CoRNR box1 sequence of NCoR (magenta ribbon). Helices H3, H4 and S3 constitutive of the interaction surface on RAR are colored in yellow. For color detail, please see color plate section.

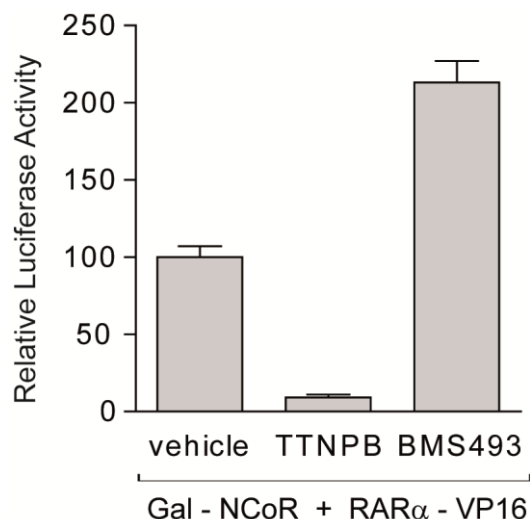


Figure 4: The chimeric luciferase-based reporter gene ((17m)5x-βGal-Luc) was transiently transfected together with vectors expressing NCoR NRID fused to the Gal DNA binding domain of the yeast *Saccharomyces cerevisiae* (Gal-NCoR) as bait and RARα LBD fused to the *Herpes simplex* VP16 acidic transcription activation domain as prey (RARα-VP16). As the VP16 domain confers constitutive transcription activation if it is brought close to a promoter, a specific gene induction is seen only if the two proteins bind to each other. In this assay, Gal-NCoR specifically binds to the “17m” DNA recognition site through the Gal DBD and can interact with the RARα LBD in the absence of ligand or in the presence of the inverse agonist BMS493. The RAR agonist TTNPB is able to decrease NCoR association (100%, basal transcriptional activity). Compounds were used at 1 μM in all two-hybrid assays. Error bars, s.e.m.

