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Analysis of the Ligand-Binding Domain of Human Retinoic Acid Receptor α by Site-Directed Mutagenesis

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Three subtypes of retinoic acid receptors (RAR), termed RARα, RARβ, and RARγ, have been described. They are composed of different structural domains, including distinct domains for DNA and ligand binding. RARs specifically bind all-*trans*-retinoic acid (RA), 9-*cis*-RA, and retinoid analogs. In this study, we examined the functional role of cysteine and arginine residues in the ligand-binding domain of hRARα (hRARα-LBD, amino acids 154 to 462). All conserved cysteine and arginine residues in this domain were mutated by site-directed mutagenesis, and the mutant proteins were characterized by blocking reactions, ligand-binding experiments, transactivation assays, and protease mapping. Changes of any cysteine residue of the hRARα-LBD had no significant influence on the binding of all-*trans* RA or 9-*cis* RA. Interestingly, residue C-235 is specifically important in antagonist binding. With respect to arginine residues, only the two single mutations of R-276 and R-394 to alanine showed a dramatic decrease of agonist and antagonist binding whereas the R272A mutation showed only a slight effect. For all other arginine mutations, no differences in affinity were detectable. The two mutations R217A and R294A caused an increased binding efficiency for antagonists but no change in agonist binding. From these results, we can conclude that electrostatic interactions of retinoids with the RARα-LBD play a significant role in ligand binding. In addition, antagonists show distinctly different requirements for efficient binding, which may contribute to their interference in the ligand-inducible transactivation function of RARα.

Retinoic acid (RA) is a biologically active form of vitamin A. All-*trans*-RA (t-RA) has effects on growth and cell differentiation, pattern formation, and tumorigenesis (7, 17, 26–28, 32, 34, 36, 38). An effect of RA on nervous system development has also been shown (15, 40, 41). The discovery of nuclear retinoic acid receptors (RARs) and retinoid X receptors (RXRs) has greatly advanced the understanding of the mechanism of action of RA. Three subtypes (α, β, and γ) constitute the RAR gene family (6, 9, 19, 24, 35, 37, 48), and three subtypes (α, β, and γ) constitute the RXR family (22, 29, 30). These receptors belong to the superfamily of steroid/thyroid hormone receptors and are in fact ligand-dependent transcriptional factors, in which both t-RA and 9-*cis*-RA are natural ligands for the RARs (1, 13), while 9-*cis*-RA and phytanic acid are natural ligands for the RXRs (16, 23, 25). The receptors are able to bind target sequence RA response elements (RAREs) for RARs or retinoid response elements (RXREs) for RXRs (12, 31).

On the basis of homology to other nuclear hormone receptors, the primary sequences of RARs and RXRs are divided into distinct domains designated A to F. The C domain constitutes the highly conserved DNA-binding domain, and the E domain confers the ligand-binding properties of each receptor. The E domains of all subtypes of RARs are highly (over 90%) conserved and contain dimerization, ligand-binding, and transactivation functions. The C-terminal part of the D domain of human RARα (hRARα) is also required for ligand binding, while the F domain and a part of the N-terminal end of the E domain can be deleted without affecting ligand binding (17a, 20). It has also been shown that the N-terminal portion of the E domain of hRARα and hRARβ is essential for the recognition of retinoids (33) and that the two hRARα residues M-406 and I-410 play an important role in 9-*cis*-RA binding to hRARα (47).

Experiments involving limited trypsin digestion of RARα have provided evidence that t-RA binding to the hRARα ligand-binding domain (LBD), as well as 9-*cis*-RA binding, induces a conformational change which may be required for the interaction of the DNA-bound RARα-RXRα heterodimer with other transcription factors (18). The RARα selective antagonist Ro 41-5253 (2) competes with agonists for RARα binding and, when bound to the receptor, induces a different conformational change as detected by limited proteolysis.

Sulfhydryl-blocking reagents have been shown to affect the ligand-binding properties of hRARα-LBD, suggesting that one or several cysteine residues of the E domain could be relevant for the interaction between t-RA and hRARα (11).

The presence of a free carboxyl group on retinoids has been demonstrated to be essential for ligand binding to RARs (3, 21). The hypothesis that basic amino acids such as arginine or lysine are involved in ligand-receptor binding has been recently confirmed. It has been shown that R-269 and K-220 of RARβ are important for the binding of RA (45, 46). Moreover, R-132 of cytoplasmic RA-binding protein II was found to be implicated in the t-RA binding (10). Recently, the structure of the cocrystal hRARγ–t-RA has been determined (39). Three arginine residues (R-274, R-278, and R-396) and one lysine residue (K-236) were shown to be involved in the ligand-binding pocket of hRARγ.

