Mutational Analysis Reveals That All-trans-retinoic acid, 9-cis-Retinoic acid, and Antagonist Interact with Distinct Binding Determinants of RARα*

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Retinoic acid (RA) has a broad spectrum of biological activities in vertebrate development and homeostasis (4–6). Due to their fundamental role in the control of cell differentiation and proliferation, RA and synthetic retinoids are clinically very useful, predominantly in the treatment of leukemia and nonmalignant hyperproliferative disorders of the skin. However, retinoids have undesirable side effects such as hypervitaminosis A syndrome and teratogenicity (4, 7–10). Two biologically active stereoisomers of RA have been identified, all-trans-retinoic acid (t-RA) and 9-cis-retinoic acid (9-cis-RA). The RXRs exclusively bind 9-cis-RA, whereas the RARs bind both isomers of RA with comparable affinity. Recently published results suggest that RARs have the same binding site for t-RA and 9-cis-RA but with different determinants (1–5). Antagonist binding on RARα has been suggested to induce distinct conformational changes in comparison with agonist binding. To elucidate the region minimally required for efficient binding of agonist (t-RA and 9-cis-RA) and antagonist Ro 41-5253 to the RARα, we generated N- and C-terminally truncated mutants of the receptor. Characterization of these deletion mutant proteins using protease mapping and ligand binding experiments revealed that different parts of the ligand-binding domain are necessary for t-RA, 9-cis-RA, and antagonist binding. Three distinct regions of the ligand-binding domain of the human retinoic acid receptor-α are required for binding of t-RA (RARα187–402), 9-cis-RA (RARα188–409), and the antagonist Ro 41-5253 (RARα226–414).

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The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; t-RA, all-trans-retinoic acid; 9-cis-RA, 9-cis-retinoic acid; hRARα, human retinoic acid receptor α; hRARα-LBD, human retinoic acid receptor α-ligand-binding domain; RARα, retinoic acid receptor α; aa, amino acid; wt, wild type.
Materials—[3H]-RA (50 Ci/mmol) was obtained from NEN Life Science Products. 9-cis-[3H]RA (25 Ci/mmol) was obtained from Amersham Corp. [3H]Ro 41-5253 (28 Ci/mmol) was obtained from NEN Life Science Products. [3H]t-RA (50 Ci/mmol) was obtained from NEN Life Science Products. 9-cis-[3H]RA (25 Ci/mmol) was obtained from Amersham Corp. [3H]Ro 41-5253 (28 Ci/mmol), unlabeled RA isomers and analogues were synthesized at F. Hoffmann-La Roche Ltd., Basel, Switzerland. All procedures using retinoids were carried out under dimmed light. The TNT® coupled reticulocyte lysate system was purchased from Promega. [3S]Methionine (>1000 Ci/mmol), 14C-methylated protein molecular weight markers, and Amplify® were obtained from Amersham. Restriction enzymes were obtained from Boehringer Mannheim. Trypsin (type I; from bovine pancreas) was purchased from Sigma.

EXPERIMENTAL PROCEDURES

Binding Determinants of RARα

RESULTS

Effect of t-RA (lanes 14–20), 9-cis-RA (lanes 21–27), and Ro 41-5253 (lanes 28–32) on limited tryptic digestion of RARα wild type (wt) and C-terminal deletion mutants. Controls for wt and truncated receptor expression are in lanes 1–13. The presence of a proteolytic resistant fragment indicates that the indicated retinoid is able to bind the truncated receptors. [In vitro synthesized [3S]methionine-labeled RARα deletion mutants were preincubated with Me2SO alone or the indicated concentrations of t-RA, 9-cis-RA, or Ro 41-5253. Trypsin solution was added giving a final concentration of 75 µg/ml. Alternatively, an equal volume of water was added. Incubations were for 10 min at room temperature. Samples were electrophoresed through a SDS-polyacrylamide gel, and the dried gel was autoradiographed. The sizes of molecular weight markers are indicated. The resistant protein fragment occurring in the presence of RA is marked by a diamond, and the resistant fragment characteristic of the antagonist is indicated by a star.

