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Purification and crystallization of the human RXRα ligand-binding domain–9-*cis*RA complex

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The purification and crystallization of the stoichiometric complex of human RXRα ligand-binding domain (hRXRα LBD) bound to its natural ligand 9-*cis* retinoic acid (9-*cis*RA) are described. A three-step purification yields a pure and homogenous complex. Based on the crystallization conditions of several other nuclear receptors, an exhaustive crystallization screening using carboxylic acids as precipitating agents was performed in association with the use of polyhydric alcohols acting as cosmotropic solutes. Crystals of the hRXRα LBD–9-*cis*RA complex grew in a tripartite mixture containing sodium formate, glycerol and propane-1,2-diol. Micro- and macroseeding were necessary to improve both the size and the quality of crystals in order to make them suitable for structure determination.

1. Introduction

Nuclear hormone receptors are ligand-activated transcription factors essential for development, cellular life and homeostasis in vertebrates. Members of the nuclear receptor (NR) superfamily share a common functional and structural organization, with six domains: a variable A/B N-terminal domain, a conserved C domain responsible for weak dimerization and specific DNA binding, a hinge D region, the C-terminal E domain (known as the ligand-binding domain) responsible for hormone binding, strong dimerization and ligand-dependant transcriptional activation (Gronemeyer & Laudet, 1995*a*,*b*) and an F domain. Physiologically, NRs can be active molecular species in monomeric, homodimeric or heterodimeric states. 9-*cis* and all-*trans* retinoic acid isomers transduce their pleiotropic biological effects through specific binding to their cognate nuclear receptors retinoid X receptors (RXRs) and retinoic acid receptors (RARs). Whereas RARs bind both the 9-*cis* and all-*trans* isomers of retinoic acid, RXRs exclusively bind the 9-*cis* isomer. Several structures of nuclear receptors in either unliganded (apo) or liganded (holo) states have been solved in the presence of agonist or antagonist compounds (Egea, Klaholz *et al.*, 2000). The ligand-binding domain (LBD) exhibits an evolutionarily conserved fold encompassing 12 α-helices and a β-turn arranged as a three-layered antiparallel α-helical sandwich. All crystallographic studies support the concept of the uniqueness of the holo-agonist conformation among all NR LBDs. In the context of agonist/antagonist design, the knowledge of the basis of RXR selectivity towards the 9-*cis* isomer is essential; the structural study of human RXRα LBD bound to its natural ligand 9-*cis* retinoic acid has been carried out to provide information about the binding site at the atomic level. Obtaining crystals suitable for structure determination required the use of micro- and macroseeding in a tripartite crystallization mixture containing a carboxylic acid and two stabilizing organic polyalcohols essential for crystallization.

2. Experimental procedures

2.1. Expression of hRXRα LBD

The human RXRα LBD encompassing residues Thr223–Thr462 was expressed as an N-terminal hexahistidine fusion in the pET15b vector (Novagen) and was overproduced in *Escherichia coli* BL21(DE3) strain (Novagen). Freshly transformed cells were grown in 2×LB medium at 310 K until the OD at 600 nm reached 0.6 and were induced for 7 h with 0.8 m*M* IPTG at 293 K. Cells were harvested, washed in 150 m*M* NaCl, 20 m*M* Tris pH 8.0 and frozen until use.

2.2. Purification of the hRXRα LBD–9-*cis*RA complex

To ensure full saturation of the receptor, 9-*cis*RA prepared as a saturated ethanolic solution was added during all steps of the purification to 0.5%(*v*/*v*) final concentration. Owing to ligand photosensitivity, all manipulations were carried out under dimmed light. Cell pellets (2 l of culture) were resuspended in lysis buffer (500 m*M* NaCl, 20 m*M* Tris

