Mass-spectrometric analysis of agonist-induced retinoic acid receptor γ conformational change

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Apo and holo forms of retinoic acid receptors, and other nuclear receptors, display differential sensitivity to proteolytic digestion that likely reflects the distinct conformational states of the free and liganded forms of the receptor. We have developed a method for rapid peptide mapping of holo-retinoic acid receptor γ that utilizes matrix-assisted laser-desorption/ionization time-of-flight MS to identify peptide fragments that are derived from the partially proteolysed holo-receptor. The peptide maps of retinoic acid receptor γ bound by four different agonists were identical, suggesting that all four ligands induced a similar conformational change within the ligand-binding domain of the receptor. In all cases, this agonist-induced conformational change promoted the direct association of retinoic acid receptor γ with the transcriptional co-activator p300 and inhibited interaction of the receptor

with the nuclear receptor co-repressor. SR11253, a compound previously reported to exert mixed retinoic acid receptor γ agonist/antagonist activities in cultured cells, was found to bind directly to, but only weakly altered the protease-sensitivity of, the receptor and failed to promote interaction of the receptor with p300 or induce dissociation of receptor–nuclear receptor co-repressor complexes. This technique should be generally applicable to other members of the nuclear receptor superfamily that undergo an induced structural alteration upon agonist or antagonist binding, DNA binding and/or protein–protein interaction.

Key words: conformation, MALDI, nuclear receptors, transcriptional co-activators, transcriptional co-repressors.

INTRODUCTION

Transcriptional activation mediated by ligand-regulated nuclear receptors is a multi-step process that is initiated by an agonist-induced conformational change which facilitates co-repressor ↔ co-activator exchange [1]. The results of crystallographic studies have shown that that agonist binding to nuclear receptors induces: (1) a large rearrangement involving ligand-binding-domain (LBD) helices 11 and 12; (2) bending of helix 3; and (3) rotation of the omega loop separating helices 1 and 2/3 [2–5]. In the case of retinoic acid receptor γ (RAR γ), the repositioned helix 12 forms the lid of the ligand-binding pocket [4]. The agonist-induced rearrangement of helix 12 also juxtaposes the core of the ligand-dependent transcriptional activation function (AF-2) in helix 12 with helices 3, 4, and 5 creating a shallow hydrophobic groove competent for interaction with the canonical LXXLL motif of transcriptional co-activators [6–10], but incompatible with co-repressor binding [11–13].

Transcriptional co-activator proteins that interact preferentially with activated nuclear receptors can be divided into two

general families, each of which contain multiple subfamilies. Transcriptional co-activators harbouring histone acetyltransferase (HAT) activity include p300/CBP [14,15], P/CAF [16,17] and the p160 family of proteins (SRC-1/NCoA-1 [7,15,18], TIF2/GRIP1/NCoA-2/SRC-2 [7,19,20] and RAC3/AIB1/PCIP/ACTR/SRC-3 [7,21–23]. By covalent modification (acetylation) and subsequent decondensation of chromatin, transcriptional co-activators possessing HAT activity are believed to enhance the access of the RNA polymerase II (Pol II) complex to the template [24]. In addition to interacting with activated nuclear receptors, p160 and p300/CBP proteins interact with each other and the latter interact with P/CAF [16,17], suggesting that the multi-protein chromatin-modifying machinery assembled on the promoter of target genes is large and complex.

Interaction of the HAT co-activator complex with the activated receptor is apparently transient, as dissociation is believed to occur as a result of acetylation of one or more co-activators on lysine residues adjacent to the signature LXXLL motif [25]. Subsequently, the activated receptor presumably recruits a second class of multi-protein transcriptional co-activator complexes to

Abbreviations used: 9-*cis*-RA, 9-*cis*-retinoic acid; AF, activation function; AGN193109, 4-{2-[5,6-dihydro-5,5-dimethyl-8-(4-methylphenyl)-2-naphthalenyl]propynyl}benzoic acid; AHPN, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (for reasons of brevity this and the other long systematic names are not redefined in the text); BMS270394, (R)-3-fluoro-4-[2-hydroxy-2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)acetylaminol]benzoic acid; chymotrypsin-TLCK, tosyl-lysylchloromethane-treated chymotrypsin; DPSA, differential proteolytic sensitivity assay; GST, glutathione S-transferase; HAT, histone acetyltransferase; LBD, ligand-binding domain; MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight MS; NCoR, nuclear receptor co-repressor; Pol II, RNA polymerase II; (h)RAR γ , (human) retinoic acid receptor γ ; RARE, retinoic acid response element; RXR, retinoid X receptor; SR11253, 1,3-dithiane of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)-2-naphthalenecarboxylic acid; SR11254, the (Z)-oxime of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)-2-naphthalenecarboxylic acid; *trans*-RA, *trans*-retinoic acid; TRAP, thyroid-hormone-receptor-associated protein; TFA, trifluoroacetic acid; trypsin-TPCK, tosylphenylalanyl-chloromethane-treated trypsin.

