Critical role of the H6–H7 loop in the conformational adaptation of all-*trans* retinoic acid and synthetic retinoids within the ligand-binding site of RAR α

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ABSTRACT

The pleiotropic effects of the natural and synthetic retinoids are mediated by the activation of the two subfamilies of nuclear receptors, the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs). At the molecular level, these events begin with the specific ligand recognition by a nuclear receptor subtype. The adaptation of ligands to the receptor binding site leads to an optimal number of interactions for binding and selectivity which justifies elucidation of the structural requirements of the ligand binding pocket. To explore the contribution of H6–H7 loop folding in the ligand-induced conformational changes explained by the mouse-trap model, four RAR α mutants were constructed. Ligand binding and transactivation studies revealed that three residues from the H6–H7 loop (Gly³⁰¹, Phe³⁰² and Gly³⁰³) are critical for the conformational adaptation of both synthetic agonists and antagonists. Model building and analysis of both RAR α –ATRA and RAR α –CD367

complexes demonstrate that accommodation of CD367 results in a less tight contact of the saturated ring of this ligand with the amino acid side chains of the receptor ligand-binding pocket compared with that of ATRA. According to the flexibility of the agonists tested (ATRA>TTNPB=Am580>CD367), we observed a decrease in binding that was dependent on ligand structure rigidity. In contrast, the binding and transactivating activities of the L266A mutant confirmed the structural constraints imposed by synthetic ligands on binding affinity for the receptor and revealed that subtle local rearrangements induced by specific conformational adaptation changes result in different binding affinities. Our results illustrate the dynamic nature of the interaction between RAR α and its ligands and demonstrate the critical role of the H6–H7 loop in the binding of both synthetic retinoid agonists and antagonists.

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INTRODUCTION

Retinoic acids exert their pleiotropic effects on vertebrate development and homeostasis by binding to nuclear receptors (Gronemeyer & Laudet 1995, Kastner *et al.* 1995, Perlmann & Evans 1997). The direct biological effects of the natural and synthetic retinoids are mediated by the activation of two subfamilies of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). Both subfamilies consist of three receptor subtypes, referred to as RAR α , β and γ and RXR α , β and γ (Chambon 1996 and references therein).

The C-terminal region of RARs and RXRs contains both the ligand-binding domain, which functions as a ligand-dependent transactivation domain (activation function 2, AF-2), and surfaces for both homo- and heterodimerization and for interaction with co-activators and co-repressors (Moras & Gronemeyer 1998, Torchia *et al.* 1998). Retinoids are used in the treatment of various skin diseases, including psoriasis and acne, and in the treatment or chemoprevention of cancers. Numerous synthetic retinoids have been synthesized with the aim of achieving greater specificity and fewer side effects in therapeutic applications, and isotype-specific

compounds may act as selective ligands through RARs and RXRs. The adaptation of ligands to the receptor binding site leads to an optimal number of interactions for binding; this justifies the elucidation of the structural requirements of the ligand binding-pocket (Douguet *et al.* 1999).

The crystal structures of apo-RXR α and holo-RAR γ have been determined (Bourguet *et al.* 1995, Renaud *et al.* 1995) and provided the first model of these nuclear receptor ligand-binding domains in the free and liganded states. In addition, the identification of an RAR γ -selective ligand exhibiting an intrinsic flexibility similar to that of 9-*cis* retinoic acid led to the description of an identical holo-ligand-binding domain protein conformation (Klaholz *et al.* 1998). Both of the natural stereoisomers, all-*trans* retinoic acid and 9-*cis* retinoic acid, bind to and induce transactivation by the RARs, whereas RXRs exclusively binds 9-*cis* retinoic acid. On the basis of the structural and functional similarities between the ligand-binding domains of the different RAR subtypes, a common folding pattern has been proposed (Wurtz *et al.* 1996). In all crystal structures available at present, the ligand is embedded within the protein, with no clear accessible entry or exit site; significant conformational changes are necessary to generate potential entry sites. This is explained in the 'mouse-trap' model in which the mobility of helix H12 opens a channel by removing the 'lid' from the ligand-binding pocket. On ligand binding, H11 is repositioned in continuity with H10, and the concomitant hingeing of H12 is accompanied by additional structural changes such as bending of H3 and folding of the H6–H7 loop. These conformational changes lead finally to the formation of a specific ligand-binding domain interface with coactivators (Darimont *et al.* 1998). In the RAR ligand-binding domain, the ligand size matches the volume of the ligand-binding pocket. This results in a tight binding, with a large number of hydrophobic contacts between the ligand and structural determinants of the receptor-binding pocket (Renaud *et al.* 1995). The RARs can bind the two isomers, all-*trans* retinoic acid and 9-*cis* retinoic acid, equally well. Crystallographic analysis of these two isomers in the RAR γ confirmed that there is a conformational change of these ligands on binding to the receptor, allowing them to bind to a common ligand-binding pocket. Using conformationally restricted RAR agonists or antagonists and mutational analysis, we now show that reducing the tight folding of the H6–H7 loop results in an overall decrease in both the ligand-binding and transactivating properties of the receptor.

