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Different Agonist- and Antagonist-Induced Conformational Changes in Retinoic Acid Receptors Analyzed by Protease Mapping

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The pleiotropic effects of retinoic acid on cell differentiation and proliferation are mediated by two subfamilies of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). Recently the synthetic retinoid Ro 41-5253 was identified as a selective RARα antagonist. As demonstrated by gel retardation assays, Ro 41-5253 and two related new RARα antagonists do not influence RARα/RXRα heterodimerization and DNA binding. In a limited trypsin digestion assay, complexation of RARα with retinoic acid or several other agonistic retinoids altered the degradation of the receptor such that a 30-kDa proteolytic fragment became resistant to proteolysis. This suggests a ligand-induced conformational change, which may be necessary for the interaction of the DNA-bound RARα/RXRα heterodimer with other transcription factors. Our results demonstrate that antagonists compete with agonists for binding to RARα and may induce a different structural alteration, suggested by the tryptic resistance of a shorter 25-kDa protein fragment in the digestion assay. This RARα conformation seems to allow RARα/RXRα binding to DNA but not the subsequent transactivation of target genes. Protease mapping with C-terminally truncated receptors revealed that the proposed conformational changes mainly occur in the DE regions of RARα. Complexation of RARβ, RARγ, and RXRα, as well as the vitamin D_3 receptor, with their natural ligands resulted in a similar resistance of fragments to proteolytic digestion. This could mean that ligand-induced conformational changes are a general feature in the hormonal activation of vitamin D_3 and retinoid receptors.

Retinoic acid is known to play a critical role in many aspects of vertebrate development and homeostasis (11, 27, 36). Retinoic acid and synthetic retinoids are clinically useful as anticancer agents and in the treatment of nonmalignant hyperproliferative disorders of the skin but have the disadvantage of being teratogenic at high concentrations (8, 27, 42). Retinoids exert their effects through two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The classification is based on differences in primary structure (14, 29, 30, 35). Both subfamilies of retinoid receptors belong to the nuclear steroid-thyroid vitamin D_3-retinoid receptor superfamily, whose members act as ligand-inducible transcription enhancer factors (12, 23, 28). On the basis of homologies, the primary sequences of RARs and RXRs can be divided into six distinct regions designated A through F. Region C constitutes the highly conserved DNA binding domain, and region E confers the ligand-binding properties of each receptor (the ligand-binding domain [LBD]).

The RAR gene family consists of three subtypes, referred to as RARα, RARβ, and RARγ (4, 5, 9, 14, 22, 35, 47). Each of the three genes encodes a variable number of isoforms produced by the usage of two promoters and differential splicing (17, 25, 48). All RARs bind RA (all-*trans* retinoic acid) as well as 9-*cis* RA (9-*cis* retinoic acid) with comparable affinities (2). RARs seem to operate effectively only as heterodimeric RAR/RXR complexes (6, 20, 32, 46, 49), but RXR-independent transactivation by RARs has also been observed (39).

The members of the second subfamily, RXRα, RXRβ, and RXRγ (24, 29, 30), do not bind RA. A natural ligand was found to be the 9-*cis* RA stereoisomer (16, 26). The RXRs are able to activate genes via homodimers (31, 50) but act predominantly as coregulators, which enhance the binding of RA, vitamin D_3, thyroid hormone, and peroxisome proliferator-activated receptors to their response elements via heterodimerization (6, 19, 20, 32, 46, 49).

Distinct tissue- and developmental-stage-specific distribution patterns of RAR and RXR subtype transcripts in adult tissues and during embryogenesis have been described (10, 29, 37, 38). These observations have suggested that RARs and RXRs mediate many of the developmental effects and regulate different target genes in several tissues in adult life.

Recently the synthetic retinoid Ro 41-5253 was demonstrated to act as a selective RARα antagonist. This compound counteracts RA effects on HL-60 cell differentiation, on B-lymphocyte polyclonal activation, and in a RARα transactivation assay (3). Elucidation of the molecular mechanism by which the antagonist blocks the action of agonists may increase the understanding of the general mechanism of retinoid action and may be helpful for the design of pharmaceuticals.

Therefore, we investigated the influence of antagonists and agonists on the binding of the RARα/RXRα complex to the retinoic acid response element of the RARβ gene (βRARE), which is known to be activated via all three subtypes of RARs (43, 45). Furthermore, we determined different agonist- and antagonist-induced changes in the resistance of RARα to limited proteolytic digestion, which suggests different ligand-induced conformational changes

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within the receptor. The data led us to propose a model for the activation of RARα by agonists and their neutralization by antagonists. The results obtained for RARα caused us to investigate possible ligand-induced changes in the proteolytic patterns of other retinoid receptors and the vitamin D_3 receptor (VDR).

MATERIALS AND METHODS

Materials. RA isomers and analogs, as well as 1,25-dihydroxyvitamin D_3, were synthesized at F. Hoffmann-La Roche Ltd., Basel, Switzerland. All-*trans* [^{3}H]RA (50 Ci/mmol) was obtained from DuPont/NEN. Retinoids were solubilized in dimethylsulfoxide (DMSO) as 10 mM stock solutions and kept at −80°C. Further dilutions were made in DMSO (gel retardation and proteolytic-digestion experiments), in phosphate-buffered saline containing 1% gelatin (Bio-Rad) and 4% DMSO (retinoid binding assay), or in Dulbecco's modified Eagle's medium (RARα transactivation assay). Some retinoids were unstable upon storage, and fresh solutions were prepared for each experiment. 1,25-dihydroxyvitamin D_3 was solubilized in ethanol as a 1 mM stock solution and kept at −80°C under nitrogen. Fresh dilutions were made in ethanol for each experiment. The transcription kit (T7 MEGAscript) was obtained from Ambion, and the nuclease-treated rabbit reticulocyte lysate translation kit was purchased from Promega. [α-^{32}P]dATP (~3,000 Ci/mmol), [^{35}S]methionine (>1,000 Ci/mmol), [^{14}C]methylated protein molecular mass markers, and Amplify were obtained from Amersham. Poly(dI-dC) · poly(dI-dC) was obtained from Pharmacia LKB. Gelatin was purchased from Bio-Rad. Restriction enzymes and elastase (from porcine pancreas) were obtained from Boehringer Mannheim. Trypsin (type I, from bovine pancreas) and chymotrypsin (type I-S, from bovine pancreas) were purchased from Sigma.

Retinoid binding assay and RARα transactivation assay. Retinoid binding and RARα transactivation assays were performed as described earlier (3).