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the template, and this latter complex, referred to as the thyroid-hormone-receptor-associated protein (TRAP) complex, appears to be similar to the SRB ('suppressor of RNA polymerase B')- and Mediator-containing cofactor (SMCC), vitamin D receptor-interacting protein (DRIP) and the yeast Mediator complexes [26]. Because the TRAP or TRAP-like complexes harbour components of the Pol II machinery and/or recruit the Pol II complex to the template, formation of the transcriptional preinitiation complex and subsequent transcription are facilitated [26]. Thus agonist-induced nuclear-receptor conformational change, which is crucial for interaction of the receptor with two distinct classes of co-activator proteins, the HAT and TRAP proteins, plays a central role in the signalling pathways of many nuclear receptors, leading to transcriptional activation.

We have developed methodology to map ligand-induced conformational change in human RAR γ (hRAR γ) using a combination of the differential proteolytic sensitivity assay (DPSA) and matrix-assisted laser-desorption-ionization time-of-flight (MALDI-TOF) MS. This technique, combined with *N*-terminal amino acid sequencing, allowed the unambiguous identification of agonist-induced hRAR γ proteolytic fragments. Together with knowledge of the three-dimensional structure of the receptors, this technique can be used to develop a dynamic map of ligand-binding-induced conformational change among retinoic acid receptors, as well as other members of the nuclear-receptor superfamily.

MATERIALS AND METHODS

Reagents

All-*trans* retinoic acid (*trans*-RA) and trypsin-TPCK (tosyl-phenylalanylchloromethane-treated trypsin) were purchased from Sigma, and 9-*cis*-[11,12- ^3H]retinoic acid (43 Ci/mmol) was purchased from Amersham. 9-*cis*-Retinoic acid (9-*cis*-RA) and the 1,3-dithiane (SR11253, formerly known as MM11253) and the (*Z*) oxime (SR11254) of 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenylcarbonyl)-2-naphthalenecarboxylic acid [27] were synthesized as previously described [28–30].

Plasmid construction and protein preparation

An hRAR γ fragment encoding Met 73 –Ala 454 followed by the natural stop codon was amplified by PCR from a template kindly supplied by Dr A. Krust and Dr P. Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France). This fragment was appropriately digested and subcloned into *Nde*I/*Bam*HI-digested pET14b (Novagen), generating a hexahistidine-hRAR γ fusion protein, (His) $_6$ -hRAR γ :

MGSSHHHHHHSSGLVPRGSHM $^{73\dots}$ hRAR γ -A 454

(the amino acids contributed from the vector are underlined). The integrity of this construct was verified by sequence analysis. The preparation of a bacterial expression vector for a glutathione S-transferase (GST)–p300 $_{1-450}$ fusion protein was previously described [31].

(His) $_6$ -hRAR γ and GST–p300 $_{1-450}$ were expressed in *Escherichia coli* BL21(DE3)pLysS and purified by nickel-chelate and GSH-affinity chromatography respectively.

DPSA studies and purification of proteolytic fragments

Purified (His) $_6$ -hRAR γ (0.1–0.2 nmol) was preincubated with ligands or vehicle (0.1%, v/v, ethanol) for 15 min at 22 °C. Trypsin-TPCK (30–50 $\mu\text{g}/\text{ml}$ final concn.) was then added and proteolytic digestions were carried out for an additional 15 min

at 22 °C. DPSA reactions were terminated by addition of 2 $\times$ Laemmli sample buffer and boiling (gel analysis) or by acidification with trifluoroacetic acid (TFA; final concentration of 0.1%, v/v) and chromatography on 10-mm-diameter $\times$ 250-mm-long C $_4$ reversed-phase HPLC column (Vydac) equilibrated in aq. 0.1% TFA and running at 2.5 ml/min (for HPLC purification). After washing, peptides were eluted from the HPLC column with a 0–100% gradient of 70% acetonitrile in 0.1% TFA over a period of 100 min. The HPLC elution profile of both proteolytic fragments, PF30 and PF31, was as determined by analysis of individual chromatographic fractions by gel electrophoresis followed by Coomassie Blue staining.