MATERIALS AND METHODS

Materials

[³H]-Labelled *trans*-retinoic acid (55.6 Ci/mmol; 1 mCi/ml) was obtained from DuPont/NEN (Le Blanc-Mesnil, France) and all-*trans*-retinoic acid (ATRA) was purchased from Sigma (St Louis, MO, USA). Synthetic retinoids were provided by U Reichert (CIRD Galderma, Sophia Antipolis, Valbonne, France). The RAR α -specific antagonist was obtained from Hoffman-La Roche Inc. (Basel, Switzerland). Restriction enzymes were from Promega (Madison, WI, USA). Isopropylthio- β -galactopyranoside, ampicillin and kanamycin were from Appligene (Strasbourg, France). Oligonucleotides were purchased from Eurogentec (Le Sart-Tilman, Belgium). The PCR-site directed mutagenesis kit was from Stratagene (La Jolla, CA, USA). Trypsin was from Boehringer (Mannheim, Germany) and all other chemicals were from Merck (Darmstadt, Germany).

Plasmid constructs and site-directed mutagenesis

Point mutations were introduced in the RAR α cDNA using the ExSite PCR-based site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). The hRAR α full-length cDNA was cloned into the BamHI and HindIII restriction sites of pQE-9 as described elsewhere (Lefebvre *et al.* 1995) and used as template for the preparation of the mutants. The GCC codon was used to encode the mutant Ala residue indicated in bold in the following mutagenic primers. Receptor mutant G301A: 5' sense 5'ATGCATAACGCT**GC**CTTCGGCCCCC3' and 3' antisense 5'CTGGGTCCGGTTCAGGGT**CAG** 3'; mutant F302A: 5' sense 5' ATGCATAACGCTGGC**CGCCG**CCCCC 3' and 3' antisense 5' CTGGGTCCGGTTCAGGGT**CAG** 3'; mutant G303A: 5' sense 5' ATGCATAACGCT**GGCT**TCGCCCCC 3' and 3' antisense 5' CTGGGTCCGGTTCAGGGT**CAG** 3'; and mutant L266A: 5' sense 5' CAAGGCT**GCCT**GCGCCGATATCC 3' and 3' antisense 5' AG GAGGGTGATCTGGTCGG**CG** 3'. The SacI–BclI fragment that contained the desired mutation was exchanged with that of pSG5–hRAR α wild-type to create each of the mutant DNA constructs. In all cases, the presence of the specific mutation and the lack of random mutations were verified by DNA sequence analysis.

Retinoid binding assays

Receptor extracts were diluted in binding buffer (50 mM Tris–HCl pH 8.00, 100 mM NaCl, 1 mM

EDTA, 10% glycerol) to a final concentration of 100 μ g total protein. Total ATRA binding was determined in the diluted extracts by adding tritiated ATRA in the concentration range 2.5–30 nM and incubating for 16 h at 4 °C. Non-specific binding was determined in the presence of 100-fold molar excess unlabelled ATRA; bound ATRA was separated from free ATRA as described elsewhere (Sablonnière *et al.* 1994). K_d values determined for the wild-type and K_i values obtained for each mutant receptor were determined by competition experiments as described previously (Lefebvre *et al.* 1995).