Plasmids. The human RARα cDNA insert of pT7-RARα (18) was subcloned as an *Msc*I-*Bam*HI fragment into pSG5-*Msc*I, giving pCA151. pSG5-*Msc*I was constructed by inserting a linker with an *Msc*I site in the *Eco*RI-*Bam*HI site of pSG5. The cDNA of RARβ was cloned by PCR from human lung mRNA and subcloned into pSG5, giving pPL205. The correctness of the sequence was confirmed by sequencing. For the construction of the corresponding clone of RARγ, the mouse RARγ cDNA was cloned by PCR from mouse F9 cells, and the correctness of the sequence was confirmed. The clone was then altered by site-directed mutagenesis to encode the human RARγ protein and was subcloned into pSG5, giving pPL144. Human RXRα in pSG5 (pLS36) (26) was provided by L. Sturzenbecker (Hoffmann-La Roche Inc., Nutley, N.J.), and human VDR in pSG5 (7) was provided by W. Hunziker (F. Hoffmann-La Roche Ltd., Basel, Switzerland). The construction of the C-terminally truncated RARαs was done by PCR. The fragments were subcloned, the correctness of the sequence was confirmed, and the fragments (*Sac*I-*Bam*HI) were exchanged in pCA151, giving pCA155 and pCA156. The resulting proteins are RARα-Δ438 and RARα-Δ420 (see Fig. 5A).

In vitro transcription and translation. pCA151 and pLS36 were linearized with *Bgl*II, and pPL144, pPL205, pCA155, pCA156, and human VDR in pSG5 were linearized by *Xba*I. The linearized templates were transcribed with T7 RNA polymerase by using the MEGAscript kit according to Ambion's instructions. Purified mRNA was in vitro translated by using rabbit reticulocyte lysates according to Promega's instructions in the presence of [^{35}S]methionine. For gel retardation experiments, unlabeled receptors were produced in the absence of [^{35}S]methionine by using a complete amino acid mixture.

Gel retardation assay. Oligonucleotides corresponding to βRARE (5'-gatcGTAGGGTTCACCGAAAGTTCACTC-3') and the vitellogenin gene estrogen response element (5'-gatcTCAGGTCACAGTGACCTGA-3') were obtained synthetically. The probes were prepared by heating with the complementary strands to 90°C and subsequent cooling to room temperature over a period of 3 h. The resulting oligonucleotides were labeled with [α-^{32}P]dATP by using the Klenow fragment of DNA polymerase. The reticulocyte lysates containing unlabeled receptors were preincubated with retinoids or DMSO alone in glass tubes for 20 min at room temperature in the dark. The final concentration of DMSO was 2.5%. In the DNA binding reaction, 4 μl of this mixture was added to a 16-μl solution containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 80 mM KCl, 2.5 mM $MgCl_2$, 5 μM $ZnCl_2$, 5% glycerol, 1 mM dithiothreitol, 0.1 μg of poly(dI-dC) · poly(dI-dC) per ml, 25 pg of ^{32}P-labeled oligonucleotide per ml (final concentrations), and, if indicated, a 30- or 100-fold molar excess of unlabeled competing nucleotides. After a 20-min incubation at room temperature in the dark, 2 μl of loading dye (50 mM HEPES [pH 7.9], 40% glycerol, 0.2% bromophenol blue, 0.2% xylene cyanol) was added. An aliquot of the mixture was loaded on a 5% nondenaturing polyacrylamide gel (acrylamide/*N*,*N'*-methylene-bisacrylamide weight ratio of 29:1). Gels were run at 4°C in 0.5× TBE (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA). Gels were dried and autoradiographed with Kodak Scientific Imaging X-Omat AR film overnight at −80°C.

Limited proteolytic digestion. The following reactions were carried out under dimmed light. Aliquots of reticulocyte lysates containing the labeled receptors were incubated in glass tubes with retinoids in DMSO or with 1,25-dihydroxyvitamin D_3 in ethanol for 20 min at room temperature. Controls were incubated with DMSO or ethanol. The final concentration of both DMSO and ethanol was 2.5%. To 9-μl aliquots, 1-μl aliquots containing different amounts of proteases in water were added. After 10 min at room temperature, the digestion was stopped by adding 40 μl of sodium dodecyl sulfate (SDS) sample buffer and boiling for 10 min. SDS gel electrophoresis was carried out as described in the instructions provided with the in vitro translation kit at 4°C with a 12% polyacrylamide gel (acrylamide/*N*,*N'*-methylene-bisacrylamide weight ratio of 29:1). The gels were incubated for 30 min or overnight in 25% isopropanol–10% acetic acid, after which they were treated for 20 min with Amplify, vacuum dried at 60°C, and autoradiographed as described for the gel retardation assay.

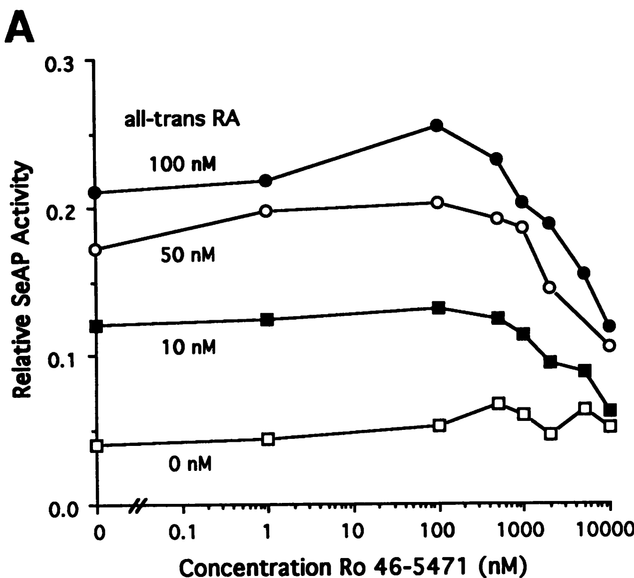
RESULTS

Ro 46-5471 and Ro 46-8515 act, like Ro 41-5253, as antagonists of RARα activation by retinoic acid. The antagonistic activities of Ro 46-5471 and Ro 46-8515 (Table 1) were measured in a RARα transactivation assay by using COS-1 cells cotransfected with a RARα-estrogen receptor chimera expression vector and a SeAP (secreted alkaline phosphatase) reporter plasmid (3). The concentration-dependent antagonistic activities of Ro 46-5471 and Ro 46-8515 at various RA concentrations are shown by the decreased

TABLE 1. RAR binding and transactivation data for retinoids

Retinoid	Binding (IC_{50}, nM)[a]			Activation (EC_{50}, nM)[b]
	RARα	RARβ	RARγ	RARα
Agonists				
Retinoic acid	14	14	14	6.7
Ro 13-7410	36	22	15	2.1
Ro 19-0645	460	26	190	67
Antagonists				
Ro 41-5253	60	2400	3300	> 1000
Ro 46-5471	27	5100	2400	> 1000
Ro 46-8515	360	2800	1400	> 1000
Antagonist related analogues				
Ro 46-5468	>10000	>10000	>10000	> 1000
Ro 41-4452	2100	>10000	>10000	> 1000

[a] Retinoid concentration required to inhibit 50% of the specific RA binding.
[b] Retinoid concentration at which 50% of maximal SeAP induction is observed in COS-1 cells transfected with RARs and the reporter plasmid vit-tk-SeAP.