Binding of t-RA to the N- and C-termally Truncated RARα Mutants—An important region for the t-RA binding (aa 186–198) was defined previously in hRARα by a N-terminally truncated receptor (41). A C-terminal deletion to position 403 resulted in a moderate decrease in affinity for the same ligand (41). On the other hand, a C-terminal deletion to position 404 of hRARα exhibited a Kd of 0.3 nM for t-RA (2). To determine the precise region of the hRARα-LBD required for an efficient t-RA binding, we used binding assays and protease mapping to probe t-RA binding to N- and C-terminally truncated hRARα-LBD (Figs. 1 and 2).

A Kd of 1.1 nM for t-RA was obtained with the hRARα-LBD (aa 155–462) (Table I). This value is in the range of the Kd published for the full-length hRARα expressed in E. coli or COS cells (0.67 nM (42), 1.7 nM (45)), and also for the hRARα-LBD expressed in E. coli (6.2 nM (46); 0.6 nM (3)).

Among the N-terminally truncated receptors, the hRARα-D187 exhibited good protection against trypsin digestion when bound to t-RA. A strongly reduced signal was observed for the digestion of the hRARα-D188 truncated receptor under the same conditions. At the C-terminal end of the receptor, the same tendency was observed with the two mutants hRARα-D402 and hRARα-D401. In regard to the binding activity of these mutant receptors, the N-terminally truncated hRARα-D187 exhibited a Kd value 4.5-fold higher than the wild type hRARα-LBD, whereas a deletion of a single further amino acid abolished the binding activity in such a way that neither Kd nor IC50 could be determined (Table II).
Similar experiments were carried out using N-terminally truncated mutants of hRARγ. We found that hRARγ-D189 (hRARγ-D189) was able to bind t-RA with an affinity comparable with the full-length hRARγ, whereas hRARγ-D190 (hRARγ-D188) showed no detectable binding activity to this ligand (data not shown). Therefore, it appears that, while the C-terminal region of the D domain of RARα (aa 188–199) is required for efficient binding of the t-RA, the C-terminal part of the E domain (aa 405–419) and the entire F domain can be deleted without affecting the ability of the RARα to bind its natural t-RA ligand (see Fig. 3).

**Binding of 9-cis-RA to the N- and C-terminally Truncated RARα Mutants**—Two contradictory results have been published concerning the binding of 9-cis-RA to C-terminal deletion mutants of hRARα. Lefebvre et al. (41) observed that a deletion up to position 403 of the hRARα expressed in E. coli resulted in a moderate decrease in affinity for 9-cis-RA (41). On the other hand, a small region (aa 405–419) within the ligand binding domain of a truncated hRARα was demonstrated to be required for the 9-cis-RA binding (2). In our study, a KD value of 1 nM was determined for the binding of 9-cis-RA to the hRARα-LBD. This value is in agreement with the range of the reported data for the truncated receptors expressed in E. coli (0.3 nM for the hRARα-D189 (2) and 1.2 nM for the hRARα-LBD (3)).

After the limited trypsin digestion of the N-terminally truncated hRARα liganded to 9-cis-RA, the corresponding protection was almost completely abolished for the mutant hRARα-D189, and a reduction of the signal was already observed for the mutant hRARα-D188 (Fig. 2). At the C-terminal end of the receptor, the digestion of the hRARα-D409 liganded to 9-cis-RA by trypsin yielded a band of the appropriate size, whereas the truncated hRARα at the next residue (hRARα-D408) exhibited only a faint band (Fig. 1). IC50 and KD determinations for the binding of the 9-cis-RA to the above mentioned truncated receptors confirmed the N- and C-terminal deletion borders of the hRARα concerning the RA isomer. No 9-cis-RA binding was detectable to the hRARα-D189, whereas a 5-fold higher IC50 value was obtained for the binding of the t-RA isomer to the hRARα-D188 in comparison with the hRARα-LBD (Table II). At the C-terminal end, a 35-fold higher KD value, was obtained for the hRARα-D409, while the hRARα-D408 bound the 9-cis-RA more than 150-fold less efficiently than the hRARα-LBD (Table I). From these results, it is clear that the C-terminal region of the D domain of RARα (aa 189–199) is required for an efficient binding of 9-cis-RA. Furthermore, the C-terminal part of the E domain (up to aa 409) is also crucial for the binding efficiency of this ligand to the hRARα (see Fig. 3).