Cell culture and transfections

COS-7 cells were transiently transfected by the polyethylenimine coprecipitation method with the reporter plasmid p(TREpal)₃ Luc as previously described (Lefebvre *et al.* 1998). Cells were treated with the different retinoids at a final concentration of 10^{−6} M for 16 h. The luciferase assay was performed with the LucLite system (Packard Instruments, Rungis, France) according to the manufacturer's instructions and activity (as relative units) was measured with a Lumi-Count reader.

In vitro transcription, translation and limited proteolytic digestion

Wild-type and receptor mutants in pSG5 were transcribed and *in vitro* translated in the presence of [³⁵S]methionine by using rabbit reticulocyte lysates (Quick T7 TnT kit, Promega). Aliquots of lysates containing the labelled receptors were incubated with 10^{−6} M ATRA for 20 min at room temperature, then samples were treated with trypsin (50 μ g/ml) for 30 min at 20 °C. Digestion was stopped by the addition of 5 μ l sodium dodecylsulphate sample buffer and boiling for 3 min. SDS/polyacrylamide gel electrophoresis and autoradiography were carried out as described previously (Keidel *et al.* 1994).

Ligand-binding pocket modelling

Sequences of hRAR α and hRAR γ ligand-binding domains were aligned and a model of hRAR α –ATRA was built by homology with hRAR γ –ATRA. As shown from the receptor coordinates (accession code: 2 lbd, Protein Data Base), Lys²³⁶ displays two equivalent possible conformations (Renaud *et al.* 1995). We chose the hRAR γ coordinates in which the NH₃⁺ of Lys²³⁶ interacts with the carboxyl group of ATRA. To mutate side chains that are different between hRAR α and hRAR γ , we used the homology module from the Insight II software

(Discover 97.0, MSI, San Diego, USA). We added hydrogen atoms, setting Arg, Lys, Asp and Glu residues from the ligand-binding pocket with charged side chains. We used the Discover software with CFF91 as forcefield. During all calculations, a cut-off radius and a switching function of 16 and 2 Å were used. The dielectric constant was distance-dependent and set to 1.0. ATRA was docked in hRAR α in the same way that it was positioned in hRAR γ . Next, we sampled side-chains rotamer and ATRA docking in the gas phase by a simulated annealing protocol from 500 K to 300 K in 50 steps of 50 fs each. Backbone atoms were initially tethered to their original position (energy constant of 50 kcal/Å²) and tethering constraints were decreased linearly. The final model was energy-minimized until the maximum derivative was less than 1.0 kcal/Å. For the docking of CD367, the ligand structure was superimposed on the docked ATRA in hRAR α and submitted to the simulated annealing docking procedure according to the Insight II affinity module software; this uses translation, rotation and flexibility of the ligand and allows some flexibility of the amino acid side chains from the ligand-binding pocket. Docking data were considered to have been achieved if they agreed with energy and root mean squared (RMS) distance criteria. With CD367, only one solution was found in a reasonable computation time.

RESULTS

Effects of H6–H7 point mutations on retinoid binding and ligand-dependent transactivation

Three site-specific mutants of the H6–H7 loop of hRAR α in which Gly³⁰¹, Phe³⁰² and Gly³⁰³ were individually replaced with an Ala residue (G301A, F302A, G303A) have been prepared. As shown in Table 1, the ATRA K_d values for wild-type and the three mutants were similar. To explore the conformational adaptation of the ligand structure further, the binding properties of the wild-type receptor were compared with those of the three H6–H7 receptor mutants. The apparent K_d values were therefore determined using competition experiments with tritiated ATRA. The retinoids tested included three synthetic RAR α agonists (TTNPB, Am580 and CD367) and seven RAR α antagonists (CD2905, CD2331, CD2815, CD2817, CD3105, CD3106 and Ro41–5253; Fig. 1). Interestingly, the three H6–H7 receptor mutants showed an overall decrease in binding for all synthetic agonists and antagonists that were tested (Table 1). The transactivation activities for ATRA and the different retinoids tested were compared between