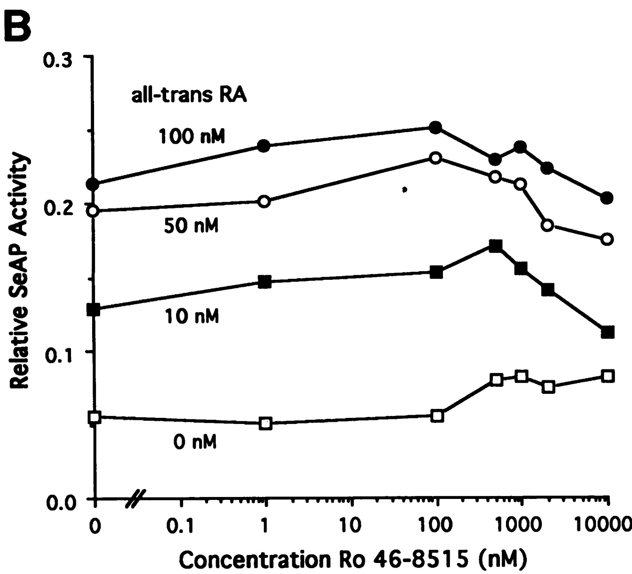


FIG. 1. Influence of Ro 46-5471 and Ro 46-8515 on transactivation induced by RA in COS cells transfected with RARα and the reporter plasmid vit-tk-SeAP. Twenty-two hours after transfection, RA at various concentrations was added to the cultures. Simultaneously, the indicated concentrations of Ro 46-5471 (A) or Ro 46-8515 (B) were added.

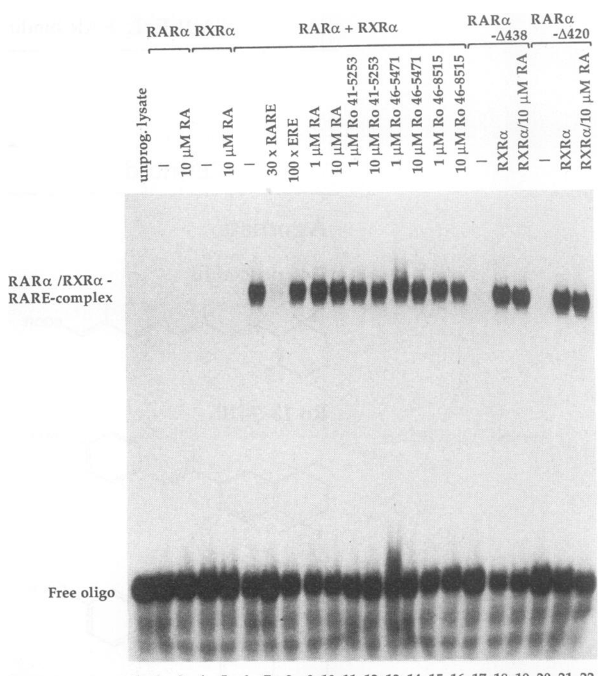


FIG. 2. Ligand effects on the binding of RARα, RXRα, and RARα deletion mutants to βRARE. In vitro-translated receptors were preincubated with the indicated concentrations of retinoids or DMSO alone (lanes 1, 2, 4, 6 to 8, 17, 18, 20, and 21) and as indicated and then tested for DNA binding activity in a gel retardation assay. A 28-bp double-stranded oligonucleotide containing a βRARE sequence was used as probe. Where indicated, a 30-fold molar excess of unlabeled βRARE or a 100-fold molar excess of unlabeled estrogen response element (ERE) was added.

induction of SeAP activity (Fig. 1). In the absence of RA, the antagonists do not activate the chimeric receptor (<10% of the activation at 100 nM RA). For Ro 41-5253 and Ro 46-5471, approximately a 5-fold molar excess over RA is needed to show an antagonistic effect, whereas in the case of Ro 46-8515 comparable effects were detectable only at approximately a 50-fold molar excess. With a 1,000-fold molar excess, Ro 46-5471 and Ro 41-5253 are able to completely suppress the transactivation induced by 10 nM RA.

The relative antagonistic potency of the three retinoids in the transactivation assay is paralleled by their ability to displace the natural ligand RA from RARα (Table 1). On the basis of our binding data (for RARs, see Table 1, no binding of the three antagonists to RXRα at 10 μM), Ro 41-5253, Ro 46-5471, and Ro 46-8515 appear to be highly selective for RARα.

Influence of RARα antagonists on the binding of RARα/RXRα heterodimers to βRARE. The influence of the RARα antagonists on RARα/RXRα binding to DNA was studied in the gel retardation or band shift assay using in vitro-synthesized receptors and a radiolabeled oligonucleotide corresponding to βRARE. Under our conditions, neither RARα nor RXRα bound to βRARE either in the absence or in the presence of 10 μM RA (Fig. 2, lanes 2 to 5). As expected (6, 20), the affinity of RARα to βRARE was greatly enhanced by the addition of RXRα (Fig. 2, lane 6). The specificity of the RARα/RXRα interaction with βRARE was examined by using unlabeled oligonucleotides as competitor. Oligonucleotides containing βRARE competed efficiently for RARα/RXRα complex binding at a 30-fold molar excess, whereas oligonucleotides containing the unrelated estrogen response element failed to compete at a 100-fold molar excess relative to the radiolabeled βRARE (Fig. 2, lanes 7 and 8).

As expected, the RARα/RXRα binding to βRARE is independent of RA (Fig. 2, lanes 9 and 10). Similarly, the presence of antagonists showed no significant effects on RARα/RXRα binding to βRARE (Fig. 2, lanes 11 to 16).

