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Nuclear receptor ligand-binding domains: reduction of helix H12 dynamics to favour crystallization

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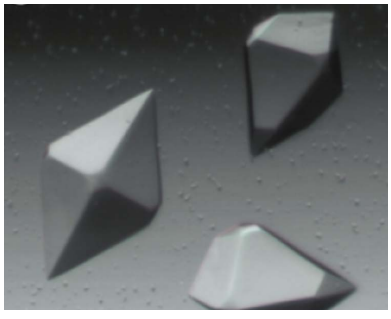


Crystallization trials of the human retinoid X receptor α ligand-binding domain (RXRα LBD) in complex with various ligands have been carried out. Using fluorescence anisotropy, it has been found that when compared with agonists these small-molecule effectors enhance the dynamics of the RXRα LBD C-terminal helix H12. In some cases, the mobility of this helix could be dramatically reduced by the addition of a 13-residue co-activator fragment (CoA). In keeping with these observations, crystals have been obtained of the corresponding ternary RXRα LBD–ligand–CoA complexes. In contrast, attempts to crystallize complexes with a highly mobile H12 remained unsuccessful. These experimental observations substantiate the previously recognized role of co-regulator fragments in facilitating the crystallization of nuclear receptor LBDs.

1. Introduction

Nuclear receptors (NRs) form an important superfamily of transcription factors which regulate various biological processes either by activating or repressing the expression of target genes (Perissi & Rosenfeld, 2005). Activation of gene transcription occurs upon binding of an agonistic ligand, resulting in a conformational change of the ligand-binding domain (LBD) and the recruitment of co-activators to DNA-bound NRs. Comparison of unliganded (apo) and agonist-bound (holo) LBD structures has led to the mousetrap model of the ligand-dependent conformational switch (Renaud *et al.*, 1995; Nettles & Greene, 2005; Pike, 2006). In apo-LBD structures different positions of the C-terminal helix 12 (H12, also called the activation helix) have been observed, indicating the presence of a great deal of flexibility in this region (Nagy & Schwabe, 2004). Upon binding of an agonist, H12 packs against the rest of the LBD in the so-called holo or active position, thereby closing the ligand-binding pocket. In this conformation NR LBDs expose an interaction surface to which co-activator proteins can bind through short helical L*xx*LL motifs (where L represents leucine and *x* represents any amino acid). Conversely, antagonists or partial agonists/antagonists permanently or partially prevent the active positioning of H12 (see movies in de Lera *et al.*, 2007).

Selective nuclear receptor modulators (SNuRMs) that specifically modulate different receptor subtypes and act in a cell-selective manner are being developed for therapeutic purposes (Pike, 2006; Nahoum & Bourguet, 2007; de Lera *et al.*, 2007). Based on the structure of the potent agonist CD3254 (Bernardon, 1997), we recently synthesized six novel retinoid X receptor α (RXRα) modulators, hereafter referred to as **2a**–**2f** (Fig. 1), ranging from partial agonists (**2a**–**2d**) to pure antagonists (**2e**–**2f**). To gain structure-based insight into the mechanism of action of these new ligands, crystallographic studies have been carried out (Nahoum *et al.*, 2007). Although attempts were made to crystallize the various RXRα LBD–ligand complexes, crystals were obtained for just three of the six ligands and only in the presence of a TIF-2 co-activator fragment containing the L*xx*LL interaction motif (CoA). In the present report, we show a clear inverse correlation between the crystallization potential of a particular RXRα LBD–ligand complex and the impact

of the bound ligand on the structural dynamics of helix H12. Moreover, our data substantiate the role of co-regulator peptides as additives facilitating NR LBD crystallization and validate the use of fluorescence spectroscopy as a predictive tool of the crystallization capacity of NR LBD complexes.

2. Materials and methods

Protein-purification and crystallization experiments have been described previously (Nahoum *et al.*, 2007). Briefly, the histidine-tagged LBD of human RXRα was purified with an Ni^{2+}-affinity column followed by a gel-filtration step. Fractions containing RXRα LBD were pooled, concentrated and mixed with a threefold molar excess of ligands **2a**–**2f**. In another set of experiments, a fivefold molar excess of the TIF-2 co-activator peptide was also added. Crystallization trials were performed by sitting-drop vapour diffusion in 96-well protein-crystallography plates (Corning) at 291 K. 18 different commercially available crystallization screens were tested and crystals were obtained using the PEGs Suite screen (Nextal) for ligands **2a**, **2b** and **2c** in the presence of CoA (for further details, see Nahoum *et al.*, 2007).