GST-pulldown experiments

GST-pulldown experiments were performed as previously described using GST–p300 $_{1-450}$ or GST–nuclear receptor co-repressor (NCoR) $_{2110-2453}$ fusion proteins and [^{35}S]methionine-labelled hRAR γ prepared by *in vitro* translation.

Radioligand binding experiments

Competition radioligand binding experiments were conducted using purified (His) $_6$ -hRAR γ . All experiments were performed at 4 °C for 1 h in a 200 μl of binding buffer (10 mM Hepes, pH 7.8, 150 mM NaCl, 0.1 mM EDTA and 0.5 mM dithiothreitol, containing 10% glycerol and 2.5 $\mu\text{g}/\mu\text{l}$ insulin) using a receptor concentration of approx. 10 nM and 1–3.5 nM 9-*cis*-[11,12- ^3H]RA (43 Ci/mmol). Bound 9-*cis*-[^3H]RA was separated from free radioligand by gel filtration on Sephadex G-50 resin, which was pre-equilibrated in binding buffer, and was quantified by liquid-scintillation counting. Non-specific 9-*cis*-[^3H]RA binding (defined as that occurring in the presence of 1 μM non-labelled 9-*cis*-RA) generally accounted for approx. 5% of total radiolabel bound. Data from competition radioligand binding experiments were analysed using a non-linear least-squares fitting routine (Prism; GraphPad) as previously described [32].

MALDI-TOF MS

HPLC-purified (His) $_6$ -hRAR γ and tryptic fragments derived from the receptor were freeze-dried, then resuspended in a small volume of acetonitrile/TFA and then diluted (1:2) into α -cyano-4-hydroxycinnamic acid (10 mg/ml) in 40% acetonitrile/60% 0.1% TFA. A 1 μl droplet of this analyte/matrix solution was deposited onto a precrystallized organic matrix and allowed to dry in air. The samples were then analysed using a custom-built TOF mass spectrometer equipped with an Na:YAG-pulsed laser (355 nm; Spectra Physics), a two-stage delayed-extraction ion source and a single-stage ion mirror (reflectron). In parallel, the N-termini of hRAR γ tryptic peptides and full-length (His) $_6$ -hRAR γ were determined by N-terminal sequencing (performed at the University of Oregon Institute of Molecular Biology by Deb McMillen). The results of both MALDI experiments and N-terminal sequencing were analysed using the ProteinProspector software package (version 3.2.1; University of California at San Francisco) to identify hRAR γ tryptic peptides.

Computational analyses

The crystal structure of the hRAR γ LBD (1FCX) was acquired from RCSB (Research Collaboratory for Structural Bioinformatics) Protein Data Bank (www.rcsb.org). CAChe software (Fujitsu, Beaverton, OR, U.S.A.) was used for conformational analyses of free ligands (AHPN, SR11253, SR11254 and BMS270394). The RAR γ -bound conformer of BMS270394 with fixed torsion

angles of 234, 255 and 235° was used [33]. Conformer geometry was optimized using the MM3 force field with the conjugate gradient method used to locate the energy minima of AHPN, SR11253 and SR11254 with hydrogen atoms included and each value of the search label varied at 30° intervals, while other labels were held at their minimum energy positions and Van der Waals interactions between atoms separated by more than 9 Å excluded. Carboxylate groups were considered rigid. Optimization was deemed complete when the energy change was less than 0.001 kcal/mol (1 kcal = 4.184 kJ). Low-energy conformers of each structure within 2 kcal of the global energy minimum were superimposed with the conformer obtained for BMS270394 by using least-squares rigid fit of the atoms corresponding to its 2- and 4 α -naphthalenyl, hydroxy-group-bearing and benzoic carbon atoms.

RESULTS

Mapping agonist-induced hRAR γ conformational change

To determine if structurally diverse hRAR γ agonists promote and/or stabilize differential receptor conformations, we assessed the protease-sensitivity of (His) $_6$ -hRAR γ when bound by three agonists: *trans*-RA, 9-*cis*-RA and the pro-apoptotic retinoid AHPN, which was first reported as the RAR γ -selective agonist CD437 [34,35]. All three agonists induced the formation of two identical partial tryptic fragments of (His) $_6$ -hRAR γ , namely PF30 and PF31 (Figure 1A, lanes 4, 7 and 10). This finding suggests that *trans*-RA, 9-*cis*-RA and AHPN likely induce and/or stabilize (an) identical, or highly related, conformation(s) of hRAR γ *in vitro*.