In the gel retardation assay, the C-terminally truncated receptors RARα-Δ438 and RARα-Δ420 showed the same properties as the full-length RARα (Fig. 2, lanes 17 to 22). This indicates, in accordance with Yu et al. (46), that region F in RARα is not necessary for heterodimerization with

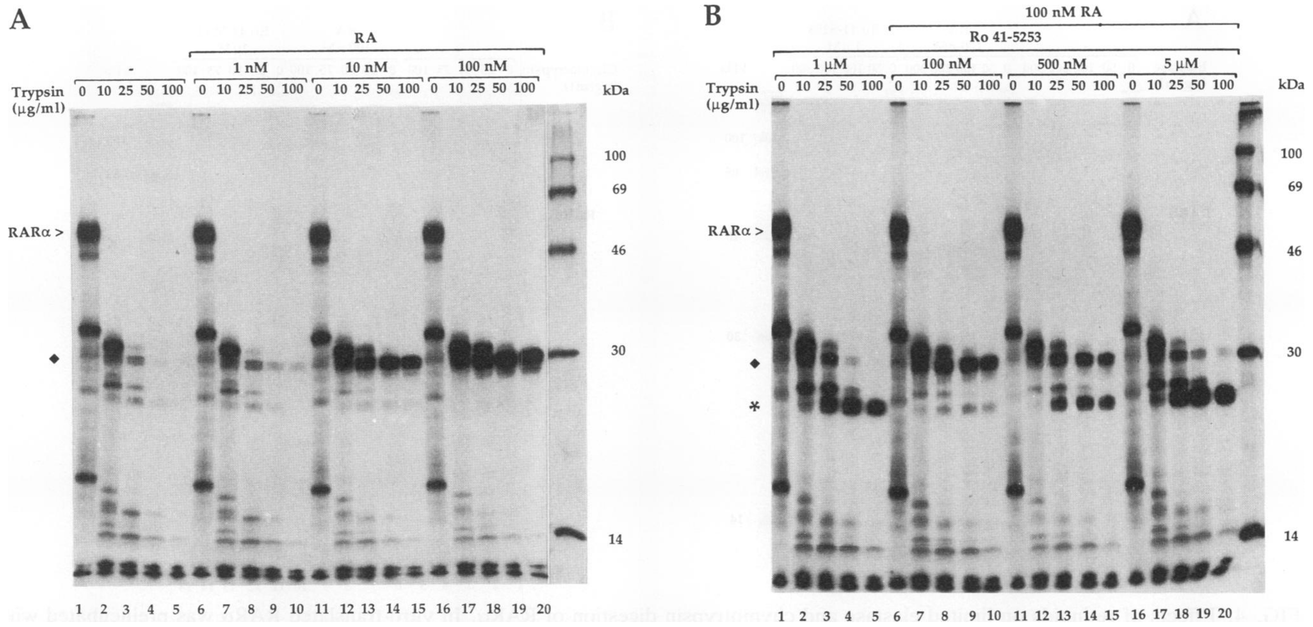


FIG. 3. Retinoid effects on limited trypsin digestion of RARα. In vitro-synthesized [^{35}S]methionine-labeled RARα was preincubated with DMSO alone (A) (lanes 1 to 5) or the indicated concentrations of RA (A) or RA and Ro 41-5253 (B). Trypsin solutions or an equal volume of water was added, giving the indicated final protease concentrations, and the mixtures were incubated for 10 min at room temperature. Samples were electrophoresed through an SDS-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 RA is marked by a diamond, and the resistant fragment characteristic of antagonists is indicated by an asterisk.

RXRα or for the binding of the resulting complex to βRARE. As expected, the molecular masses of the resulting RARα-mutant/RXRα/βRARE complexes are lower than in the case of full-length RARα.

Limited proteolytic digestion of RARα suggests different conformational changes induced by agonists and antagonists. To investigate possible agonist- and antagonist-induced conformational changes in RARα, we used a limited proteolytic-digestion assay. This method has often been used to study protein conformations (1, 21, 40, 44).

[^{35}S]methionine-labeled RARα translated in vitro was incubated with retinoids or DMSO alone and then digested with several concentrations of trypsin or other proteases. The digestion products were analyzed by SDS-polyacrylamide gel electrophoresis. Figure 3A shows that RARα is completely digested to peptides smaller than 14 kDa at trypsin concentrations of 50 or 100 μg/ml within 10 min at room temperature (lanes 4 and 5). This was expected on the basis of the location of 52 possible cleavage sites. At lower trypsin concentrations, we observed several proteolytic fragments around 30 kDa (lanes 2 and 3). The proteolysis of one of these fragments was greatly diminished when RA-complexed RARα was digested under the same conditions, suggesting a ligand-induced conformational change or a stabilization of a particular conformation of the receptor. The effect was observed in the presence and in the absence of βRARE (compare Fig. 7 with Fig. 3, Fig. 4B, and Fig. 6B and C). The proteolytic resistance of the 30-kDa fragment is dependent on the RA concentration (Fig. 3A, lanes 6 to 20). The first effects were observed at concentrations around 5 nM RA. Treatment of RARα with the agonistic retinoid Ro 13-7410, Ro 19-0645 (Table 1), or 9-*cis* RA induces a digestion pattern analogous to that of RA (data not shown). As expected from the binding and transactivation data (Table 1) (for 9-*cis* RA, see reference 2), the concentration of 9-*cis* RA or Ro 13-7410 required to diminish the proteolysis of the 30-kDa fragment is similar to that of RA, whereas approximately 50-fold-higher concentrations of Ro 19-0645 are required for the same effect.

Analogous digestion experiments carried out with the RARα antagonists Ro 41-5253, Ro 46-5471, and Ro 46-8515 showed that these retinoids also altered the degradation of the receptor such that a fragment became resistant to tryptic proteolysis (Fig. 3B, lanes 1 to 5) (data not shown for Ro 46-5471 and Ro 46-8515). However, this fragment has a molecular mass of 25 kDa. This suggests a conformational change induced by antagonists which is different from that occurring in the presence of agonistic retinoids. When RARα is simultaneously treated with 100 nM RA and increasing amounts of the antagonist Ro 41-5253, the RA-induced digestion pattern changes to the one characteristic of antagonists (Fig. 3B, lanes 6 to 20). In accordance with the binding and transactivation data (Table 1), at least 100 nM Ro 41-5253 or Ro 46-5471 and 1 μM Ro 46-8515 are needed to induce a proteolytic resistance pattern characteristic of antagonists, and a 50-fold excess of Ro 41-5253 or Ro 46-5471 over RA is needed to change the RA-induced agonist-specific digestion pattern into the one specific for antagonists, whereas a 100-fold molar excess of Ro 46-8515 over 100 nM RA is not sufficient to change the digestion pattern completely. There is no difference in the digestion pattern when RARα is preincubated for 10 min with the antagonist before addition of the agonist or with 10 min of preincubation with the agonist prior to addition of the antagonist (data not shown). The suggested conformational effects caused by RARα agonists and antagonists seem therefore to be reversible.