TABLE 1. K_d values for RARα and four receptor mutants obtained in saturation experiments with ATRA and in competition with other retinoids. Values are the mean ± s.d. of at least three independent experiments

Compound	In vitro binding K_d (nM)				
	RARα	G301A	F302A	G303A	L266A
ATRA	3.5 ± 0.5	15.4 ± 2.0	16.2 ± 2.0	36 ± 3.5	22 ± 2.0
TTNPB	24 ± 2.0	181 ± 14	510 ± 70	89 ± 7.0	1540 ± 80
Am580	12 ± 1.5	1700 ± 100	347 ± 23	872 ± 60	>3000
CD367	14 ± 2.0	2000 ± 70	543 ± 40	448 ± 35	326 ± 20
CD2905	120 ± 8.0	1677 ± 75	>3000	>3000	>3000
CD2331	766 ± 50	1762 ± 80	>3000	>3000	2000 ± 70
CD2815	950 ± 60	>2600	>2700	>3000	396 ± 35
CD2817	888 ± 80	2029 ± 120	>2700	>3000	1551 ± 95
CD3105	108 ± 8.0	236 ± 18	>2700	469 ± 35	>3000
CD3106	29 ± 3.0	>2600	937 ± 55	>3000	450 ± 40
Ro41-5253	24 ± 2.5	1520 ± 70	>3000	2387 ± 200	12 ± 0.5

the wild-type receptor and the four receptor mutants (G301A, F302A, G303A and L266A). As shown in Fig. 2, the three H6–H7 loop mutants exhibited a reduced transactivation level compared with the wild-type receptor when the cells were

treated either with ATRA or with the three synthetic retinoid agonists. Interestingly, for most ligands of the antagonist series, we found a reduction in the transactivation activity to the same degree as for the agonist series, except for CD3105,

FIGURE 1. Structures of the different retinoids tested.

FIGURE 2. Ligand-dependent transactivation of wild-type and mutants of hRAR α , showing the transcriptional responses obtained in the presence of RAR α or the receptor mutants G301A, F302A, G303A and L266A with a retinoid concentration of 10^{-6} M on a TREpal-Luc reporter. Retinoid-induced luciferase activation is expressed as a percentage of maximal activation obtained with 10^{-6} M ATRA on the wild-type receptor. Data represent the average of three experiments.

CD3106 and Ro41-5253, which exhibited a similar or an increased transactivation activity compared with the wild-type receptor.

Effects of L266A mutation on retinoid binding and transactivation

Mutation of the L266 side chain can provide additional information on the conformational adaptation of RAR antagonists as it interacts directly with the 7-methenyl-group of ATRA and is also located in the ligand-binding pocket (Fig. 3). This effect appeared to be variable according to the structure of the ligand tested: whereas the affinity for ATRA was slightly decreased, that for the three

agonists decreased 20- to 250-fold. Interestingly, the affinity of some antagonists (CD2905, CD2331, CD2817 and CD3105) was significantly decreased, whereas a slight increase in affinity was observed for Ro41-5253 and, to a lesser degree, in the case of CD2815. When the ability of the L266A mutant to transactivate the reporter gene was tested in COS-7 cells, this mutant was found to retain about 70–100% of the wild-type activity in response to stimulation by ATRA or by the three synthetic agonists (Fig. 2). However, compared with ATRA, a decrease in transactivation efficiency was observed for L266A when it was activated by the different antagonists tested, except for CD3105 and CD3106.

FIGURE 3. ATRA modelled into the ligand-binding pocket of the hRAR α ligand-binding domain. Amino acid side chains bearing at least one atom within less than 5 Å of ATRA are depicted. Side chains of Phe³⁰² and Leu²⁶⁶ are red; the ATRA structure is blue.

Antagonistic activity of CD2905, CD2331, CD2815, CD2817 and Ro41–5253 in the RAR α transactivation assay

The concentration-dependent antagonistic activities of these ligands at an ATRA concentration of 10^{-8} M were demonstrated by the decreased induction of luciferase activity (Fig. 4). For each antagonist tested, the excess of each ligand over ATRA that was required to produce an antagonistic effect was similar between wild-type and the receptor mutants.