**Binding of Ro 41-5253 to the N- and C-terminally Truncated RARα Mutants**—The antagonist Ro 41-5253 has been reported to induce a different conformational change when bound to the hRARα (40). It has also been shown recently that Ro 41-5253, as well as other antagonists, exhibit distinctly different requirements for efficient binding to hRARα (3). In the present study, we determined an IC50 value of 16 nM for the binding activity of the antagonist Ro 41-5253 to the hRARα-LBD (Table I). Limited trypsin proteolysis of the N- and C-terminally truncated hRARα bound to Ro 41-5253 led to the definition of the N-terminal deletion border between the residue D226 and the residue Lys-227 and the C-terminal deletion border between the residue L414 and M413 of the E region of the hRARα-LBD.

Concerning the binding activity of Ro 41-5253 to the truncated hRARα, 24- and 15-fold higher IC50 values for hRARα-D227 and hRARα-D413 have been observed, respectively, in comparison with the hRARα-LBD (Tables I and II). Remarkably, the hRARα-D226 and the hRARα-D414 mutant exhibited only a 2- and 3.5-fold reduction in IC50, respectively, in comparison with the hRARα-LBD. These results evidence distinctly different requirements of the N- and C-terminal regions of the ligand-binding domain of the hRARα for binding Ro 41-5253 compared with the two other ligands t-RA and 9-cis-RA (see Fig. 3).
DISCUSSION

It has been shown that the binding of both t-RA and 9-cis-RA induces a different conformational change in hRARα to that observed with the binding of the antagonist Ro 41-5253 (40). At present, several arguments allow one to think that, depending on the chemical structure of the ligands, distinctly different determinants are required for the efficient binding to the RARs. Recently published results also showed distinct determinant requirements for the binding of different ligands to the estrogen receptor (47). The recently described crystal structure of the RARγ ligand-binding domain bound to t-RA has clarified the ligand binding interactions. Modeling 9-cis-RA in the hRARγ binding pocket revealed that the binding site of this receptor was likely to be very similar to that of t-RA (1). In addition, three residues have been shown to play a significant role in ligand binding (agonist and antagonist) to hRARα, whereas Cys-235, Arg-217, and Arg-294 have been shown to be important residues of the hRARα-LBD, specifically for antagonist interactions (3). Furthermore, Arg-269 of the hRARβ (Arg-276 of hRARα and Arg-278 of hRARγ) has been shown to be a crucial residue for RA binding (48, 49). These findings and the highly conserved amino acid homology of the E domain of the RARαs (92%) (23) suggest that the binding conditions of t-RA to the three subtypes of receptors RARα, -β, and -γ are very similar. It also indicates that these ligands may compete for a unique binding site in the RARs.

In the present study, the 30-fold higher Kd value for 9-cis-RA binding to the C-terminal deletion hRARα-D409 reinforces the role of the residue Ile-410 in the 9-cis-RA binding as shown by Tate and Grippo (2). We have provided evidence by this study that the complete F domain can be removed without disturbing the ability of the hRARα to bind any ligand. The C-terminal part of the E domain of the hRARα (to aa 414) is sufficient for the efficient binding of the antagonist Ro 41-5253, whereas further deletion of the E domain of the hRARα to positions 409 or 402 for 9-cis-RA and t-RA, respectively, does not disturb the ability of the receptor to bind these two ligands. Interestingly, t-RA and 9-cis-RA require the C-terminal part of the D domain of the hRARα (from aa 187 and 188, respectively), while the D domain and the N-terminal part of the E domain (aa 155 to 226) are not required for the efficient binding of the antagonist Ro 41-5253.