RARα is nearly completely digested to peptides smaller

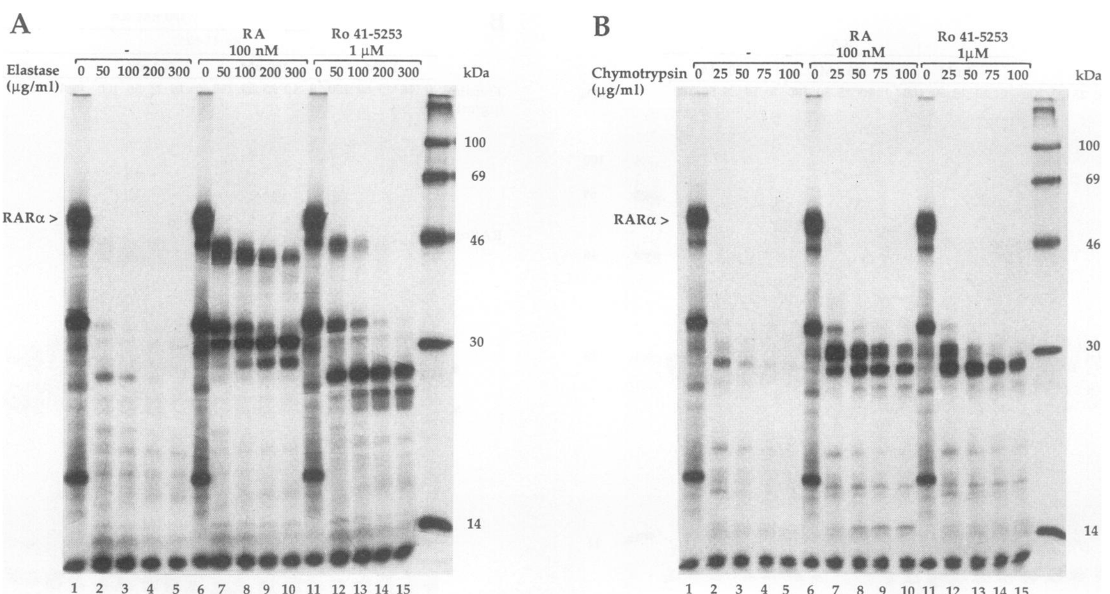


FIG. 4. Effects of retinoids on limited elastase and chymotrypsin digestion of RARα. In vitro-translated RARα was preincubated with DMSO alone (lanes 1 to 5) (dashes), 100 nM RA, or 1 μM antagonist Ro 41-5253 and then digested with the indicated concentrations of elastase (A) or chymotrypsin (B). Samples were analyzed as described in the legend to Fig. 3.

than 14 kDa with elastase at concentrations greater than 50 μg/ml within 10 min at room temperature (Fig. 4A, lanes 3 to 5). As in the case of trypsin, some fragments became resistant to proteolysis when RARα was treated with 100 nM RA (Fig. 4A, lanes 6 to 10). The molecular masses of the two main fragments are 30 and 41 kDa. Complexation of RARα with 1 μM Ro 41-5253 followed by elastase digestion resulted in a different pattern, with a major resistant fragment of 26 kDa (Fig. 4A, lanes 11 to 15).

Chymotrypsin at concentrations greater than 50 μg/ml almost completely digests RARα (Fig. 4B, lanes 4 to 5). Incubation of RARα with 100 nM RA results in a diminished proteolysis of a 28-kDa fragment (Fig. 4B, lanes 6 to 10). Chymotrypsin digestion of RARα in the presence of the antagonist Ro 41-5253 results in a proteolytic pattern significantly different from that obtained in the presence of the agonist RA (Fig. 4B, lanes 11 to 15). Similar degradation patterns in the presence of RA and Ro 41-5253 were obtained when RARα was digested with dispase and endoproteinase GluC (data not shown).

As a control for nonspecific effects, the influence of the structurally related retinoids Ro 46-5468 and Ro 41-4452, which bind with very low affinity to the receptor (Table 1), was investigated by the described proteolysis experiments with trypsin, elastase, and chymotrypsin. As expected, no change of the degradation pattern compared with the control was observed (data not shown).

The proposed conformational change occurs mainly in the ligand-binding domain of RARα. To examine which part of RARα constitutes the proteolysis-resistant fragments, proteolytic degradation experiments were performed with the C-terminally truncated receptors RARα-Δ438 and RARα-Δ420 (Fig. 5A and B). As expected, the molecular masses were 49 kDa for RARα-Δ438 and 47 kDa for RARα-Δ420 in comparison with 51 kDa for the full-length RARα (Fig. 5B, lanes 1 to 3). As summarized in Table 2, in the presence of RA the molecular masses of the proteolysis-resistant fragments are the same for RARα and RARα-Δ438, whereas the corresponding fragment of RARα-Δ420 has a molecular mass about 1 kDa lower with all proteases. This result indicates that the C terminus of the RA-induced resistant fragments is around amino acid 430. In the case of trypsin, Arg-432 is therefore the most probable C terminus, and calculated from the molecular weight of the fragment, the N-terminal cleavage site is probably Arg-192 or Lys-193 or Lys-207. The C terminus of the antagonist-induced resistant fragment appears, in the case of trypsin digestion, to be the same as it is for RA, whereas with elastase or chymotrypsin as protease, the C terminus of the resistant fragment is expected to be around amino acids 450 to 462 (Fig. 5C). The interpretations are consistent with immunoprecipitation experiments in which polyclonal antiserum raised against the last 19 amino acids of RARα and precipitating the full-length receptor was unable to precipitate the resistant fragments occurring after trypsin digestion of RA- or Ro 41-5253-complexed RARα (data not shown). Note that the fragments of RARα-Δ420 generated in the presence of an antagonist are less resistant to trypsin and chymotrypsin degradation than those from RARα-Δ438 or RARα. Therefore, a part of region F seems to be necessary for the generation of the proteolysis-resistant conformation induced by the antagonist in contrast to the resistant conformation induced by RA (the same resistance of the RA-induced fragments derived from RARα and RARα-Δ420).

RARβ, RARγ, RXRα, and VDR also show ligand-dependent protease sensitivities. Receptors were translated in rabbit reticulocyte lysates with [^{35}S]methionine and treated with their natural ligands or vehicle. Proteolytic digestion and analysis were carried out as described for RARα.