Fragments PF30 and PF31 were eluted together in a well-separated peak from a reversed-phase HPLC column with a retention time of approx. 130 min, corresponding to an acetonitrile concentration of 62.5% (Figure 1B). This fraction was then subjected to MALDI-TOF MS analysis to determine the mass of each peptide. Doublet peaks were observed for the singly (MH^+), doubly (MH^{2+}), triply (MH^{3+}) and quadruply (MH^{4+}) charged species, corresponding to both PF31 and PF30 (Figure 1C). On the basis of the mean ($\pm$ S.E.M.) of four independent MALDI-TOF determinations, the masses of PF31 and PF30 were calculated to be 31058 ± 96 and 30714 ± 40 Da respectively. The N-termini of both PF31 and PF30 were sequenced by Edman degradation and found to correspond to Glu 170 and Glu 173 of hRAR γ respectively (results not shown). These findings, together with the known cleavage specificity of trypsin, indicate that PF31 corresponds to hRAR γ -(Glu 170 -Lys 451)-peptide (calculated mass 31088 Da), whereas PF30 corresponds to hRAR γ -(Glu 173 -Lys 451)-peptide (calculated mass 30733 Da).

MALDI-TOF analyses ($n = 3$) revealed that the mass of full-length unproteolysed (His) $_6$ -hRAR γ was 44969 ± 32 Da (mean $\pm$ S.E.M.) compared with a predicted mass of 45089 Da (results not shown). This difference was accounted for by the lack of the (His) $_6$ -hRAR γ initiator methionine (Met 1 ; molecular mass 149 Da), and this was confirmed by N-terminal sequence analysis (results not shown). These findings indicate that the preparation of (His) $_6$ -hRAR γ used for DPSA studies possessed an intact C-terminus corresponding to codon 454 (Ala 454) and that cleavage at Lys 451 occurred as a consequence of protease treatment.

SR11253 fails to induce hRAR γ structural alterations at a concentration of 1 μ M

DPSA experiments were conducted to determine if (His) $_6$ -RAR γ bound by the reported mixed agonist/antagonist SR11253 [36,37]

Figure 1 Identification of agonist-induced (His) $_6$ -hRAR γ partial tryptic fragment

(A) Differential proteolytic sensitivity assay. Purified (His) $_6$ -hRAR γ (lane 1) was incubated with increasing concentrations of AHPN (10, 100 and 1000 nM respectively; lanes 2–4), *trans*-RA (lanes 5–7), 9-*cis*-RA (lanes 8–10) or vehicle (0.1%, v/v, ethanol; lane 11) and then digested with trypsin-TPCK (30 μ g/ml) as indicated. Fragments were separated by SDS/12.5%-(w/v)-PAGE and identified by staining with Coomassie Blue. The positions of two ligand-induced fragments, PF31 and PF30, and that of trypsin-TPCK are indicated. (B) Purification of PF30 and PF31 by reversed-phase HPLC. Partial tryptic digests of (His) $_6$ -hRAR γ (as shown in A) were injected on to a 10 mm diameter $\times$ 250 mm long C $_4$ reversed-phase column equilibrated in 0.1% TFA and running at 2.5 ml/min. After washing, peptides were eluted with a 0–100% gradient of 70% acetonitrile in 0.1% TFA (% B) over 100 min. PF30 and PF31 were eluted together in a single peak at approx. 130 min (62.5% acetonitrile) as determined by SDS/PAGE and Coomassie Blue staining of individual fractions. No other peptides were observed in the PF30/31 fraction. (C) Determination of the mass of PF30 and PF31 by MALDI-TOF MS. Doublet peaks corresponding to singly (MH^+), doubly (MH^{2+}), triply (MH^{3+}) and quadruply (MH^{4+}) charged species of both PF31 and PF30 are indicated. Averaging four independent MALDI-TOF determinations, the masses of PF31 and PF30 were calculated to be 31057.5 ± 96 and 30713.8 ± 40 Da respectively. These findings, together with N-terminal sequence analysis (see the text), indicate that PF31 corresponds to hRAR γ Glu 170 -Lys 451 (theoretical mass of 31088 Da), and PF30 corresponds to residues Glu 173 -Lys 451 (theoretical mass of 30733 Da).