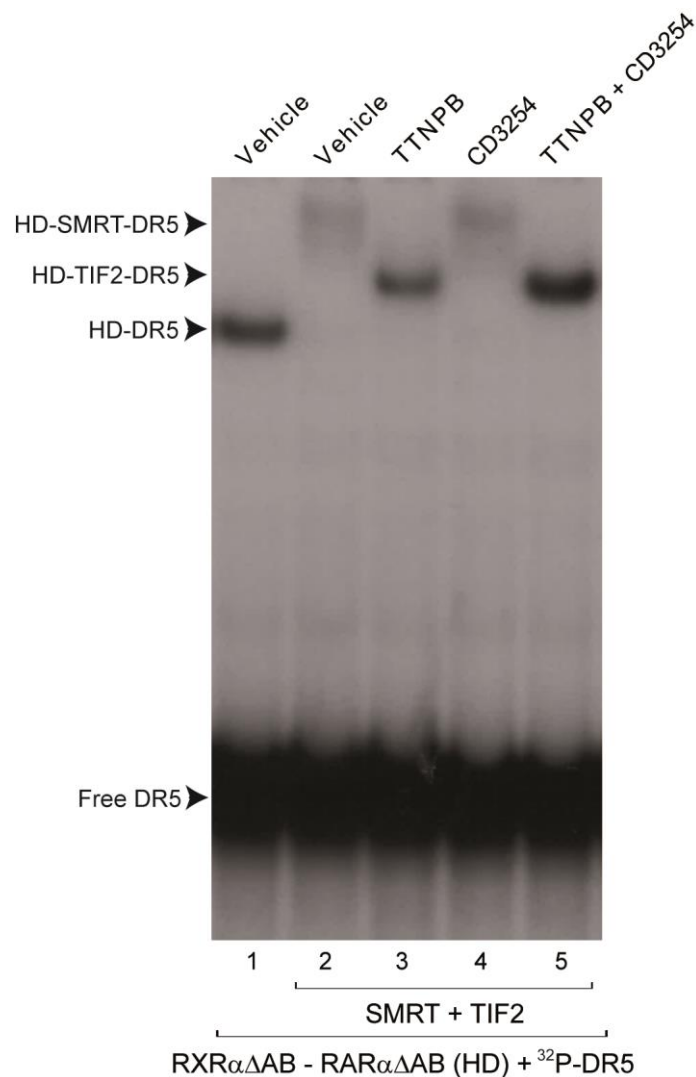


Figure 5: EMSAs demonstrating ligand-dependent co-regulator recruitment by the RXR α Δ AB-RAR α Δ AB heterodimer (HD). Incubation of RXR α Δ AB-RAR α Δ AB heterodimer bound to the DR5 DNA sequence with a mix of SMRT and TIF2 produced heterodimer-SMRT complexes detectable as a super-shift. SMRT, which dissociated and was replaced by TIF2 in the presence of the RAR agonist TTNPB, remained bound to a heterodimer when exposed to the RXR agonist CD3254 alone despite the presence of TIF2, in accordance with the subordination principle. DR5 oligonucleotide, HD (0.4 μ l of TNT reaction products added per assay), SMRT (1 μ g per assay), and TIF2 (0.5 μ g per assay) were co-incubated in the absence or in the presence of saturated amounts of TTNPB used alone or with CD3254.

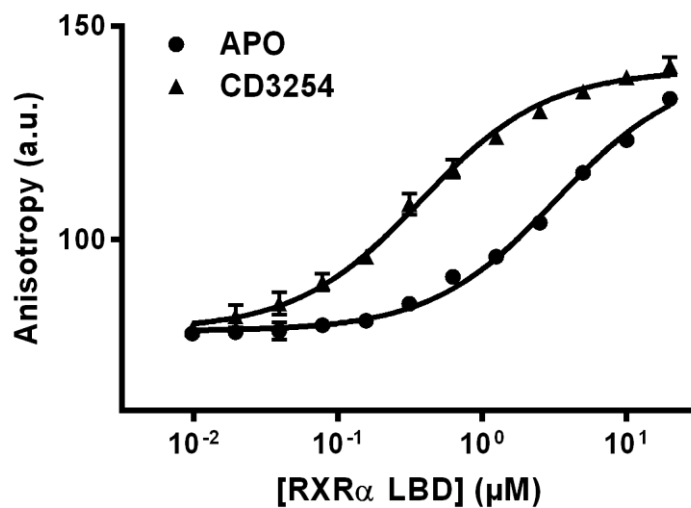


Figure 6: Titration of fluorescein-labelled PGC-1 α peptide by RXR α LBD, in the absence of ligand (APO) or in the presence of the full RXR agonist (CD3254), followed by fluorescence anisotropy. As expected, the synthetic agonist, CD3254, strongly enhances the recruitment of the coactivator peptide, since in the apo condition the K_d is around 3.3 μ M against 0.4 μ M in the presence of CD3254.

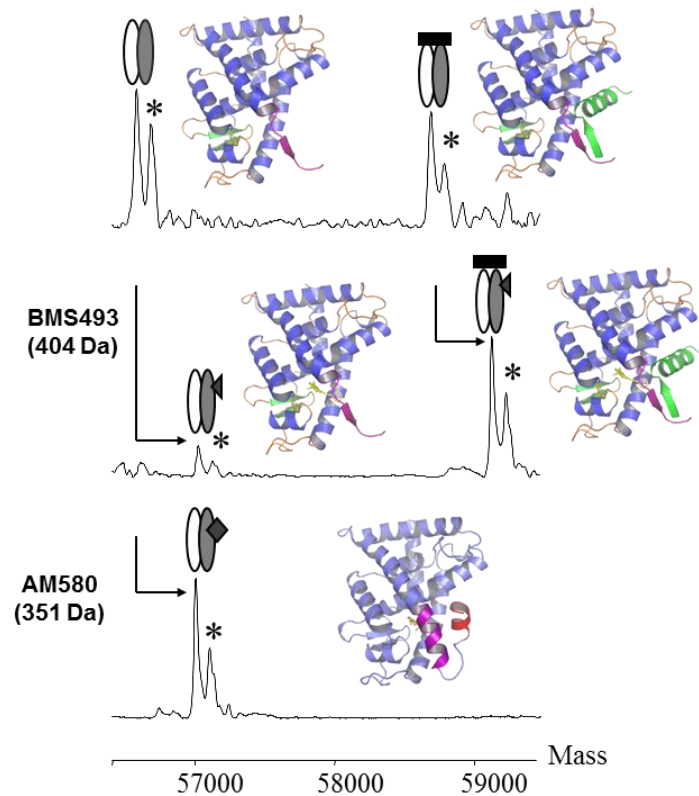


Figure 7: Positive ESI mass spectra of RXR–RAR–CoRNR1 complexes in the absence of ligand or in the presence of the inverse agonist BMS493 or the agonist AM580. Peaks labeled with an asterisk correspond to species with an additional N-terminal methionine. Whereas the inverse agonist strongly stabilizes the interaction between the heterodimer and CoRNR1, the AM580 induces a quantitative release of the peptide.