Model building and analysis of hRAR α –ATRA and hRAR α –CD367 complexes

We built three-dimensional models of these ligand–receptor complexes, in the gas phase, by homology with the crystallographic structure of hRAR γ –ATRA (Renaud *et al.* 1995), focusing on the ligand-binding pockets of these models, using the modelling procedure reported in the Methods section. When hRAR α –ATRA and hRAR γ –ATRA were compared (Fig. 5), it was evident that the side-chain rotamers of those amino acids that are identical in both receptors were positioned in the same way inside the ligand-binding pocket, with the exception of Leu²⁶⁹ (in hRAR α), which showed a C α –C β rotation to accommodate the ‘up-repositioning’ of ATRA caused by Ser²³². For RAR-specific side chains, the hydroxyl group of Ser²³² provide a hydrogen bond to the N-4 main-chain carbonyl group, whereas Ile²⁷⁰ and Val³⁹⁵ led only to a few perturbations in their immediate neighbourhood. The ATRA carboxylate participates in the same network of hydrogen bonds with Lys²³⁴, Arg²⁷⁶ and Ser²⁸⁷ side chain/backbone hydrogen-bond donor functions. When we compared the hRAR α –ATRA and hRAR α –CD367 complexes (Fig. 6) we found that CD367 docking in hRAR α resulted in a superposition of the two ligands that underlined their similar curvature. The choice of CD367 because of its limited flexibility gave an unambiguous docking of this synthetic ligand. In the hRAR α –CD367 complex, neither the side-chain rotamers nor the carboxylate hydrogen-bonding network were different from those already described for the hRAR α –ATRA complex. Slight torsions were observed, mainly in aromatic side chains (Phe²⁸⁶, Phe³⁰²), to fit with the increased bulk of the CD367 rings. The main difference came from the shortest length of CD367 resulting in less tight contact of the saturated ring of CD367 with the corresponding side chains, which point towards C2, C3 and C4 of the ATRA cyclohexene ring.

Comparison of the ligand-dependent protection of L266A receptor mutant against tryptic digestion between CD2915, CD2817 and Ro41–5253

As shown in Fig. 7, limited proteolysis of the L266A mutant in the absence of ligand resulted in a complete digestion of the receptor protein (lane 2), whereas in the presence of ATRA we obtained a reduced proteolysis with a main 30 kDa band protected (lane 3); after preincubation with the antagonist Ro41–5253, a main 26 kDa band was protected (lane 4). Interestingly, this specific antagonist-induced protection was not observed with CD2815 and CD2817 synthetic ligands, which gave the same tryptic digestion pattern as ATRA (lanes 5 and 6).

DISCUSSION

The utility of non-selective retinoids in the treatment of human disease has been limited by the toxicity associated with these compounds (Chandraratna 1999). Several classes of RAR-specific ligands have been described and RAR subtype selectivity and specificity have now been achieved (Alam *et al.* 1995, Johnson *et al.* 1996, Yu *et al.* 1996). To elucidate how differently shaped ligands could modulate the activity of the same receptor and to reveal the molecular basis for receptor selectivity, ideally, the crystal structures of each ligand–receptor ligand-binding domain are required. To date, only the crystal structures of apo-hRXR α and holo-hRAR γ have been determined (Bourguet *et al.* 1995, Renaud *et al.* 1995). According to these data, the ligand lies in the retinoid binding site in a position in which it is buried in a predominantly hydrophobic pocket formed by several residues located in H1, H3, H5, the β -turn, loop H6–H7, H11, loop H11–H12 and H12. Among the numerous residues that make contact with ATRA and were deduced from the diagram of the van der Waal's contact (4.5 Å cut-off) between the protein and the ligand, are Phe³⁰² which is in close contact with the 5,5-dimethyl group of ATRA, and Leu²⁶⁶ which makes contact with the adjacent C7 methenyl group (Renaud *et al.* 1995). In previous studies, two residues in the H6–H7 loop region, Gly³⁰¹ (Durand *et al.* 1994) and Gly³⁰³ (Saitou *et al.* 1994) were shown to be critical for RAR α -specific interactions with ATRA. In their vicinity and positioned towards the tetraene chain of ATRA are some residues of helix H5. The importance of Met²⁷² in RAR γ has been demonstrated and the