To further clarify the interaction of the LBD of hRARα with t-RA, 9-*cis*-RA, and other agonists and antagonists, we have prepared site-specific mutants of the hRARα-LBD. We have characterized these mutants by using blocking reactions, ligand-binding experiments, transactivation assays, and protease mapping. We have come to the conclusion that the cysteine residues of the hRARα-LBD play no direct role in the ligand-receptor interaction whereas two single arginine residues,

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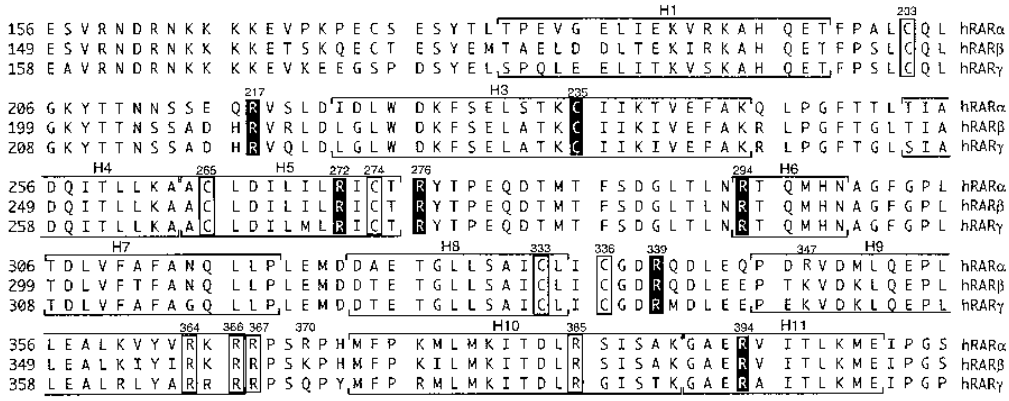


FIG. 1. Alignment of the LBD of hRARα, hRARβ, and hRARγ. All mutated cysteines and arginines are boxed. Mutations which showed a weak effect are indicated by an open box, and mutations with a strong effect are indicated by a solid box.

R-394 and R-276, do play a significant role, since mutations into alanine at these residues led to a dramatic decrease in ligand binding. The same influence of these mutations was observed for t-RA, 9-*cis*-RA, and synthetic retinoids (agonists and antagonists). The mutations R217A and R294A increase the ability of hRARα to bind antagonists, while the mutation C235G specifically decreases antagonist binding.

MATERIALS AND METHODS

Materials. t-RA, 9-*cis*-RA, and synthetic retinoids were synthesized at F. Hoffmann-La Roche Ltd., Basel, Switzerland. t-[^{3}H]RA (53.9 Ci/mmol; 1 mCi/ml) was obtained from DuPont/NEN. 9-*cis*-[^{3}H]RA (47 Ci/mmol; 0.2 mCi/ml) was obtained from Amersham. The retinoids were solubilized in dimethyl sulfoxide as 10 mM stock solutions and kept at −20°C. The blocking reagents 2,3-butanedione, *N*-ethylmaleimide, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Fluka. Sequencing reactions were performed with the Sequenase plasmid-sequencing kit (U.S. Biochemicals), and α-^{35}S-dATP (1 mCi) was obtained from Amersham. The in vitro transcription-translation reactions were performed with a TNT coupled reticulocyte lysate system (Promega).

Site-directed mutagenesis. The method used here has been previously described (5). All mutants were produced by two PCRs, the second one consisting of two steps. The first PCR product was obtained with a 5′ mutagenic primer and 3′ primer (positions 1389 to 1369) on plasmid DNA containing hRARα-LBD (positions 466 to 1389) as the template. The amplified fragment was eluted from an agarose gel. Together with a 5′ primer (positions 466 to 486), this first PCR fragment was used in the second PCR as a 3′ megaprimer, and a complete hRARα-LBD was obtained after 11 cycles with the now 3′-truncated hRARα (positions 1 to 1305) as template. To increase the yield of mutagenic hRARα-LBD, the 3′ primer (positions 1389 to 1369) was added for the last 16 cycles. The fragment was cleaved with *Eco*RI and *Bam*HI (located in the 5′ primer and the 3′ primer, respectively), purified from an agarose gel, and cloned in T7 *Escherichia coli* expression vector pET-3a (44) in which the original *Eco*RI site was destroyed and a new *Eco*RI site was inserted directly after the ATG of the *Nde*I site. For all clones, the correctness of the sequence was confirmed by sequencing. The efficiency of the mutagenesis observed was between 65 and 100%.