Trypsin digestion of RARβ and RARγ (Fig. 6A, lanes 1 to 5 and 16 to 20) leads to small fragments similar to those of RARα (Fig. 3A, lanes 1 to 5). Incubation of RARβ with 100 nM RA altered the digestion pattern such that a 35-kDa fragment became resistant to tryptic digestion (Fig. 6A, lanes 6 to 10). Complexation of RARγ with 100 nM RA resulted in the resistance of a 33-kDa fragment (Fig. 6A,

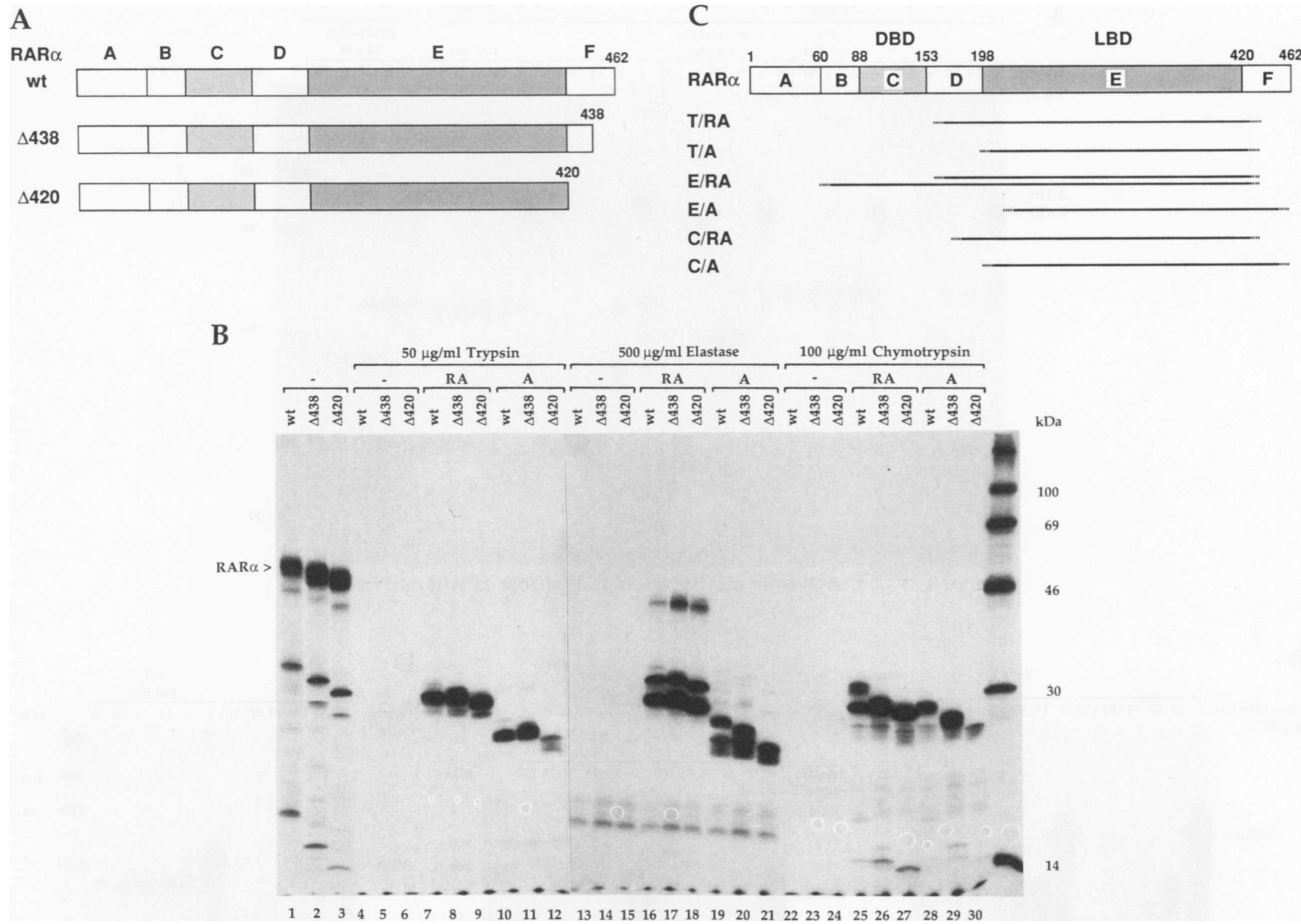


FIG. 5. Determination of the RARα regions contained in the protease-resistant protein fragments. (A) C-terminally truncated receptors used in this study (RARα-Δ438 [Δ438] and RARα-Δ420 [Δ420]) compared with the full-length RARα (wt). (B) In vitro-translated full-length RARα (wt), RARα-Δ438 (Δ438), or RARα-Δ420 (Δ420) was preincubated with DMSO (lanes 1 to 6, 13 to 15, and 22 to 24) (dashes), 100 nM RA, or 1 μM Ro 41-5253 (A) and then treated with the indicated concentrations of protease or an equal volume of water (lanes 1 to 3). Probes were analyzed as described in the legend to Fig. 3. (C) RARα regions contained in the main fragments occurring after treatment of RARα with RA or Ro 41-5253 (A) followed by digestion with 50 μg of trypsin (T) per ml, 500 μg of elastase (E) per ml, or 100 μg of chymotrypsin (C) per ml. DBD, DNA-binding domain.

lanes 21 to 25). The concentration dependence of this effect of RA is, as expected from the binding data, exactly the same as that shown for RARα (data not shown). Treatment of RARβ with 10 μM Ro 41-5253 resulted in the proteolytic resistance of a 30-kDa fragment (Fig. 6A, lanes 11 to 15). Incubation of RARγ with 10 μM Ro 41-5253 led to a slight

TABLE 2. Molecular mass of proteolysis-resistant fragments from RA- or antagonist-complexed full-length (wt) or C-terminally truncated RARα