FIGURE 4. Comparison of the relative antagonistic potencies of the different synthetic antagonists between RAR α and F302A or between RAR α and L266A. Transcriptional responses were measured with hRAR α and the F302A or L266A receptor mutants. Retinoid-induced luciferase activation of the TREpal-Luc reporter was expressed as a percentage of maximal activation obtained with 10^{-8} M ATRA and increasing concentrations (0 – 10^{-5} M) of each synthetic ligand tested. Each point represents the average of three experiments.

FIGURE 5. Stereoview of the superimposition of the hRAR γ -ATRA (grey) and hRAR α -ATRA (black) complexes. Amino acid side chains bearing at least one atom within less than 5 Å of ATRA are depicted. RMS deviation=0.81 Å.

possible role of Cys²⁶⁷ in RAR β also reported (Wolfgang *et al.* 1997, Ostrowski *et al.* 1998).

Because the β -ionone moiety has been replaced by a more bulky and often polycyclic structure in many synthetic retinoids, we wanted to examine how these ligands may accommodate the receptor-ligand binding pocket of hRAR α . Comparison of hRAR α -ATRA and hRAR α -CD367 ligand-binding domain complexes revealed only limited differences. It appears that accommodation of CD367 results in a less tight contact of the saturated ring of this ligand with the corresponding amino acid side chains of the receptor-ligand binding pocket, as compared with ATRA. This might explain the conserved affinity of this ligand for hRAR α . To assess the conformational adaptation of the different ligands of our series, both Phe³⁰² and the respective adjacent residues, Gly³⁰¹ and Gly³⁰³ were mutated and individually replaced with an Ala residue; these three mutants are located in the receptor H6-H7 loop. The result of each of these mutations could

be expected to be to enlarge the size of the ligand-binding pocket in the area where the cyclohexene ring of ATRA is located. G301A and G303A bring a methyl group that can limit the tight folding of the H6-H7 loop, resulting in a possible widening of this area of the binding cavity, whereas F302A removes a phenyl ring, also contributing to enlarging the ligand-binding pocket. If the four most similar ligands of our series are graded according to their flexibility – ATRA>TTNPB=Am580>CD367 – we observe that the more the ligand is constrained the more its binding properties are decreased. This is in agreement with the conclusions drawn from the crystallographic comparison of hRAR γ -ATRA and hRAR γ -9-*cis* retinoic acid (Klaholz *et al.* 1998) showing the constraints imposed by the receptor protein on those natural ligands. Synthetic ligands are able to fit correctly to the wild-type receptor but appear unable to undergo suitable conformational adaptations. With Am580, for which the selective RAR α

FIGURE 6. Stereoview of the superimposition of the hRAR α -CD367 (grey) and hRAR α -ATRA (black) complexes. Amino acid side chains bearing at least one atom within less than 5 Å of ATRA are depicted. RMS deviation=0.81 Å.

FIGURE 7. Effect of ATRA (lane 3), Ro41–5253 (lane 4), CD2815 (lane 5) and CD2817 (lane 6) on limited tryptic digestion of the L266A receptor mutant. The *in vitro*-translated L266A receptor mutant was preincubated with dimethyl sulphoxide alone (lanes 1 and 2) or with 10^{−6} M of the indicated retinoids (lanes 3–6). Samples were then digested with trypsin (50 µg/ml) for 30 min at 20 °C and analysed by electrophoresis in a 10% SDS-polyacrylamide gel, and the dried gel autoradiographed. The sizes of the molecular mass markers are indicated.

binding properties rely on a specific hydrogen bond with Ser²³², the optimization of the fit is even more pronounced, as assessed by the marked loss of binding of the three mutants tested. This latter observation may be compared with the more reduced loss of binding obtained with TTNPB, the apolar analogue of Am580.