For preparation of the fluorescent protein, RXRα LBD was expressed as a fusion with an inducible self-splicing intein and a chitin-binding domain using the vector pTYB1 (New England Biolabs). The crude extract was applied onto chitin beads (New England Biolabs) and incubated overnight at 277 K in the presence of 4%(*m*/*v*) 2-mercaptoethanesulfonic acid (MESNA) in 10 m*M* Tris–HCl, 500 m*M* NaCl, 1 m*M* EDTA pH 8.0 (buffer *A*). Fractions containing the cleaved RXRα LBD were obtained by elution with buffer *A*, dialyzed against 10 m*M* Tris–HCl, 150 m*M* NaCl pH 7.5 (buffer *B*) and incubated overnight at 277 K with 4 m*M* cysteine-fluorescein and 150 m*M* MESNA in buffer *B*. The fluorescent RXRα LBD was further purified using a Superdex 75 26/60 gel-filtration column (GE Healthcare) equilibrated with buffer *C* (10 m*M* Tris–HCl, 5 m*M* DTT, 150 m*M* NaCl, 1 m*M* EDTA, 10% glycerol pH 7.5). The 5-(L-cysteinylamido-acetamido)-fluorescein was synthesized as described previously (Mekler *et al.*, 2002). Fluorescence-anisotropy assays were performed using a Safire^2 microplate reader (TECAN) at a protein concentration of 0.265 μM. The excitation wavelength was set at 470 nm, with emission measured at 530 nm. Assays were performed by adding ligands and the human TIF-2 peptide (686-KHKILHRLLQDSS-698) to a final concentration of 10 μM.

3. Results and discussion

A large screening of crystallization conditions was carried out for all the LBD–**2a**–**2f** complexes either in the absence or in the presence of a TIF-2 co-activator peptide (CoA). Crystals of RXRα LBD in complex with ligands **2a**, **2b** or **2c** were obtained in the presence of CoA (Fig. 2). They belong to the tetragonal space group $P4_32_12$ and diffract X-rays to 1.8, 2.2 and 2.2 Å resolution, respectively. The structures of the complexes have been solved (PDB codes 2p1t, 2p1u and 2p1v, respectively) and provide insights into the structural basis of ligand-dependent regulation of NRs (Nahoum *et al.*, 2007). In contrast, we failed to obtain crystals for the corresponding complexes in the absence of CoA. Moreover, attempts to crystallize RXRα LBD with the remaining ligands (**2d**, **2e** and **2f**), either in the presence or in absence of CoA, remained unsuccessful.

We monitored the mobility of the activation helix H12 of RXRα in the presence of the newly synthesized RXRα modulators. Experiments were carried out by steady-state fluorescence-anisotropy measurements of a fluorescein moiety that had been specifically attached to the C-terminus of helix H12 *via* intein chemistry (Chong *et al.*, 1997). This spectroscopic method is based on the observation that the anisotropy of the fluorescence signal varies according to the mobility of the protein region to which the fluorophore is attached: the lower the mobility, the higher the anisotropy and *vice versa*. Fig. 3 shows that the various ligands have different effects on H12 dynamics. Whereas the agonist SR11237 induces the highest anisotropy value within the series, indicative of the lowest H12 mobility, ligands **2a**–**2f** induce weaker anisotropy, suggesting greater dynamics of the activation helix. In keeping with these data, none of the binary RXRα LBD–**2a**–**2f** complexes could be