Binding assay and K_D and IC_{50} determination. Mutated and wild-type (wt) hRARα-LBD were expressed in *E. coli* BL21(DE3)/pLysS (44), after isopropyl-β-D-thiogalactopyranoside (IPTG) induction. The protein extractions were performed from 250-ml cultures by lysis of the cells washed in 50 mM Tris (pH 7.8)–30 μM dithiothreitol. After centrifugation for 10 min at 3,400 × *g* and 4°C, the bacteria were incubated for 1 h on ice in lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0], 75 mM glucose, 2 mM dithiothreitol) together with 2 mg of lysozyme per ml; 100 mM NaCl and 2 mM phenylmethylsulfonyl fluoride were then added (final concentration), and the samples were freeze-thawed twice. After addition of 0.15 U of aprotinin and 20 μg of leupeptin per ml (final concentration), the samples were sonicated on ice four times for 30 s at level 3 to 4 with a Branson Sonifier 250. After centrifugation for 10 min at 24,000 × *g* and 4°C, the supernatant was recovered. The protein concentration was determined by a Bio-Rad protein assay, and 15 μg of total protein was loaded on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel to verify the presence of the expressed hRARα-LBD in the crude protein extracts. For each protein extract, a series of dilutions were made and used in binding assays to define the appropriate amount of extract to use for the 50% inhibitory concentration (IC_{50}) and K_D determinations. The binding assay and the calculation of the IC_{50} were done as described previously (4). Scatchard analysis was performed by the method of Scatchard (43).

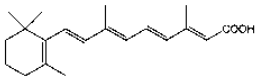
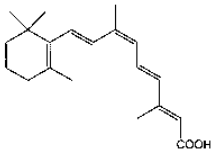
Transactivation assay. For construction of an RARα expression vector for eukaryotic cells and for in vitro translation (see below), the N-terminal part of hRARα (positions 1 to 465) was cloned into pSG5 (14). For this, the *Eco*RI-*Bam*HI fragment of pSG5 was replaced by a linker containing *Msc*I, *Eco*RI, and *Bam*HI sites (5′-AATTGCCACCATGGCCAGACGAATTCTCAGGATC-3′) and cleaved with *Msc*I and *Eco*RI, and a *Msc*I-*Eco*RI PCR product corresponding to hRARα (positions 1 to 465) was inserted. The correctness of the sequence was confirmed by sequencing. In a second step, the C-terminal part of RARα (wt or mutated form) was inserted as an *Eco*RI-*Bam*HI fragment, resulting in a full-length receptor with mutants at each cysteine or arginine in the E domain. The modification of the first two amino acids of the D domain due to the insertion of the *Eco*RI cloning site did not affect the transactivation efficiency, and we observed the same 50% effective concentration (EC_{50}) for the last construct in as that for the wt hRARα. The RAR reporter plasmid used in transactivation assays was constructed with a synthetic oligonucleotide containing three copies of the RAR response element from the RARβ promoter (12) in front of the basal promoter of thymidine kinase and the luciferase coding region in the plasmid pGL2-basic (Promega) βRARE-thymidine kinase-luciferase. The transfection was performed by the calcium phosphate coprecipitation method (42). CV-1 cells were transiently transfected as described previously for COS cells (4). After addition of t-RA or Ro 13-7410 and incubation for 36 h, the luciferase activity was measured and the EC_{50} was calculated.

In vitro transcription, translation, and limited proteolytic digestion. Human RARα (wt or mutated) in pSG5 was transcribed and in vitro translated in the presence of [^{35}S]methionine by using rabbit reticulocyte lysates as specified by Promega. The limited proteolytic digestions were performed as described elsewhere (18).

RESULTS

Cysteine point mutations in the E domain of hRARα. From the results of blocking reactions with cysteine-specific reagents, it has been previously postulated that one or several sulfhydryls are involved in ligand-hRARα interactions (11). We analyzed the effect of *N*-ethylmaleimide and DTNB on t-RA binding with wt hRARα-LBD. We found the same effect of t-RA on the wt receptor, consisting of a decrease in binding when increasing amounts of blocking reagent were used (data not shown). The K_D determination of wt hRARα-LBD resulted in a value of 0.6 nM for t-RA, which establishes the functionality of the procaryotic expression and extraction methods. The K_D correlates well with that determined previously for the full-length receptor (1). Each cysteine residue in the LBD of hRARα has been changed to glycine (C-203, C-235, C-274, C-333, and C-336 [an overview is presented in Fig. 1]). Only the single C265G change showed a decrease of binding (15-fold for t-RA and 95-fold for 9-*cis*-RA) as determined by Scatchard analysis (Table 1). C-265 has also been changed to alanine. This mutated receptor showed an IC_{50} of 6 nM and an