Figure 2 SR11253 does not alter the protease-sensitivity of (His)₆-RAR γ

(A) DPSA experiments were conducted as described in the legend to Figure 1 using (His)₆-RAR γ and *trans*-RA, the RAR γ -selective agonist SR11254, and the reported RAR γ mixed agonist/antagonist SR11253 (all tested at a concentration of 1 μ M). Shown is a Coomassie Blue-stained gel that is representative of those obtained in six similar experiments. The positions of proteolytic fragments PF30 and PF31, trypsin-TPCK and molecular-mass markers (first lane) are indicated. The concentration of trypsin-TPCK used for these studies was 30 μ g/ml. (B) Competition radioligand binding experiments using purified (His)₆-RAR γ . These experiments were conducted as described in the Materials and methods section. The data represent the mean $\pm$ S.E.M. ($n = 3$) of the percentage of 9-*cis*-[11,12-³H]₂RA binding to (His)₆-RAR γ that was inhibited by unlabelled *trans*-RA, SR11254, and SR11253 (all tested at 1 μ M). (C) GST-pulldown experiments using GST-p300₁₋₄₅₀ and [³⁵S]methionine-labelled RAR γ in the absence and presence of ligands. [³⁵S]Methionine-labelled RAR γ was preincubated with the indicated ligands (all used at a concentration of 1 μ M) or vehicle (0.1%, v/v, ethanol) for 15 min at 4 $^{\circ}$ C prior to the addition of GST-p300₁₋₄₅₀. The autoradiograph shown is from a single experiment that is representative of five (GST-p300₁₋₄₅₀) and three (GST) identical experiments.

exhibited a pattern of proteolytic sensitivity that differed from that of either the aporeceptor or receptor bound by the three agonists tested above. SR11253 failed to alter the proteolytic sensitivity of hRAR γ at a concentration of 1 μ M (Figure 2A, lane 5), whereas both *trans*-RA and a RAR γ -selective agonist, SR11254 [37,38], strongly induced formation of PF31 and PF30 at this concentration (Figure 2A, lanes 3 and 4 respectively). Radioligand binding experiments were then conducted to verify direct binding of SR11253 to purified (His)₆-RAR γ . SR11253, *trans*-RA and SR11254 inhibited the binding of 9-*cis*-[³H]RA to (His)₆-hRAR γ to a similar extent (Figure 2B), demonstrating that all three compounds bound directly to the purified receptor *in vitro*.

Agonists promote interaction of hRAR γ with the transcriptional co-activator p300

To determine the consequence of induced hRAR γ conformational change *in vitro*, we assessed the ability of RAR γ agonists and SR11253 to promote interaction of the receptor with the transcriptional co-activator p300 [39]. All agonists enhanced association of hRAR γ with GST-p300, although AHPN was consistently weaker than other agonists tested (compare lane 4 with lanes 3, 6 and 7 of Figure 2C). The effect of the mixed agonist/antagonist SR11253 on hRAR γ -p300 interaction was indistinguishable from that of vehicle at a concentration of 1 μ M (Figure 2C, lanes 5 and 2 respectively; see also below), indicating that SR11253 did not promote or stabilize a hRAR γ conformation that was permissive for interaction with p300. Neither vehicle nor any of the ligands tested promoted interaction of the receptor with GST bound to GSH-Sepharose (Figure 2C, lanes 8–13), indicating the interaction specificity of holo-hRAR γ .

High concentrations of SR11253 induce agonist-like hRAR γ structural alteration

SR11253 bound directly to purified (His)₆-hRAR γ (Figure 2B), yet neither altered the protease-sensitivity of the receptor in a detectable manner, nor promoted interaction of the receptor with p300 when tested at a concentration of 1 μ M. Competition radioligand binding experiments revealed that SR11253 inhibited the binding of 9-*cis*-[³H]RA to purified (His)₆-hRAR γ with a K_i of 424 ± 29 nM ($n = 4$; Figure 3A). Thus DPSA experiments conducted using a concentration of SR11253 of 1 μ M (Figure 2A) resulted in a fractional occupancy of the receptor of approx. 0.7. Because this degree of fractional occupancy may have been insufficient to observe alterations in the protease-sensitivity of the SR11253-bound receptor, DPSA studies were repeated using SR11253 over a higher concentration range (up to 5 μ M, corresponding to a fractional occupancy of approx. 0.92). In the present study, SR11253 and 9-*cis*-RA were found to induce the formation of similar RAR γ tryptic fragments, although the former was much weaker in this respect (compare lanes 3–6 and 7–10 of Figure 3B). To verify a SR11253-induced alteration in (His)₆-hRAR γ structure, DPSA experiments were also conducted using tosyl-lysylchloromethane-treated chymotrypsin (chymotrypsin-TLCK), a protease with cleavage specificity completely different from that of trypsin-TPCK. SR11253 and 9-*cis*-RA induced the formation of multiple and identical chymotryptic fragments of hRAR γ (compare lanes 3–6 and 7–10 of Figure 3C). Again, SR11253 was much weaker than 9-*cis*-RA with regard to the induction of these chymotryptic fragments, consistent with an affinity difference of nearly 200-fold between these two compounds. Because SR11253 appears to possess RAR γ