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pH 8.0) and sonicated at 281 K. The soluble extract, clarified by ultracentrifugation (45 000 rev min^{-1} at 277 K for 2 h), was loaded onto a cobalt-chelating affinity column (Clontech). The column was washed with 500 m*M* NaCl, 10 m*M* imidazole and 20 m*M* Tris pH 8.0. Protein was eluted with 125 m*M* imidazole, 500 m*M* NaCl and 20 m*M* Tris pH 8.0. The affinity pool was concentrated using a Centricon-30 (Amicon) and injected onto a Superdex200 26/60 (Pharmacia) gel-filtration column in 500 m*M* NaCl, 20 m*M* Tris pH 8.0. Proteins eluted as two distinct well resolved peaks corresponding to an apo RXRα tetramer and the holo hRXRα dimer bound to 9-*cis*RA. The main peak corresponded to the complex, but despite the use of a saturating concentration of ligand, the presence of substantial amounts of unliganded tetramer was always observed. The hexahistidine tag was removed with bovine thrombin (Sigma). The complex was repurified on a Superdex200 10/30 (Pharmacia) gel-filtration column in 50 m*M* NaCl, 10 m*M* Tris pH 8, 0.5 m*M* EDTA, 10 m*M* DTT and 0.1 m*M* diisopropyl fluorophosphate (DIFP). Fractions containing the protein were pooled, concentrated and used for crystallization without the addition of an excess of ligand. Homogeneity was checked by SDS and native PAGE (Fig. 1).

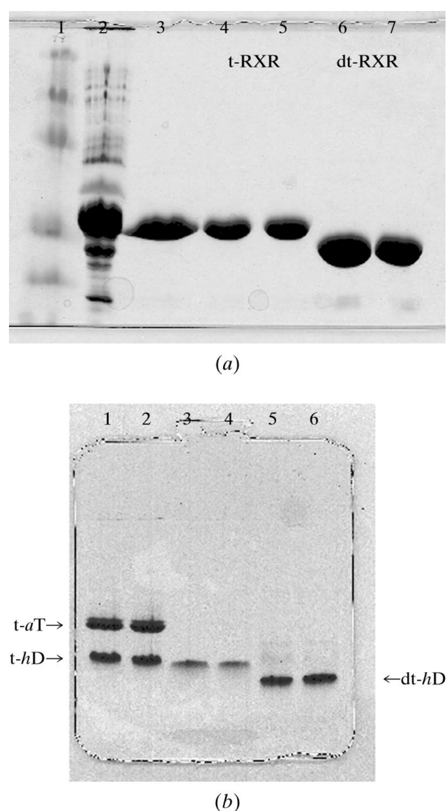

Figure 1
Purification of hRXRα LBD–9-*cis*RA complex. (*a*) SDS–PAGE 12.5% gel. Lane 1: molecular-weight markers (112, 84, 53, 35, 29 and 21 kDa). Lane 2: total soluble extract from *E. coli*. Lane 3: cobalt-chelating column pool. Lanes 4 and 5: first gel-filtration pools for tetrameric and dimeric tagged (t-RXR) species. Lanes 6 and 7: second gel-filtration pools for tetrameric and dimeric detagged species (dt-RXR) after tag removal by thrombinolysis. (*b*) Native PAGE 8–25% gradient gel. Lanes 1 and 2: affinity pool containing tagged apo tetramer (t-*a*T) and holo dimer (t-*h*D) species. Lanes 3 and 4: first gel-filtration pool corresponding to the holo dimer before tag removal by thrombinolysis. Lanes 5 and 6: final detagged holo dimer (dt-*h*D) after tag removal by thrombinolysis.

2.3. Crystallization of the hRXRα LBD–9-*cis*RA complex

2.3.1. *De novo* crystallization by direct spontaneous nucleation. Screening for crystallization conditions using the commercially available kits from Hampton Research (Crystal Screens 1 and 2, Natrix and MembFac) failed in our case. We initiated a systematic screening considering the available crystallization conditions of NR-LBDs. Carboxylic acids have been described as efficient crystallization agents for RXR (Bourguet, Ruff, Bonnier *et al.*, 1995) and RAR (Rochel *et al.*, 1997). Systematic grid screens using the carboxylic acids citrate, acetate and formate were performed at several pHs using sodium and ammonium ions as counter-cations. Protein precipitates obtained in acetate and formate salts and, to a lesser extent, in citrate salts were reversible. Nevertheless, no crystals were obtained under such conditions using a carboxylic acid as the sole crystallization agent.