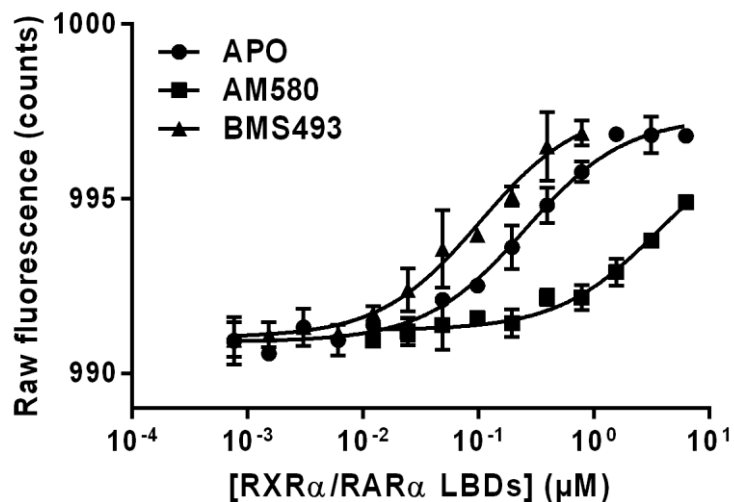


Figure 8: Interaction study between the heterodimer RXR α -RAR α LBDs and the fluorescently labeled nuclear interaction domain of NCoR (NCoR_{NID}) followed by MST, in the presence of a full RAR α agonist (AM580) or a RAR α inverse agonist (BMS493). The raw fluorescence (in counts) is plotted against the concentration of heterodimer for analysis of thermophoresis. The protein shows a significant decrease in fluorescence in the bound than in the unbound state. The K_d is fitted to 0.2 μ M (apo complex), more than 5 μ M (complex in the presence of AM580) and 0.1 μ M (complex in the presence of BMS493) (Cordeiro et al., 2019). Error bars represent standard error of n=3 measurements.

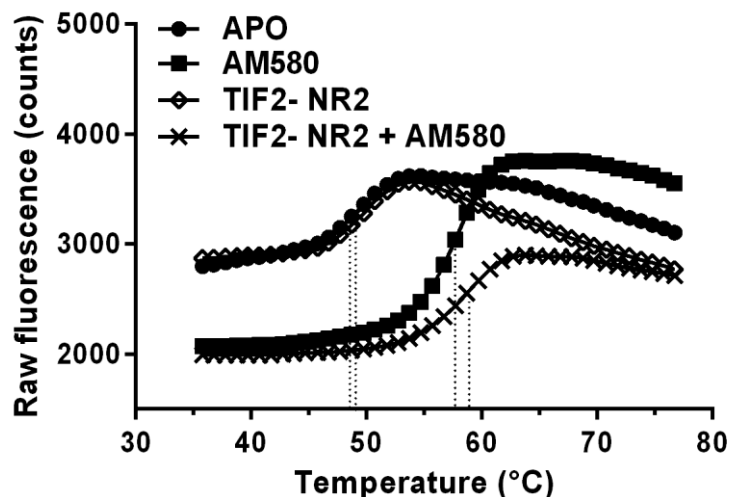


Figure 9: Interaction study between RAR α LBD and a peptide corresponding to the NR2 motif of the coactivator TIF2 (TIF2 NR2) followed by thermal shift assay, in the presence of a full RAR α agonist (AM580). Thermal denaturation curves of RAR α LBDs, in its unliganded form (APO), in the presence of two molar excess of AM580, in the presence of two molar excess of TIF2-NR2 and in the presence of the ligand and peptide. From these curves, fitting using a Boltzmann sigmoidal equation allows to deduce the melting temperature (T_m) of each complex (dashed lines). The RAR α LBD ($T_m=48.77^\circ\text{C}$ in its unliganded form) is strongly stabilized by the addition of the ligand AM580 ($T_m=57.4^\circ\text{C}$) and further stabilized by the concomitant addition of the peptide SCR1-NR2 ($T_m=58.2^\circ\text{C}$), confirming the formation of the RAR-coactivator complex in the presence of an agonist ligand.