TABLE 1. t-RA- and 9-*cis*-RA-binding properties of wt hRARα-LBD and selected mutants

Chemical structure	hRARα-LBD	Binding Activity K_D (nM)[a]
t-RA 	wt	0.6
	C265G	9.3
	R217G	37
	R272G	71
	R276G	90
	R294G	34
	R339G	28
	R394G	n.d[b]
9-*cis* RA 	wt	1.2
	C265G	115
	R217G	48
	R272G	58
	R276G	93
	R294G	15
	R339G	8.1
	R394G	n.d[b]

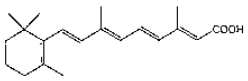
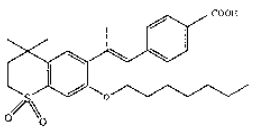
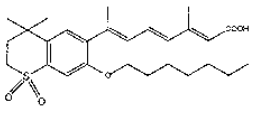
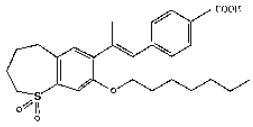
[a] The K_D values were obtained by Scatchard analysis. The K_D values and IC_{50} are the means of two single experiments.
[b] n.d., not determined because of low binding activity.

EC_{50} of 15 nM for t-RA in binding and transactivation experiments, thus showing no difference from the wt. For the double mutant hRARα-C265G/C274G, a K_D of 120 nM has been obtained for t-RA, which is 200-fold higher than for the wt. The double mutant hRARα-C333G/C336G did not show any difference from the wt (data not shown). In all cases, the same tendency was observed when 9-*cis*-RA or synthetic agonists were used as ligands in the binding assays (data not shown).

Role of hRARα C-235 in ligand binding. Interestingly, the mutant C235G showed a dramatic reduction in binding for all antagonists that have been tested (Table 2). The analogs Ro 41-5253, Ro 46-5471, and Ro 46-6464 all indicate a role for C-235 of hRARα-LBD in antagonist interaction. Ro 41-5253, which has a phenyl group in the upper side chain, exhibited a 60-fold higher IC_{50} for the mutant C235G than the wt. In the other antagonists, changing from a six- to a seven-membered carbon ring (Ro 46-6464) or removing the upper side chain phenyl group (Ro 46-5471), also resulted in a lower binding efficiency (about 10-fold lower than the wt). The use of other antagonists, in which the oxygen from the lower hydrophobic side chain was removed, gave similar results (data not shown), suggesting that C-235 does not interact with the side chain oxygen.

Arginine point mutations in the E domain of hRARα. Since the free carboxyl group on retinoids has been demonstrated to be essential for ligand binding to RARs (3, 21), a positively charged arginine or lysine residue interaction is very likely. We first used 2,3-butanedione as a specific arginine-blocking reagent and found a decrease of binding of t-RA for wt hRARα-LBD in response to increasing amounts of the blocking reagent (data not shown). Subsequently, a triple mutation (R364G, R366G, and R367G) of arginines to glycines in the hRARα-LBD was made. This mutated receptor exhibited no difference in binding compared with the wt (Fig. 2A). All other arginines except R-347 and R-370, which are the only arginine residues that are not conserved among the subtypes α, β, and γ (Fig. 1), were also mutated to glycine. By Scatchard analysis of mutations with t-RA and 9-*cis*-RA ligands, we identified R217G, R272G, R276G, R294G, R339G, and R394G, which showed a 50- to 100-fold reduction in binding compared with the wt (Table 1), whereas R385G did not affect binding activity at all (data not shown). The binding activity of the R394G mutant was very low, and we were able to define an IC_{50} value only as higher than 3,000 nM for both t-RA and 9-*cis*-RA. Figure 2B shows an example of K_D determination (for R294G).

TABLE 2. Effect of mutation of residues R-217, R-276, R-294, R-394, and C-235 on binding of t-RA and the antagonists Ro 41-5253, Ro 46-5471, and Ro 46-6464

Structure	IC_{50} of hRARα-LBD mutants: wt	C235G	R217A	R276A	R294A	R394A
t-RA 	4	5	5	1200	6	2500
Ro 41-5253 	48	3100	13	410	4	9200
Ro 46-5471 	56	650	14	6800	16	6900
Ro 46-6464 	140	1500	17	2300	11	6900