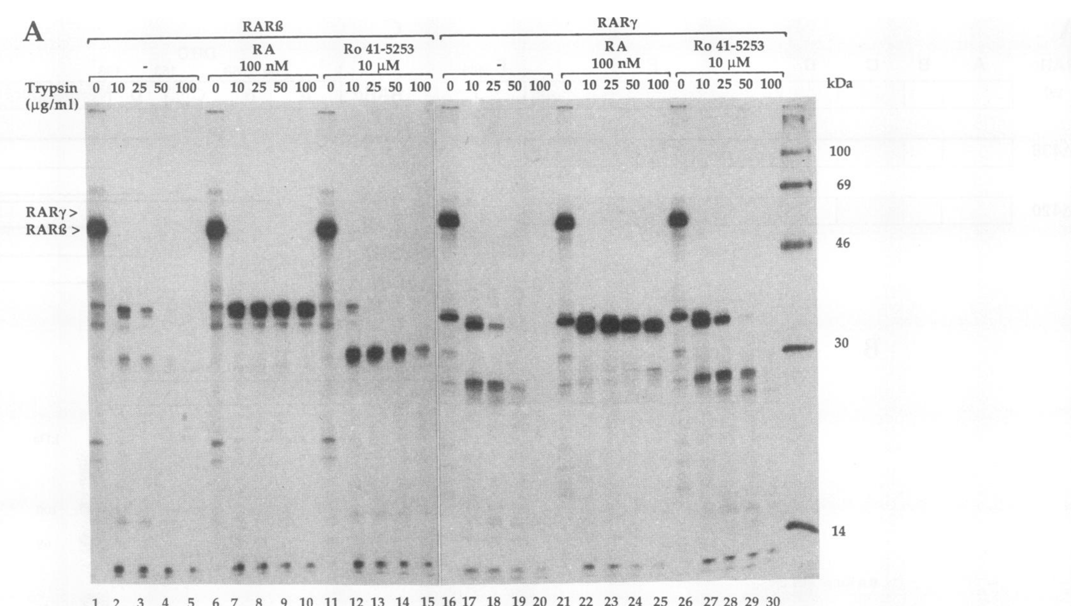
Protease	Retinoid	Molecular mass (kDa) of fragment from:		
		RARα wt	RARα-Δ438	RARα-Δ420
Trypsin	RA	30	30	29
	Ro 41-5253	25	25	24
Elastase	RA	30, 41	30, 41	29, 40
	Ro 41-5253	26	25	24
Chymotrypsin	RA	28	28	27
	Ro 41-5253	28	27	26

resistance to proteolysis of a 27-kDa fragment (Fig. 6A, lanes 26 to 30).

Chymotrypsin digestion of RXRα leads to the proteolytic pattern shown in Fig. 6B (lanes 1 to 6). A 23-kDa fragment became resistant to proteolysis when RXRα was incubated with its ligand 9-*cis* RA (1 μM) (Fig. 6B, lanes 7 to 12). Trypsin digestion of RXRα results in a digestion pattern yielding a remarkably resistant 32-kDa fragment, interestingly, both in the absence and in the presence of 9-*cis* RA (Fig. 6C).

Tryptic and chymotryptic digestion of a mixture of RARα, RXRα, and βRARE leads in the presence of 9-*cis* RA or Ro 41-5253 to a simple addition of the proteolytic patterns of both of the receptors when RARα and RXRα are radioactively labeled (compare Fig. 7 with Fig. 3, Fig. 4B, and Fig. 6B and C). In accordance with our binding data (no binding of Ro 41-5253 to RXRα at 10 μM), 10 μM Ro 41-5253 shows no influence on the tryptic and chymotryptic RXRα digestion patterns.

The VDR is nearly completely digested to peptides smaller than 14 kDa at trypsin concentrations higher than 10 μg/ml (Fig. 6D, lanes 4 to 6). The proteolysis of several fragments



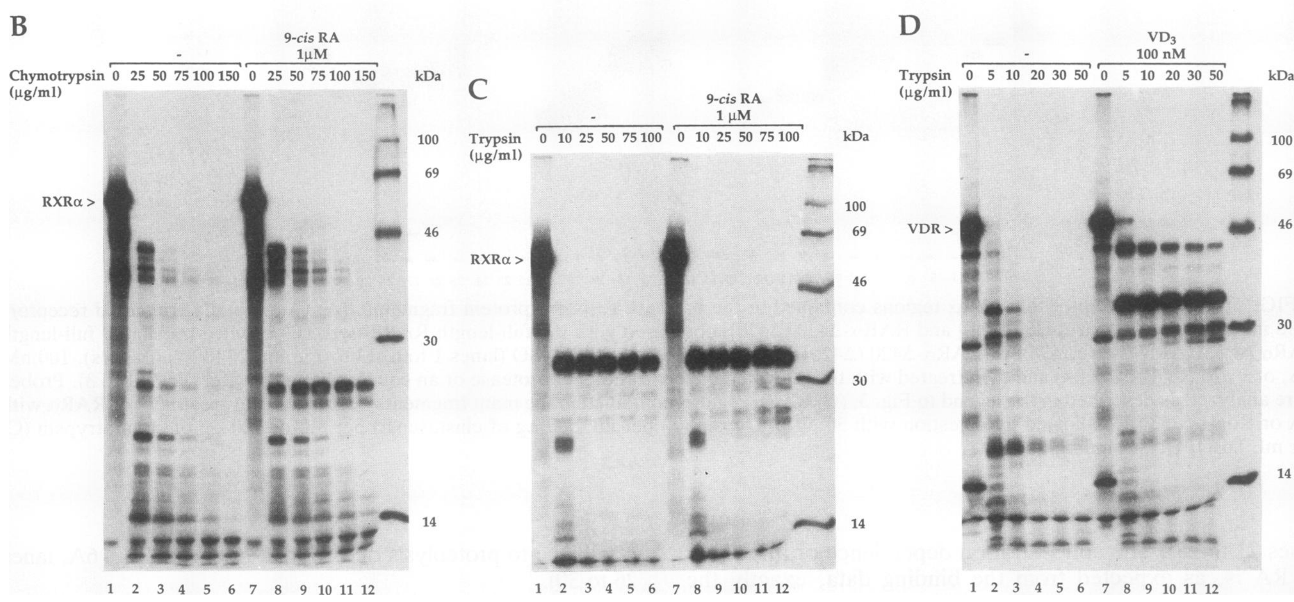


FIG. 6. Ligand-dependent proteolytic patterns of RARβ, RARγ, RXRα, and VDR. In vitro-synthesized retinoid receptors were preincubated with DMSO alone (lanes 1 to 5 and 16 to 20 in panel A and lanes 1 to 5 in panels B and C) (dashes) or the indicated concentrations of retinoids. In vitro-translated VDR was preincubated with ethanol alone (lanes 1 to 6 in panel D) (dash) or 100 nM 1,25-dihydroxyvitamin D_3 (VD_3). The indicated concentrations of proteases or an equal volume of water was added. Samples were analyzed as described in the legend to Fig. 3. (A) Trypsin digestion of RARβ and RARγ; (B) chymotrypsin digestion patterns of RXRα; (C) trypsin digestion of RXRα; (D) trypsin digestion patterns of VDR.

at trypsin concentrations of 5 and 10 µg/ml (Fig. 6D, lanes 2 and 3) was greatly diminished when the VDR was incubated with 100 nM 1,25-dihydroxyvitamin D_3 (Fig. 6D, lanes 7 to 12). The molecular mass of the main resistant fragment was found to be 33 kDa.