Non-volatile organic alcohols such as glycerol have previously been described as effective protein stabilizing agents. The successful use of such organic cosmotropic cosolvents for crystallization of biological macromolecules has been well documented (Sousa & Lafer, 1990; Jeruzalmi & Steitz, 1997). The effects of glycerol and ethylene glycol (considered as additives) in association with 2-methylpentane-2,4-diol (MPD), hexane-1,6-diol (HXD) and propane-1,2-diol (PPD) (considered as coprecipitants) were investigated. The ternary trial mixtures were based on the association of an additive compound (ethylene glycol or glycerol) and a coprecipitant (MPD, PPD or HXD) for a given carboxylic acid precipitant (acetate, formate or citrate). The first crystals of the complex were obtained at 295 K by the vapour-diffusion method in hanging drops with a tripartite crystallization mixture containing sodium formate, glycerol and PPD. They appeared as fuzzy clusters of densely packed needles (Fig. 2*a*). The reservoir contained 3.5–4.5 *M* sodium formate, 12.5–22.5% PPD, 5–10% glycerol and 50 m*M* Tris pH 7.5–8.5 and drops were produced by mixing equal amounts of protein and reservoir solutions (Table 1). Similar crystals could be grown at 277 K. Attempts to grow RXRα LBD–9-*cis*RA single crystals of reasonable quality and size by direct nucleation failed despite intensive and extensive screening over a wide range of parameters such as pH, temperature and the concentrations of the protein and the three compounds.

2.3.2. Optimization of crystals by micro- and macroseeding. We increased the crystal

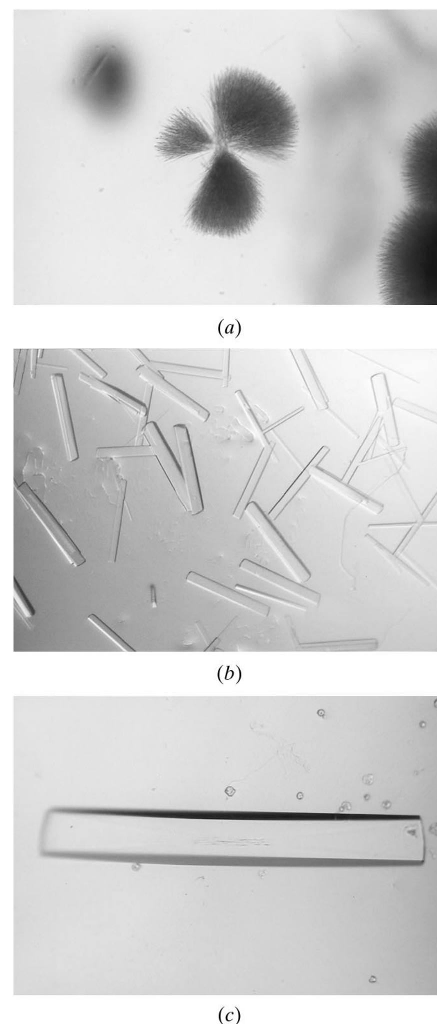

Figure 2
Crystallization of hRXRα-LBD–9-*cis*RA complex. (*a*) Clusters of needles obtained by direct nucleation in hanging drops. (*b*) Single monocrystals obtained by microseeding in sitting drops using a seed mixture of crushed needles. Crystal dimensions are about 100 × 10 × 10 µm. (*c*) Single monocrystal obtained by macroseeding in sitting drops. A similar crystal was used for structure determination. Crystal dimensions are about 700 × 70 × 70 µm.

Table 1
Summary of crystallization conditions of hRXR*α* LBD–9-*cis*RA complex.

Step	Protein concentration (mg ml^{-1})	Drop characteristics	Reservoir
De novo nucleation	5–15	10 µl hanging drop, 295 and 277 K, 1:1 ratio	3.5–4.5 *M* Na formate, 12.5–22.5% PPD, 5–10% glycerol, 50 m*M* Tris pH 7.5–8.5
Microseeding	5–10	20 µl sitting drop, 295 K, 1:1 ratio	3.5–4.5 *M* Na formate, 15–20% PPD, 5–10% glycerol, 50 m*M* Tris pH 8.0
Macroseeding	20–25	30–40 µl sitting drop, 295 K, 1:1 ratio	2.0–2.6 *M* Na formate, 15–20% PPD, 5–10% glycerol, 50 m*M* Tris pH 8.0

size and quality by micro- and macroseeding (Stura & Wilson, 1990). To obtain larger crystals, all seeding steps, especially in the case of macroseeding, were performed at 295 K in sitting drops and in increasing drop volumes. Microseeding was performed using a seed-stock mixture (Luft & DeTitta, 1999) of crushed needles briefly washed in a solution containing 2 *M* sodium formate, 10% glycerol, 10% PPD and 50 m*M* Tris pH 8.0. Seeds from serial dilutions were introduced into freshly made drops. The reservoir contained 3.5–4.5 *M* sodium formate, 15–20% PPD, 5–10% glycerol and 50 m*M* Tris pH 8.0; 10–20 µl drops were produced by mixing equal amounts of protein and reservoir solution (Table 1). Microseeded crystals appeared within 24 h and grew to optimal size and shape in about 1–2 weeks (Fig. 2*b*). For macroseeding, single crystals were washed and transferred to freshly made drops. The reservoir contained 2–2.6 *M* sodium formate, 15–20% PPD, 5–10% glycerol and 50 m*M* Tris pH 8.0; 30–40 µl volume drops were produced by mixing equal amounts of protein and reservoir solutions (Table 1). Macroseeded crystals grew to their optimal size and shape in about 2–4 weeks (Fig. 2*c*). As the crystallization liquor is a cryoprotectant, crystals were harvested in nylon cryoloops and directly flash-frozen in liquid propane for data collection.