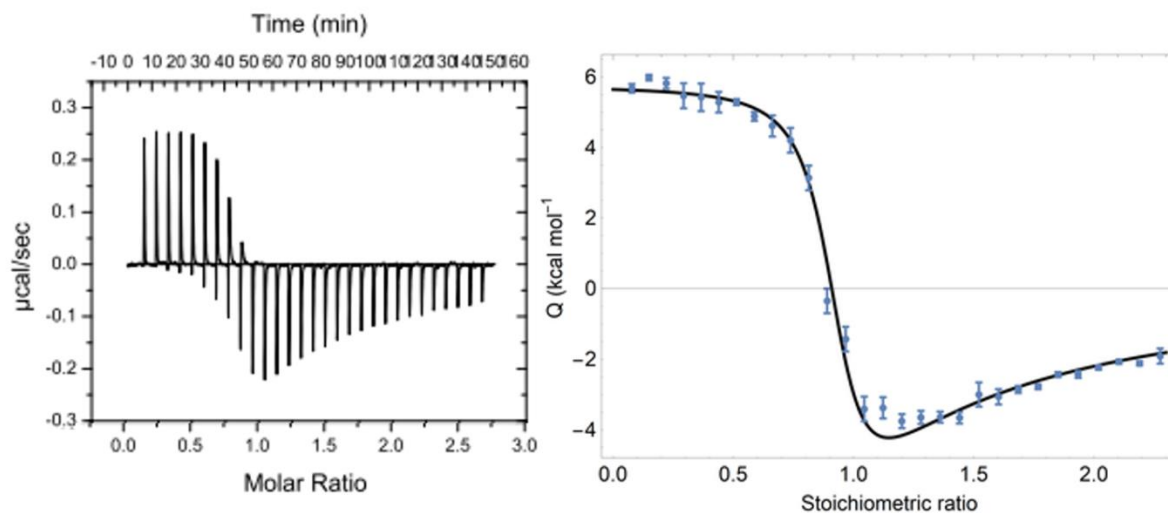


Figure 10: Thermometric evaluation of the affinity between RXR α and THR α LBDs. ITC thermograms were obtained by titration of RXR α into THR α LBD. Isothermal calorimetric enthalpy change (left panel) and resulting binding isotherm (right panel) are shown. Two events take place: a first dissociation of the homodimer RXR α -RXR α LBDs ($K_d=29.7 \mu\text{M}$) and a formation of the heterodimer RXR α -THR α LBDs ($K_d=65 \text{ nM}$) in agreement with measurement of this affinity by MST (le Maire et al. submitted).

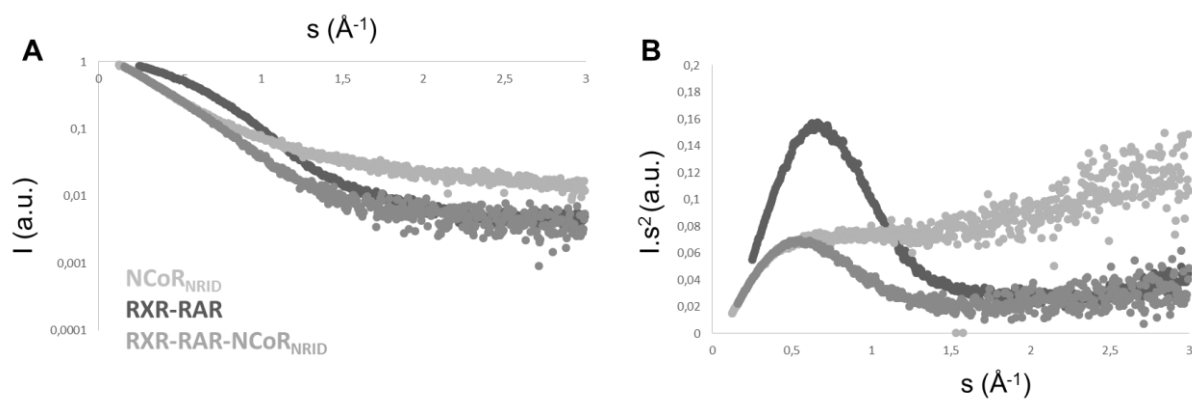


Figure 11: SAXS measurements and analysis to study the complex between the heterodimer $\text{RXR}\alpha$ - $\text{RAR}\alpha$ LBDs and the NRID of NCoR ($\text{NCoR}_{\text{NRID}}$). (A) The experimental SAXS profile for $\text{RXR}\alpha$ - $\text{RAR}\alpha$ in solution (black circles), for $\text{NCoR}_{\text{NRID}}$ (light gray circles) and for the complex (dark gray circles). (B) Kratky representations of SAXS intensity versus momentum transfers for $\text{RXR}\alpha$ - RAR in solution (black circles), for $\text{NCoR}_{\text{NRID}}$ (light gray circles) and for the complex (dark gray circles). It confirms the disordered nature of $\text{NCoR}_{\text{NRID}}$ and the globular shape of the heterodimer. The complex has both features.