Figure 3 High concentrations of SR11253 induce agonist-like RAR γ structural alterations

(A) Titration of 9-*cis*-[³H]RA (1.7 nM) binding to purified (His)₆-RAR γ by SR11253. The points shown represent the means $\pm$ S.E.M. for four independent determinations, each conducted in triplicate. The pooled data were analysed using an iterative curve-fitting routine (Prism, GraphPad) that yielded an IC₅₀ for SR11253 of 724 nM, corresponding to a K_i of 424 nM (based on an affinity of the receptor for 9-*cis*-[³H]RA of 2.4 nM [50] and assuming a single class of binding sites). The theoretical line drawn is based on the EC₅₀ parameter estimate. Non-

agonist-like activity at high concentrations in DPSA experiments when examined using different proteases, we reconsidered the possibility that this compound may promote interaction of the receptor with the co-activator, p300. Thus *in vitro* protein-protein interaction experiments were conducted using GST-p300₁₋₄₅₀, [³⁵S]hRAR γ and a high concentration of SR11253 (5 μM). This concentration was chosen because it appeared to confer a maximal effect in DPSA studies using trypsin-TPCK (compare 1 and 5 μM SR11253 in lanes 5 and 6 of Figure 3B) and it approaches the upper limit of SR11253 solubility in aqueous buffer. 9-*cis*-RA (also 5 μM), which was used as a positive control for these experiments, strongly induced interaction of [³⁵S]hRAR γ with GST-p300₁₋₄₅₀ (Figure 3D, lane 3). However, SR11253 again failed to promote interaction of the receptor with GST-p300 (compare the effects of vehicle and SR11253 in lanes 2 and 4 respectively of Figure 3D). Interaction of [³⁵S]hRAR γ with GST was not promoted by vehicle or either ligand (Figure 3D, lanes 5–7). These results demonstrate that SR11253 bound directly to hRAR γ and weakly induced agonist-like structural alterations, but failed to promote interaction of the receptor with a transcriptional co-activator fragment harbouring two LXXLL motifs. However, we cannot exclude the possibility that these co-activator recruitment studies simply lacked the sensitivity to detect effects of the weak mixed agonist-antagonist SR11253. Alternatively, or additionally, SR11253 may promote interaction of RAR γ with transcriptional co-activators other than p300, but we have no experimental evidence to support this hypothesis at present.

9-*cis*-RA and SR11254, but not SR11253, inhibit RAR γ -NCoR interaction

In contrast with classical RAR γ agonists that induce dissociation of RAR γ -NCoR complexes, AGN193109 increases the affinity of the receptor for this co-repressor [40,41] and, as such, is known as an inverse agonist. GST-pulldown experiments were conducted to address the possibility that SR11253 may function as an inverse agonist. 9-*cis*-RA and SR11254 strongly decreased association of the receptor with GST-NCoR, and this effect was most dramatic when the reaction was performed at 22 °C (compare lane 2 with lanes 3 and 5 of the upper panel of Figure 4), but was also observed at 30° and 37 °C (lanes 7, 9, 11 and 13). In contrast, SR11253 did not appear to influence the interaction of RAR γ with NCoR at any temperature tested (Figure 4, lanes 4, 8, and 12). These experiments were conducted using a ligand concentration of 5 μM and in the absence of both the RAR γ dimerization partner retinoid X receptor α (RXR α) and a retinoic acid response element (RARE). However, identical results were obtained when RAR γ -co-repressor dissociation studies were conducted in the presence of RXR α and a RARE consisting of a directly repeated motif (AGGTCA) separated by 5 bp (results

specific binding was defined as that occurring in the presence of 1 μM unlabelled *trans*-RA and was negligible. (B) [³⁵S]RAR γ DPSA studies carried out using trypsin and a high concentration range of SR11253 and 9-*cis*-RA (10, 100, 1000 and 5000 nM). Note that the concentration of trypsin-TPCK used for these studies was reduced to 25 μg/ml to facilitate detection of SR11253-induced proteolytic fragments. The positions of PF31 and PF30 are indicated. (C) [³⁵S]RAR γ DPSA studies carried out using chymotrypsin (10 μg/ml) and a high concentration range of SR11253 and 9-*cis*-RA (10, 100, 1000 and 5000 nM). The positions of 9-*cis*-RA-specific fragments are indicated by an asterisk to the right of the autoradiograph. (D) GST-pulldown experiments using GST-p300₁₋₄₅₀ and [³⁵S]methionine-labelled RAR γ were conducted as described in the legend to Figure 2(C), except that the concentration of 9-*cis*-RA and SR11253 was 5 μM. The autoradiographs shown in (B)–(D) correspond to representative experiments that were repeated three to five times.