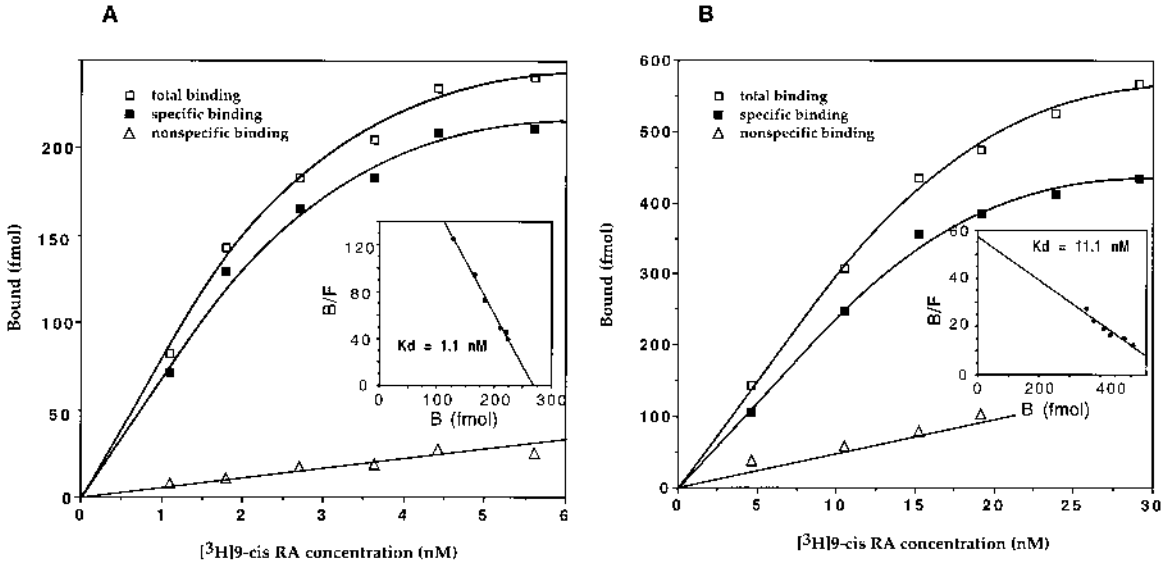


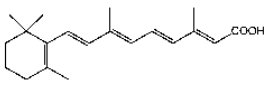
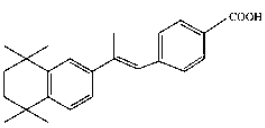
FIG. 2. Saturation-binding analysis of 9-*cis*-[^{3}H]RA binding to the triple mutant hRARα-LBD-R364G/R366G/R367G (A) and to the single mutant hRARα-LBD-R294G (B). Several dilutions of protein extract containing the hRARα-LBD from *E. coli* BL21 were used to test the binding activity to define the appropriate concentration for K_D determination. The nonspecific binding was determined with a 100-fold molar excess of unlabeled RA. For one analysis, each point represents the average of three single values.

R276 and R394 are crucial for retinoid binding and transactivation. Since glycine is the smallest amino acid (no side chain), we mutated all the residues mentioned above, which showed a reduction in binding affinity when changed to polar glycine, to the nonpolar alanine. For the mutants (R217A, R294A, and R339A), no influence was detectable compared with the wt, in contrast to what was seen for the glycine changes above. The binding and transactivation activities for t-RA and Ro 13-7410 on the other mutants are shown in Table 3. Only a 10-fold reduction in binding efficiency was observed for mutant R272A, whereas 300- and 600-fold reductions were seen for R276A and R394A, respectively. The R394 mutant has in addition been mutated to serine or glutamic acid, and more or less the same 600-fold decrease was observed (Table 3). Using CV-1 cells for cotransfection of a plasmid containing the hRARα cDNA mutated at the appropriate residues, together with a reporter plasmid containing a luciferase gene under the influence of the βRARE promoter, we were able to determine the transactivation activity for hRARα. The calculated EC_{50}s are in good agreement with the above-mentioned binding results (Table 3). We have tested all mutants for their ability to bind the antagonist Ro 41-5253 (2), as well as to other antagonists. For most mutations, we found no effect or a reduction in the binding affinity to the same degree as for agonists (data not shown). We found the strongest effect for mutations R276A and R394A (Table 2). The mutation R272A on hRARα exhibited only a slight effect. For the antagonist Ro 41-5253, we observed an IC_{50} of 210 nM for the mutant R272A in contrast to 48 nM for wt hRARα.

Mutations R217A and R294A in hRARα-LBD specifically increase the binding efficiency of antagonists. While testing all arginine mutants for their ability to bind antagonists we observed two mutants, hRARα-R217A and hRARα-R294A, which showed a higher binding affinity for the antagonists Ro 41-5253, Ro 46-5471, and Ro 46-6464, than did the wt receptor (Table 2). The same results were obtained with several other antagonists which we have also tested (data not shown). For all antagonists, the increase in affinity was between 2- and 12-fold for both mutants with respect to the wt.