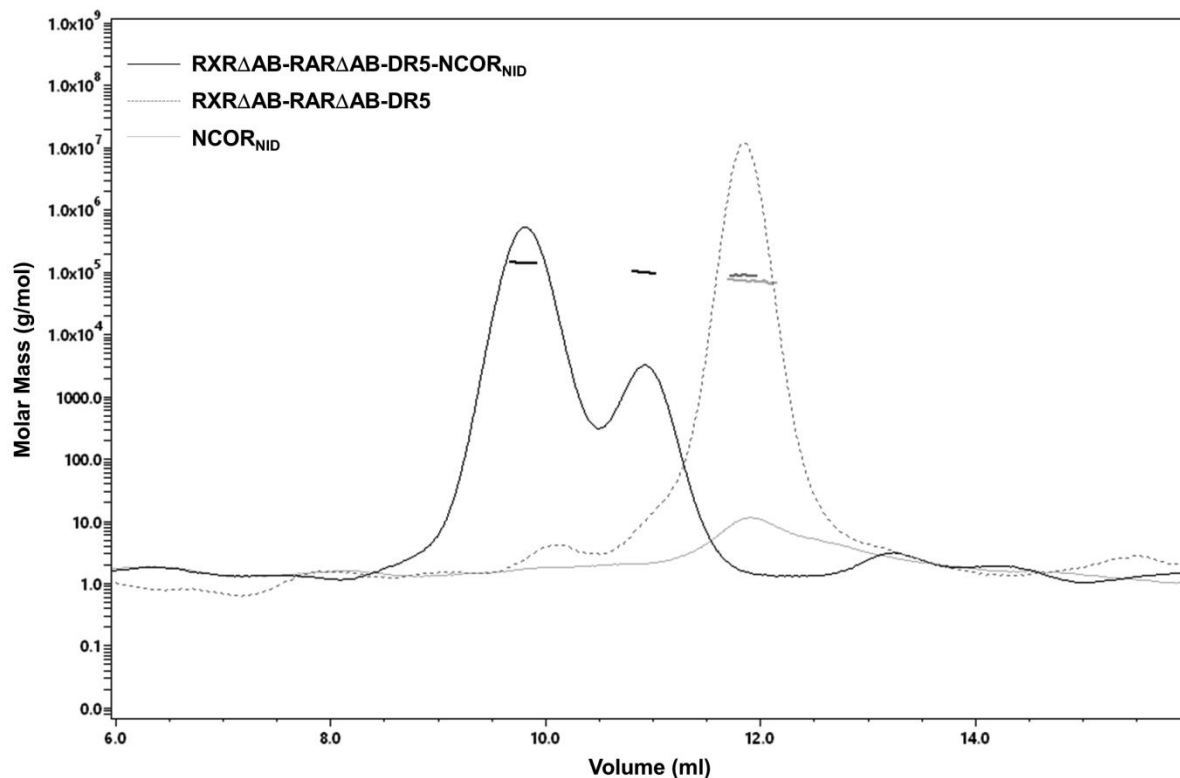


Figure 12: SEC-MALS elution profile of RXR Δ AB-RAR Δ AB-DR5, NCOR_{NID} and the full complex of RXR Δ AB-RAR Δ AB-DR5-NCOR_{NID} after large-scale purification. The protein samples were separated on a size exclusion chromatography (SEC) column (Superdex 200 Increase 10/300 GL) equilibrated with MALS buffer. The horizontal lines crossing the SEC profile indicate molecular masses of the protein and complexes determined by connected in-line multi-angle light scattering (MALS) and refractive index (RI) detectors. The molecular weights deduced from these experiment fit well with the theoretical values: RXR Δ AB-RAR Δ AB-DR5, $MW_{\text{MALS}}=91$ kDa and $MW_{\text{theo}}=82$ kDa; NCOR_{NID}, $MW_{\text{MALS}}=70$ kDa and $MW_{\text{theo}}=30$ kDa (dimer); RXR Δ AB-RAR Δ AB-DR5-NCOR_{NID}, $MW_{\text{MALS}}=140$ kDa and $MW_{\text{theo}}=126$ kDa.

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