Figure 4 SR11253 neither facilitates nor inhibits RAR γ interaction with NCoR

GST-pull-down experiments were conducted using GST-NCOR₂₁₁₀₋₂₄₅₃ (upper panel) or an equal molar amount of GST (lower panel) and [³⁵S]methionine-labelled RAR γ . These experiments were conducted at 22, 30 and 37 °C as indicated and all ligands were used at a final concentration of 5 μ M. Shown is a representative experiment that was replicated twice.

not shown). These findings suggest that SR11253, unlike AGN 193109, is not a RAR γ inverse agonist.

DISCUSSION

Partial tryptic digestion of (His)₆-RAR γ bound to *trans*- and 9-*cis* isomers of RA was found to result in the appearance of two identical proteolytic fragments, PF30 and PF31, both of which span nearly the entirety of the LBD. This result was expected, since Moras' group has previously shown that *trans*- and 9-*cis*-RA assume a similar shape within the ligand-binding pocket of RAR γ [3]. Moreover, the similarity in the conformation of the RAR γ -bound naturally occurring isomers of RA translated to the structure of the ligand-binding pocket itself, leading to similar overall protein conformations and identical transcriptional regulatory activities [3]. These results are in complete agreement with the protease-mapping studies presented herein. In addition, we found that the binding of two RAR γ -selective agonists, AHPN and SR11254, induced structural alterations in the receptor that were indistinguishable from those induced by *trans*- and 9-*cis*-RA. These findings indicate that all four compounds induce the formation of a tightly compacted protease-resistant conformation of the receptor LBD. Presumably, this conformation corresponds to that of the holoreceptor in which helix 12 is placed in juxtaposition with helices 3, 4 and 5, forming the cleft of the co-activator-docking motif [3,4,8–10]. Consistent with this hypothesis, these agonists similarly promoted interaction of the receptor with the co-activator p300 *in vitro*. Considered together, these findings suggest that the binding of AHPN by RAR γ is unlikely to result in the formation of a receptor conformation that differs grossly from that of either the *trans*-RA- or 9-*cis*-RA-bound form of the receptor, as was previously suggested [42].

This interpretation is supported by the modelling studies presented in Figure 5(A), in which low-energy conformers of RAR γ -selective agonists AHPN and SR11254 are shown after least-squares close-fitting with the crystal structure of the RAR γ -selective agonist BMS270394 [33]. The carboxy group of BMS270394 was reported to be hydrogen-bonded to Leu²³³ of helix 3, helix 1 residues Arg²⁷⁸ and Ser²⁸⁹ and a water molecule, while the hydroxy group on the bridge of BMS270394 was reported to be hydrogen-bonded to the sulphur atom of M-272 in helix 5 [33]. The overlap of AHPN, SR11254 and BMS270394 low-energy conformers indeed revealed that the hydroxy-group oxygen atoms of these agonists are all in close proximity to the Met²⁷²

sulphur atom (4.1, 2.5 and 3.3 Å respectively; Figure 5A). Interestingly, these distances are inversely proportional to the ability of these agonists to activate RAR γ on the (TREpal)₂-*tk*-CAT reporter in CV-1 cells (EC₅₀ values of 69, 22, and 30 nM respectively; X.-k. Zhang and M. I. Dawson, unpublished work). The overlap shown in Figure 5(A) also suggests that the 1-adamantyl group at the 3-position of AHPN does not prevent the receptor from assuming an agonist-induced conformation because this moiety occupies the same space as that taken by the 5,6,7,8-tetramethylnaphthalene rings of BMS270394 and SR11254. This finding is consistent with the agonist properties of AHPN in DPSA experiments (Figure 1A) and co-activator recruitment studies (Figure 2C).