Structural interpretation of the effects of mutations on the binding of the other molecules tested here (partial agonist or antagonist ligands) is not easy to perform, as the type of docking cannot be extrapolated from the hRARα-CD367 model. The

three mutants of loop H6–H7 tested exhibit a decreased binding activity that is in accordance with their reduced transcriptional activity. Similar results have previously been described with other receptor mutants such as G301R and G303E of hRARα (Durand *et al.* 1994; Saitou *et al.* 1994). According to the hypothesis of an enlargement of the ligand binding pocket by our mutations in the H6–H7 loop, for those ligands that bear a bulky adamantyl group (CD2331 and CD2905), the decrease in affinity could mean that the adamantyl moiety is not located near the H6–H7 loop. Instead, the adamantyl could lie in the opposite direction and interact with helix 12, but may interfere with the proper alignment of this helix, leading to the inactivation of the AF-2 transactivation domain, thus explaining its antagonistic potency. This explanation is supported by the conserved antagonistic potency of the F302A mutant when bound to CD2905 and CD2331 compared with that of the wild-type receptor (Fig. 4). A similar hypothesis was proposed in the case of BMS-185411, another RARα antagonist (Ostrowski *et al.* 1998). The marked decrease in binding affinity for CD3106 observed with the three mutants G301A, F302A and G303A could suggest that this antagonist is positioned inside the ligand-binding pocket in a way similar to that for BMS-185411. Considering the position of the side chain of Leu²⁶⁶ inside helix 5 and in the vicinity of Ile²⁷⁰ (which is required to fit with the hRARα-selective agonists), mutation of this residue may result in negative interactions between the receptor and retinoid agonists. The decreased affinity observed for those ligands (CD367, Am580 and TTNBP) but not for ATRA may be explained by the better flexibility of ATRA inside the ligand-binding pocket as suggested above in the case of H6–H7 loop mutants. In contrast, the slight increase in affinity observed with the L266A mutant for the two antagonists Ro41–5253 and CD2815 may suggest a common type of interaction of these molecules within the ligand-binding site. This hypothesis was contradicted by the results of limited tryptic digestion, in which a different digestion pattern was found for these two ligands (Fig. 7). It is, however, striking that CD2815 and CD2817, which differ only by one methylene group in their side chains, exhibit distinct binding affinities for the L266A mutant whereas they display nearly identical affinity for the wild-type receptor. This suggests that subtle local rearrangements induced by the ligand conformational adaptation may result in different binding and transactivation properties of the receptor. An indirect consequence of these peculiar local changes concerns the recruitment

of coactivators. Agonists support ligand-binding domain interactions with coactivators and it has been demonstrated that helix H12 is involved in the receptor–coactivator interface, raising the possibility that direct subtle changes in the position of conserved residues of helices H3, H4 and H5 during ligand binding to nuclear receptors may influence the position of H12 in the ‘agonist conformation’ (Darimont *et al.* 1998; Feng *et al.* 1998). In fact, it seems possible that Leu²⁶⁶ which is near the LL sequence (260–261), may also modulate the affinity of the interaction between the ligand-binding domain and the coactivator, glucocorticoid receptor-interacting protein 1 (GRIP1). In this context, it will be interesting to pursue this matter with future studies of further mutational analyses and their consequence on coactivator binding.

These results can be compared with those of Lamour *et al.* (1996), who found an increased binding affinity of the antagonist Ro41–5153 when it was bound to R294A and R217A RAR α mutants. Limited flexibility of constrained synthetic ligands may reduce the conformational adaptation of molecules, especially with antagonists that bear bulky groups. The previous data obtained with Ro41–5253 or BMS-185411 did agree with the hypothesis of a unique conformational perturbation of H12 positioning, leading to an impaired function of the AF-2 transactivation domain. Other studies performed with different antagonists of the glucocorticoid receptor (Modaress *et al.* 1997) or of the oestrogen receptor (Ekena *et al.* 1997) have already demonstrated that the induced conformational changes after binding are different.

Our results confirm the dynamic nature of the interaction between RAR α and its ligands and demonstrate the critical role of the H6–H7 loop of RAR α in the conformational adaptation of both synthetic agonists and antagonists.

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