The limited proteolytic digestion pattern of the R217A mutant for antagonists is different from that of the wt, but antagonist function is retained. To verify the influence of the mutations on ligand function, we first checked the protection specificity by protease mapping with trypsin, chymotrypsin, and elastase. We did not notice any difference for hRARα-C235G and hRARα-R294A compared with the wt (data not shown). Only for trypsin digestion of hRARα-R217A did we find a different pattern in the presence of the antagonist Ro 41-5253

TABLE 3. Effect of mutation of residues R-272, R-276, and R-394 on binding and transactivation activity for t-RA and the agonist Ro 13-7410

Chemical structure	hRARα	Transactivation Activity EC_{50} (nM) [a]	Binding Activity IC_{50} (nM) [b]
t-RA 	wt	5.5	4
	R272A	50	45
	R276A	160	1200
	R394A	110	2500
	R394S	140	2300
	R394E	195	3400
Ro 13-7410 	wt	1.2	4
	R272A	29	26
	R276A	130	600
	R394A	33	1500
	R394S	65	2100
	R394E	175	2300

[a] For transactivation, CV-1 cells were cotransfected with one of the hRARα expression vectors (wt or mutant) and the βRARE-luciferase reporter. After addition of t-RA or Ro 13-7410 and incubation for 36 h, the luciferase activity was measured and the EC_{50} was calculated. EC_{50} is defined as the retinoid concentration required to obtain 50% of the maximal response (at 1,000 nM).

[b] IC_{50} is the retinoid concentration required to inhibit 50% of specific RA binding.

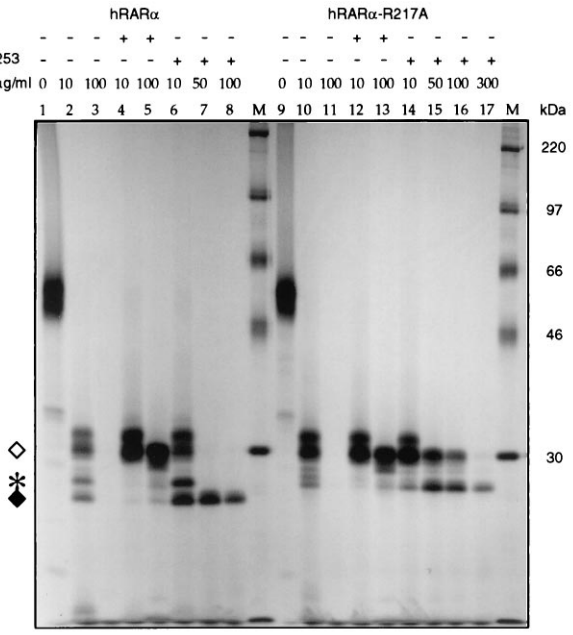


FIG. 3. Effect of t-RA and the antagonist Ro 41-5253 on limited trypsin digestion of wt and hRARα-R217A. The in vitro-translated hRARα (lanes 1 to 8) and hRARα-R217A (lanes 9 to 17) were preincubated with DMSO alone (lanes 2, 3, 10, and 11) or with 1 μM t-RA (lanes 4, 5, 12, and 13) or 1 μM Ro 41-5253 (lanes 6 to 8 and 14 to 17) and then digested with the indicated concentration of trypsin. The mixtures were incubated for 10 min at room temperature. Samples were electrophoresed through an SDS–12% polyacrylamide gel, and the dried gel was autoradiographed. The sizes of molecular mass markers are indicated. The resistant protein fragment occurring in the presence of t-RA is marked by a open diamond, the resistant fragment characteristic of the antagonist is indicated by a solid diamond, and the newly occurring fragment is indicated by an asterisk.

(Fig. 3). Protease mapping of the wt hRARα in the absence of ligand results in a complete digestion of the receptor (lanes 2 and 3), whereas in the presence of t-RA we saw a 30-kDa band protected (lanes 4 and 5) and in the presence of antagonist Ro 41-5253 a 25-kDa band was protected (lanes 6 to 8) as previously shown (18). Interestingly, this specific antagonist-induced protection changed with the hRARα-R217A mutant. At low trypsin concentrations (lane 14), we were able to see mainly a 30-kDa band corresponding to the agonist band and a very weak lower band. At higher trypsin concentrations (lanes 15 to 17), we saw a dose-dependent conversion of the 30-kDa agonist band to the lower 26-kDa band, which is around 1 kDa larger than the protected band in the experiments with the wt receptor. Using the antagonist Ro 46-5471, we found the same result (data not shown).