From the crystal structure of the hRARγ-LBD bound to t-RA and the modeled 9-cis-RA in the hRARγ binding pocket, less distance was expected between the ligand 9-cis-RA and the C-terminal region of the hRARγ (helix-11 and helix-12) in comparison with the t-RA spatial disposition (1). It was also clear that the C-terminal part of helix-1 overlaps with the
C-terminal part of helix-3. The importance of residue Cys-235 of the hRARα in antagonist binding (3) indicates a role of the helix-3 in antagonist binding. Remarkably, all of helix-1 can be deleted without disturbing antagonist binding, whereas this completely abolishes agonist binding. An explanation for this could be that the C-terminal part of helix-1 may stabilize the spatial location of helix-3 required specifically for the binding of the agonists.

Taken together, these results indicate that three different fragments of hRARα are minimally required for the binding of t-RA, 9-cis-RA, and Ro 41-5253. The data shown here suggest that distinct determinants of hRARα are either directly involved in the binding of these three ligands or, alternatively, they are needed to maintain the binding pocket in an appropriate conformation as a prerequisite for the binding of each of the isomers of RA as well as Ro 41-5253.

Limited trypsin digestion of either wild type or truncated receptor yielded the same digested peptides corresponding to the potential cleavage sites (data not shown). The undigested mutant receptors showed positions in the gel that were in accordance with their calculated molecular weights (Fig. 1, lanes 2–13; Fig. 2, lanes 2–10). Also, the molecular weights of the fragments as determined from the SDS gel were in good accordance with the magnitude of the truncation of the mutants (Figs. 1 and 2). This indicates that the binding of either the agonists t-RA and 9-cis-RA or the antagonist Ro 41-5253 to the truncated receptors induces the same conformational change in all the mutants as that observed with the full-length RARα. In this study, we demonstrate that with trypsin digestion of 9-cis-RA-bound full-length hRARα, the resulting 30-kDa fragment is less protected against further proteolysis than the one derived from the analogous experiment with t-RA. This was evidenced by the time course of trypptic digestion of RARα bound to t-RA versus 9-cis RA-bound receptor (Fig. 4). These results were further confirmed by trypptic proteolysis with increasing protease concentrations (data not shown). These differences in the proteolytic resistance cannot be explained by distinct affinities of the RA isomers to the RARα, because both exhibit $K_d$ values in the range of 1 nM (Table I). The decreased stability of the 30-kDa fragment obtained from RARα bound to 9-cis-RA could reflect the higher off-rate of 9-cis-RA from RARα in comparison with the t-RA. Displacement assays have demonstrated that 9-cis-RA exhibits about 2-fold higher off-rates from murine RARα than t-RA (38). Time course experiment using Ro 41-5253 as ligand yielded also a decreased stability of the 25-kDa antagonist characteristic fragment compared with the stable 30-kDa fragment observed with t-RA (Fig. 4). Incubation of the receptor with Ro 41-5253 or 9-cis-RA instead of t-RA in the digestion assay could result in more unliganded receptors, or fragments, for a short period of time. During this time, they might change to a more relaxed conformation, more accessible to the protease.

In conclusion, we have evidenced that three distinct regions of the of the hRARα-LBD are required for the efficient binding of t-RA, 9-cis-RA, and the antagonist Ro 41-5253. t-RA binding requires the region of the hRARα-LBD from amino acid 187 to amino acid 402, 9-cis-RA requires the region of the hRARα-LBD from amino acid 188 to amino acid 409, while the antagonist requires the region of the hRARα-LBD from amino acid 226 to amino acid 414.

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