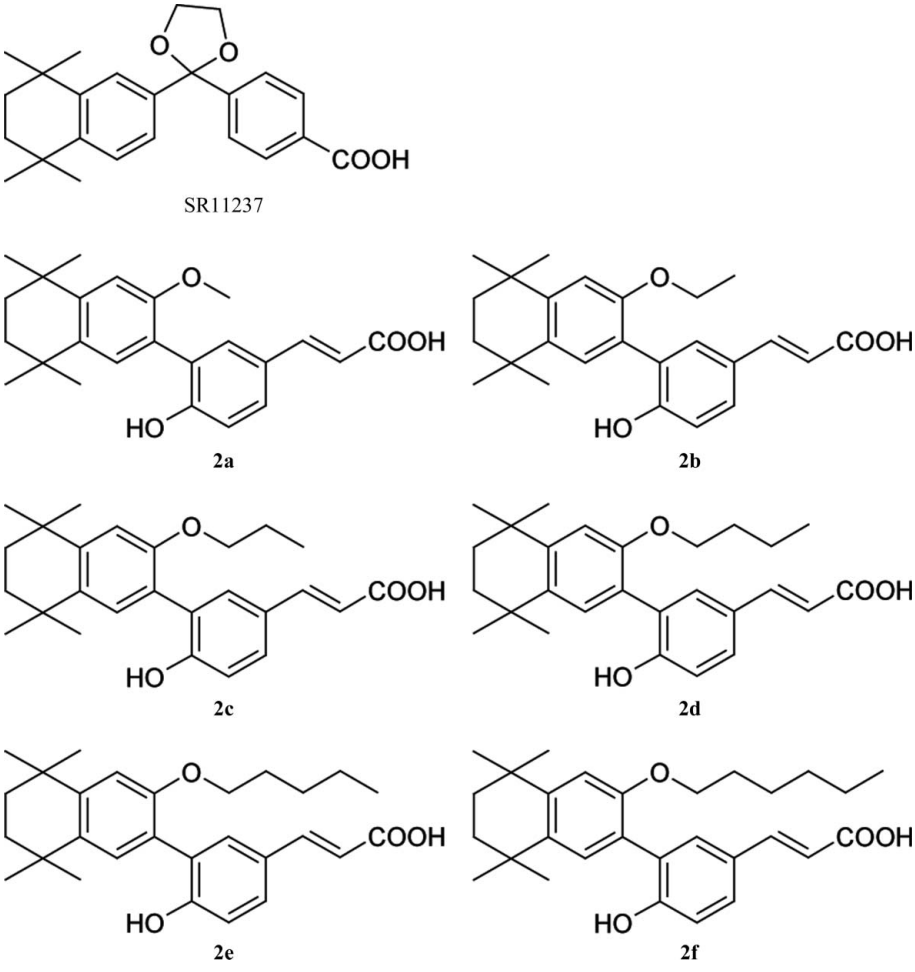
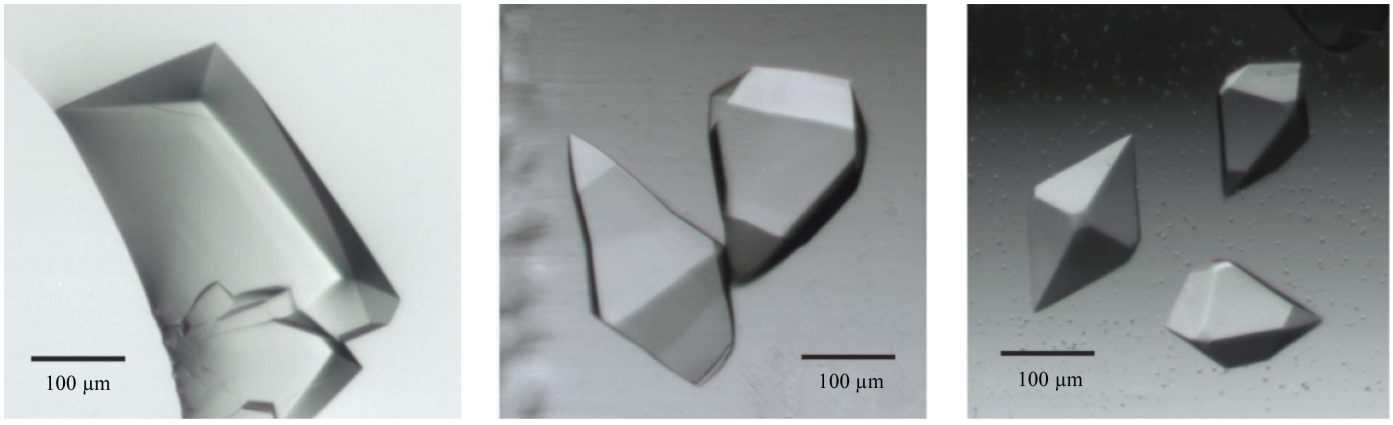


Figure 1
Chemical structures of the ligands used in the present study.