To answer the question whether the mutation hRARα-R217A changed the function of an antagonist into that of an agonist, we have performed transactivation experiments to verify the ability of the two antagonists to antagonize the t-RA induced transactivation of a reporter system. The results for Ro 41-5253 are shown in Fig. 4 (with wt and hRARα-R217A). When the antagonist was used alone, for both wt and mutant receptors there was no induction of the luciferase; at three different concentrations of t-RA (1, 10, and 100 nM), and increasing amounts of Ro 41-5253, the antagonist was able to antagonize the t-RA effect with either wt or mutant receptor.

DISCUSSION

The results of the present work are consistent with the crystal structure of the holo-hRARγ-LBD defined by Renaud et al. (39) and provide new information about this receptor family. A high homology has been observed between the holo-hRARγ-LBD structure and the recently reported structure of apo-hRXRα-LBD (8). In this latter receptor, two arginine residues (R-274 and R-278) integrate a cluster of positively charged residues which play a role in electrostatic guidance. R-278 interacts specifically with the carboxyl group of t-RA. Here, we have demonstrated that changing R-276 of hRARα-LBD (R-278 of hRARγ) into either a small polar glycine or small nonpolar alanine residue results in a decreased binding affinity to the mutant receptor for all ligands tested.

For all ligands tested, we have also seen a small reduction in binding affinity for the hRARα mutants at position R-272

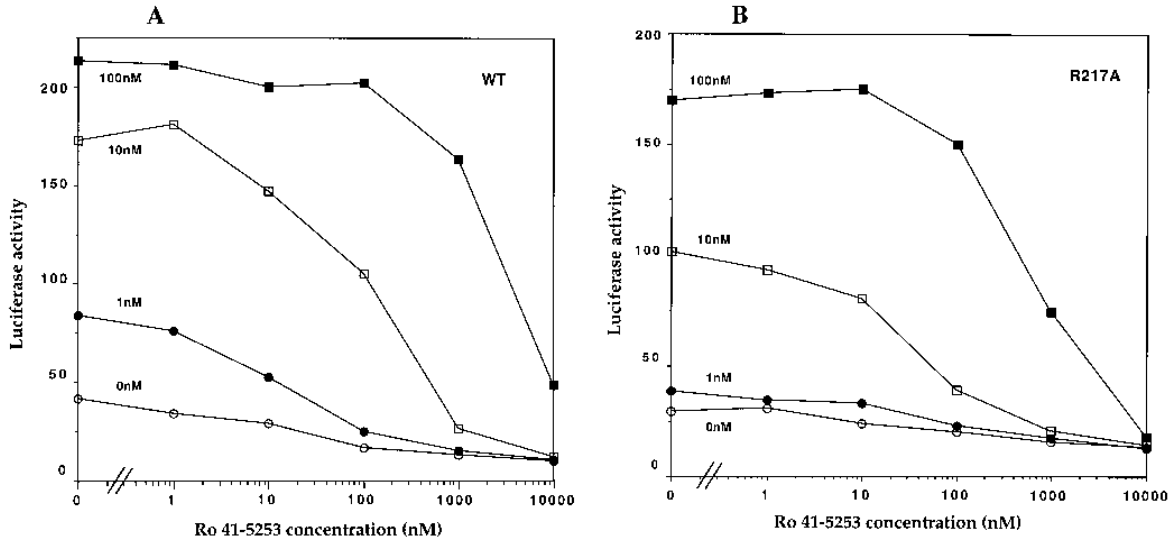


FIG. 4. Influence of the antagonist Ro 41-5253 on transactivation induced by t-RA in CV-1 cells transfected with hRARα (A) or hRARα-R217A (B) and the βRARE-luciferase reporter plasmid. At 24 h after transfection, 0, 1, 10, and 100 nM t-RA and increasing amounts of the antagonist Ro 41-5253 were added to the cultures; after a 36-h incubation, the luciferase activity was measured.

(R-274 of hRARγ). In addition, transactivation experiments with t-RA or Ro 13-7410 showed an increase in EC_{50} values for both ligands, consistent with the binding activity. This effect of residue R-272 on binding and transactivation provides functional evidence of its involvement in ligand binding.

We have clearly demonstrated that R-394 of hRARα (R-396 of hRARγ) is a crucial residue for the binding of all ligands tested. A mutation of the next residue, A-397 of hRARγ, to threonine caused only a slight effect on binding activity (39). This decrease of binding could be due to the perturbation of the R-396 spatial position. It appears that not all the residues involved in the binding pocket have the same importance for ligand binding. R-394 of hRARα is involved in the part of the ligand-binding site close to the ring of t-RA. Since changing R-394 into residues with different characteristics (G, S, A, and E) yielded the same dramatic effect and considering the spatial location of this position in the ligand-binding pocket, there are two possible explanations for the results. It could be that this change in α-helix 11 disturbs its structural function in such a way that α-helix 12 can no longer play the "lid role" to close off the binding cavity. Arguing against this hypothesis are the facts that it is still possible to transactivate the mutated receptors and that the normal agonist band is still protected in protease-mapping studies (data not shown). A more credible explanation could be that the R394 interacts via its hydrophobic portion with the t-RA ring and in this way it contributes to the high-affinity binding of the retinoid.