3. Results and discussion

3.1. Purification of the hRXR*α* LBD–9-*cis*RA complex

The previously reported purification of apo RXR*α* LBD demonstrated the existence of an equilibrium between two forms of the apo RXR*α* LBD. Apo RXR*α* was first crystallized as a dimer (Bourguet, Ruff, Chambon *et al.*, 1995) and subsequently as a tetramer (Gampe *et al.*, 2000). RXR*α* is the only receptor that displays such a complex behaviour with an equilibrium between several species. As we thought that the existence of this equilibrium could preclude crystallization of the liganded RXR*α* LBD, we designed a purification minimizing the presence of unliganded RXR species. The purification yielded substantial amounts of pure and soluble complex (5–10 mg protein per liter of culture) as shown in Fig. 1(*a*). Use of a ligand excess in the early steps of the purification was essential to minimize the amount of apo species consisting mainly in a tetramer. The first gel filtration was used to separate apo RXR tetramer from holo RXR dimer as shown in Fig. 1(*b*); this chromatographic step is essential for crystallization. The resulting complex is stable, homogenous and monodisperse; in particular, the purified holo complex does not show detectable reassociation towards the apo tetrameric form. The homogeneity and monodispersity of the complex were investigated using biophysical techniques. A solution study combining small-angle scattering and analytical ultracentrifugation (Egea *et al.*, 2001) demonstrated (i) the homogeneity and monodispersity of the purified hRXR*α* LBD–9-*cis*RA complex and (ii) that the solution species was a holo homodimer.

3.2. Crystallization of the hRXR*α* LBD–9-*cis*RA complex

Crystals of hRXR*α* LBD–9-*cis*RA complex were grown using a carboxylic acid (sodium formate) as a reversible precipitant in combination with two organic polyalcohols (glycerol and propane-1,2-diol) that acted as stabilizing and solubilizing co-solvents. Despite much effort, it was not possible to grow single macrocrystals by direct nucleation. Crystals were optimized by seeding over two successive rounds of micro- and macroseeding. In the macroseeding step, optimal crystal growth appeared to be extremely sensitive to sodium formate concentration. The content of washed single crystals was checked by electrospray ionization mass spectrometry under both denaturing and native conditions (Potier *et al.*, 2000) and confirmed the presence of the stoichiometric non-covalent hRXR*α* LBD–9-*cis*RA complex (MW = 27 536.6 ± 0.8 Da) by comparison with the apo hRXR*α* LBD monomer (MW = 27 234.3 ± 0.9 Da).

Data were collected at the ESRF (Grenoble, France) on beamlines BM14 and ID14; the diffraction limit is 2.2 Å. Crystals belong to the orthorhombic space group $P2_12_12_1$ (unit-cell parameters a = 66.1, b = 86.3, c = 90.6 Å, $\alpha = \beta = \gamma = 90°$). There are two monomers in the asymmetric unit, with a solvent content of 51% and a relative density of 2.5 Å^3 Da^{-1}. The structure was solved by molecular replacement (Egea, Mitschler *et al.*, 2000).

4. Concluding remarks

We have reported the purification and crystallization of a stoichiometric complex between hRXR*α* LBD and its natural ligand 9-*cis* retinoic acid. The use of micro- and macroseeding was essential for the optimization of crystal size and quality. The crystal structure of holo RXR*α* LBD bound to 9-*cis*RA has been solved and allowed us to visualize (i) the structural basis of retinoid acid receptor selectivity towards isomers of retinoic acid and (ii) the conformational changes induced upon ligand binding. Since all crystallographic studies carried on NR LBDs support the concept of the uniqueness of the holo agonist conformation, the purification and crystallization schemes described above can be generalized to the case of other synthetic RXR agonist–RXR complexes of pharmaceutical interest.

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