(*a*) (*b*) (*c*)

Figure 2
Cocrystals of RXRα LBD with compounds **2a** (*a*), **2b** (*b*) and **2c** (*c*). Crystals were obtained when a co-activator peptide was added at a fivefold molar excess to the LBD at the crystallization step. Crystals were grown at 291 K and appeared after a few days.

crystallized despite the large number of crystallization conditions screened (n = 1728). The structure of RXRα LBD bound to SR11237 in the absence of CoA has not been reported to date and no attempts were made to crystallize this binary complex in the present study. However, many agonist-bound NR LBD structures have been reported, indicating that, in agreement with our fluorescence data, agonists are able to efficiently stabilize H12 on their own. Upon addition of 10 µ*M* CoA to the RXRα–ligand mixtures, an increase in the anisotropy values is observed (Fig. 3). However, the presence of CoA affects H12 mobility with a magnitude that varies according to the bound ligand. Whereas the addition of CoA causes only a slight increase in the anisotropy of RXRα LBD bound to the agonist SR11237, it allows the anisotropy values of RXRα LBD bound to **2a**–**2c** and to a lesser degree **2d** to reach anisotropy levels that are similar to that measured with SR11237. In contrast, with ligands **2e** and **2f** CoA failed to efficiently stabilize H12, as reflected by the low anisotropy values. Parallelling these data, crystals have been obtained for the ternary RXRα LBD–SR11237–CoA (Egea *et al.*, 2002) and RXRα LBD–**2a**–**2c**–CoA complexes (Nahoum *et al.*, 2007) but not for those containing ligands **2e** and **2f**. Concerning ligand **2d**, for which we failed to obtain crystals even in the presence of CoA, the apparent discrepancy between fluorescence-anisotropy data and crystallization results most probably arises from the different molar excess of peptide used in the two experiments (see §2).

The analysis of the crystallization results in the light of our fluorescence data reveal a clear inverse correlation between the 'crystallizability' of a given receptor–ligand complex and the mobility of helix H12. Moreover, we experimentally demonstrate that, as initially reported (Stehlin *et al.*, 2001), the addition of co-regulator fragments can help to stabilize a particular conformation of NR LBDs, thereby facilitating their crystallization. Finally, it appears that our fluorophore-labelled RXRα LBD could be viewed as a molecular sensor that is predictive of the crystallization potential of RXRα LBD complexes and we suggest that this system could be applicable to other NR LBDs.

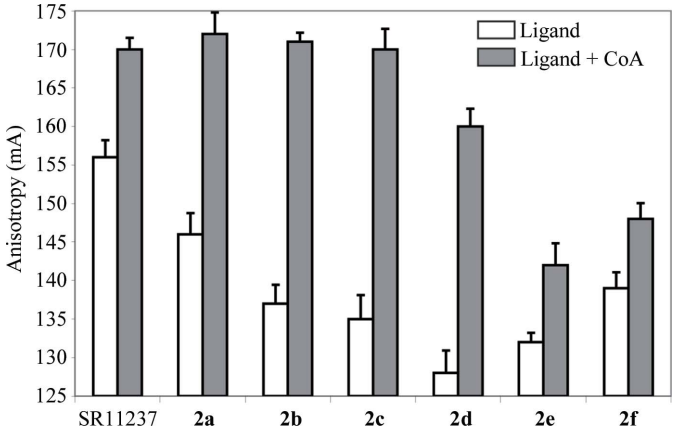


Figure 3
Monitoring of the mobility of RXRα helix H12 by steady-state fluorescence anisotropy. Anisotropy values were measured in the presence of saturating concentrations of the various ligands (agonist SR11237 and compounds **2a**–**2f**) with or without a TIF-2 co-activator fragment (CoA). The receptor in its apo form could not be used as a reliable reference as it has been demonstrated previously that bacterially expressed RXRα LBD captures fatty acids from the host (Bourguet *et al.*, 2000).

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