From our binding results of the hRARα cysteine point mutant, no change in binding efficiency for single mutants was noticeable except for the mutation C265G. For this residue, although the single alanine mutation did not significantly change the affinity of hRARα-LBD for ligands, we did observe a strong effect for the double mutant C265G/C274G. Interestingly, these two cysteines are both involved in α-helix 5, which in hRARγ contains hydrophobic residues interacting with the ligand-binding pocket. We assume that the modification of both cysteines disturbs the α-helix too severely whereas each single mutation has a less dramatic effect.

In addition, a specific influence of C-235 of hRARα (C-237 of hRARγ) on antagonist binding has been established. For agonists, the binding affinity was the same for the C235G mutant as for the wt, whereas with antagonists, a tremendous decrease in binding affinity was shown (Table 2). It is possible that a putative interaction involving the hydrophobic side chain, common to all the antagonists yet absent from agonists, might be disrupted by this mutation in α-helix 3. Furthermore, since α-helix 3 may be involved in the transconformation of α-helix 12, which is necessary for agonist activity, the interaction of the antagonist side chain with α-helix 3 may be part of the antagonistic mechanism.

We have found that the mutation to alanine of either of two residues, R-217 and R-294 of hRARα, causes a specific increase of antagonist-binding efficiency. This provides evidence for the distinctive implication of these two residues in antagonist binding. According to the hRARγ structure, R-217 should be located in the Ω-loop just before the beginning of α-helix 3 and R-294 would start α-helix 6. From the apo-hRXRα and holo-hRARγ crystal structures, a mousetrap mechanism has been postulated to sequester the ligand and transactivate target genes via conformational changes (39). This change would involved the Ω-loop flipping over α-helix 6, which would also shift position to result in a more compact structure. Using trypsin digestion, a specific protease-mapping pattern has been found for antagonists, giving a 25-kDa protected fragment for hRARα versus a 30-kDa protected fragment for bound agonists (18). Strikingly, at the low trypsin concentrations used, the pattern of antagonist bound to hRARα-R217A showed two bands protected, consisting of a main 30-kDa agonist band and a new band about 1 kDa heavier than the antagonist-specific band observed with the wt receptor. It has been previously demonstrated that the C-terminal cleavage site of the antagonist band is located between amino acids 420 and 438 (18). To obtain a protected fragment of 25 kDa, the N-terminal cleavage site has to be approximately at residue 210. The residue R-217 is located in the flexible Ω-loop, which should be an accessible site for trypsin cleavage. All other sites would be less accessible for trypsin, according to the three-dimensional structure of hRARγ. We postulate that by mutating R-217, the N-terminal trypsin cleavage site is destroyed. In the protease mapping experiments, the next most accessible site would be preferentially cleaved at low trypsin concentration, which would correspond in our case to the agonist band (Fig. 3). At higher concentrations, the less accessible site, located 10 residues upstream (K-207) would be cleaved, yielding the 1-kDa larger band observed. Competition experiments for transactivation between t-RA and antagonists (Ro 41-5253 or Ro 46-5471) have established that hRARα-R217A still allows these compounds to retain their antagonist activity. It means that the antagonists are most probably still able to induce the specific antagonist conformational change. Then, R-217 is probably the N-terminal cleavage site of trypsin in wt hRARα-bound-antagonist protection.

In addition to the role of R-394, three residues, C-235 (related to the binding pocket), R-217, and R-294 (both of which are not related to the binding pocket), are implicated specifically in antagonist binding. Interacting in stirring regions according to the three-dimensional structure of hRARγ, the antagonists could block the transitional step required for transactivation activity. We also observed a 60-fold decrease of Ro 41-5253 antagonist-binding activity for the hRARα-C235G, whereas the R276A mutation caused only an 8-fold reduction. Noticeably, the antagonist Ro 46-5471 showed an 11-fold reduction in binding for the C235G mutation while R276A caused a 110-fold decrease of binding activity (Table 2). Therefore, it seems that the roles of these two residues are counterbalanced in the binding pocket depending on the chemical structure of the antagonist, giving a certain flexibility to the binding pocket and thus allowing the binding of different ligands.

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