The findings presented herein demonstrate that SR11253 occupies the ligand-binding pocket of RAR γ and alters the tryptic-sensitivity of the receptor, albeit weakly, but in a manner consistent with that of all other agonists tested (Figure 3B). In addition, the effects of SR11253 and 9-*cis*-RA on the chymotryptic-sensitivity of RAR γ were indistinguishable (Figure 3C), again suggesting that SR11253 possesses weak agonist-like activity. However, SR11253 induced neither dissociation of NCoR from RAR γ (Figure 4) nor recruitment of p300 to the receptor in the manner of classical agonists, even at concentrations that were sufficient to induce agonist-like structural alterations (Figures 3B and 3C). These findings are consistent with modelling studies presented in Figure 5(B), suggesting that the steric bulk of the 2-substituted 1,3-dithianyl ring of SR11253 may prevent the M-272 side chain, and hence helix 5, from stably assuming the classical agonist-induced conformation that is required for induced dissociation of co-repressors and facilitation of interaction with transcriptional co-activators. The distances measured between the dithianyl 4- and 5-methylene carbon atoms and the Met²⁷² sulphur atom are 2.1 and 1.4 Å respectively. The latter distance indicates that steric hindrance by the 5-methylene group would be appreciable. The dithianyl ring of SR11253 may also interfere with the agonist-induced positioning of the phenyl ring of Phe³⁰⁴ in the loop between RAR γ helices 6 and 7 [33,43], as the distances between the *para*-carbon atom of the Phe³⁰⁴ phenyl ring and the dithianyl 4-methylene carbon atom and the 3-sulphur atom are 2.5 and 2.1 Å respectively. A similar rationale may explain the RAR γ antagonist activity of SR11302 [44]. In both compounds, a bulky group located in the bridge region of the retinoid skeleton that links the hydrophobic and hydrophilic termini is present and may function similarly to prevent the receptor from adopting the classical agonist-induced conformation.

Figure 5 Modelling the interactions of selective ligands with RAR γ

Close-fitting of lowest-energy conformers of RAR γ -selective agonists AHPN and SR11254 (**A**) and the RAR γ -selective, mixed agonist/antagonist SR11253 (**B**) with the structure of the RAR γ bound by the agonist BMS270394. For these analyses, all ligands were assumed to bind in a RAR γ ligand-binding pocket identical with that occupied by BMS270394. The peptide backbone and side chains that line this pocket (see [33]) are shown in cyan, with oxygen, nitrogen atom and sulphur atoms labelled in red, blue and yellow respectively, while the retinoids are colour-encoded as follows: BMS270394 (magenta), AHPN (blue), SR11254 (green) and SR11253 (yellow).

Alternatively, it is also possible that the SR11253-bound receptor may exist in a distinct conformation(s) that was (were) simply not revealed by partial digestion with either trypsin or chymotrypsin. Keidel and colleagues [45] previously used DPSA methodology to demonstrate that binding of the RAR α -selective antagonist Ro 41-5253 to hRAR α resulted in a pattern of proteolytic sensitivity that differed from that of either the apo- or the *trans*-RA-bound receptor. More recently, crystallographic studies have revealed that the binding of the retinoid antagonist BMS614 to the RAR α LBD results in the repositioning of helix 12 into the 'antagonist' conformation [46] similar to that of oestrogen receptor β bound by the anti-oestrogen raloxifene [47] or the partial agonist genistein [48], and oestrogen receptor α bound by the anti-oestrogen tamoxifen [49]. In these examples, binding of antagonists (or the partial agonist genistein) causes juxtapositioning and subsequent interaction of helix 12 with structural motifs in helices 3 and 4 that contribute to the co-activator interaction interface. In contrast with the agonist-bound form of the receptors, however, the antagonist-induced repositioning of helix 12 does not enhance interaction of the receptors with any of the known transcriptional co-activator proteins possessing LXXLL motifs.

Although the possibility that SR11253 induces the re-positioning of hRAR γ helix 12 into an antagonist conformation is, on the basis of the precedent of antagonist-induced conformational change among other nuclear receptors, an appealing idea, we have no experimental evidence to support this possibility. Rather, DPSA studies suggest that SR11253 induces a RAR γ conformation that resembles that of the holo-receptor, albeit much

less efficiently and possibly with a lesser degree of stability relative to that induced by classical agonists. Importantly, the SR11253 did not induce dissociation of NCoR from the RAR γ , nor promote interaction of the receptor with the transcriptional co-activators p300. Considered together with previous studies demonstrating that SR11253 functions as a mixed agonist/antagonist of RAR γ in cultured cells [36,37], the results of the present *in vitro* studies suggest that the pharmacological properties of SR11253 may be most consistent with those of a weak partial agonist of RAR γ that binds directly to the receptor but fails to promote efficient co-repressor dissociation or co-activator recruitment. Thus, depending on relative affinities, SR11253 may antagonize activation of the receptor by other compounds such as RAR γ -selective agonists SR11389 and SR11254 [37].

In summary, we have developed a method of rapidly mapping ligand-induced conformational change within the LBD of RAR γ using a combination of DPSA and MALDI-TOF MS. This technique provides a proteolytic fingerprint of the agonist-bound form(s) of the receptor and should be generally applicable to other RARs as well as other members of the nuclear-receptor superfamily of ligand-dependent transcription factors.

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