Retinoids and 1,25-dihydroxyvitamin D_3 do not inhibit the activity of the proteases. This was shown by proteolytic digestion of VDR in the presence of retinoids and by digestion of RARα in the presence of 1,25-dihydroxyvitamin D_3. In both cases, the compounds did not influence the digestion pattern (data not shown).

DISCUSSION

In addition to the retinoid Ro 41-5253 recently described (3), we have identified two further compounds, Ro 46-5471 and Ro 46-8515, as RARα-selective antagonists (Table 1 and Fig. 1). All three of these antagonistic retinoids contain a heptoxy side chain. Ro 46-5468, an analog of Ro 41-5253, contains only a methoxy side chain and shows very low binding affinity to RARα. The *Z* isomer of Ro 41-5253, compound Ro 41-4452, also shows low binding affinity. Therefore, a long side chain at the indicated position and an

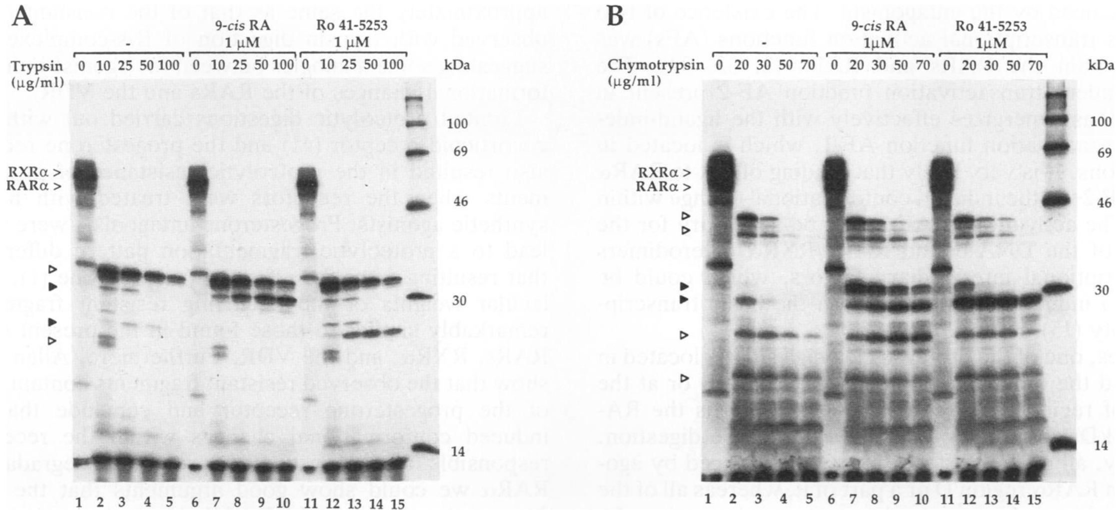


FIG. 7. Effect of heterodimerization and DNA binding on the digestion patterns of RARα and RXRα. A mixture of in vitro-translated RARα and RXRα was preincubated in the presence of 2 ng of βRARE per μl with tracer or retinoids and digested with the indicated concentrations of trypsin (A) or chymotrypsin (B). Samples were analyzed as described in the legend to Fig. 3. Both receptors are radioactively labeled. The resistant fragments derived from RARα are marked by solid triangles, and the resistant fragments derived from RXRα are indicated by open triangles.

E conformation of the molecule seem to be important for RARα binding of antagonistic retinoids of this type.

Receptor-binding data, competition experiments, and the structural similarity of RARα antagonists and agonists suggest that the antagonists replace the RA bound to RARα. Our investigations addressed why the antagonist-complexed RARα does not activate gene transcription. Recently it was shown that the antiestrogen ICI 164,384 inhibits DNA binding of the estrogen receptor by blocking the receptor dimerization (13). We demonstrated that the specific, high-affinity binding of RARα/RXRα heterodimers to βRARE is not influenced by the RARα antagonists or RA (Fig. 2). Therefore, the different effects of agonists and antagonists on gene transactivation activity must be the consequence of events after heterodimerization of RARα with RXRs and binding to the response element.

We report here that the presence of the agonistic ligand RA changes the proteolytic degradation pattern of RARα such that a 30-kDa fragment became resistant to tryptic digestion (Fig. 3A). These results are consistent with those obtained by Allan et al. (1). These authors identified the same fragment after tryptic digestion of in vitro-translated RARα as we did when the receptor was preincubated with RA. The fragment was not detectable following digestion of untreated RARα. We observed similar RARα digestion patterns with various other proteases, such as chymotrypsin, elastase (Fig. 4), dispase, and endoproteinase GluC. This suggests a ligand-induced conformational change or a stabilization of a particular conformation of the receptor. We also found that the degree of tryptic resistance of the fragment is dependent on the concentration of RA (Fig. 3A) and that complexation of RARα with several other agonistic retinoids induces a digestion pattern analogous to that obtained with RA. The data obtained reveal that the concentration-dependent ability of retinoids to induce a digestion pattern characteristic of that induced by an agonist is in accordance with their ability to bind to RARα as well as their activity in the RARα transactivation assay (Table 1).

Antagonist binding to RARα resulted in a pattern of proteolytic fragmentation different from that induced by agonist binding, regardless of the protease used. For example, trypsin digestion of antagonist-bound RARα resulted in the proteolytic resistance of a 25-kDa fragment, whereas tryptic digestion of agonist-bound RARα resulted in a resistant 30-kDa fragment (Fig. 3). This suggests that antagonist binding results in a structural alteration in RARα different from that induced by the agonists. The antagonists compete with RA and are able to change the digestion pattern from the one characteristic of agonists to one that is characteristic of antagonists (Fig. 3B). This occurs in a concentration-dependent manner, which is correlated to the RARα-binding activity of the antagonists and to their antagonistic activity as determined in a competitive RARα transactivation assay. The proposed agonist- and antagonist-induced conformational changes in RARα seem to be reversible.

The RARα digestion patterns induced by agonists are identical in the absence and the presence of the heterodimerization partner RXRα and βRARE. This is also the case for the antagonist-induced RARα digestion pattern. The digestion pattern of 9-*cis* RA-liganded RXRα is also the same in the absence of RARα and βRARE as it is in their presence (compare Fig. 7 with Fig. 3, Fig. 4B, and Fig. 6B and C). These findings show that the proposed retinoid-induced conformational changes in RARα and RXRα are independent of heterodimerization and DNA binding. The results suggest furthermore that under the present conditions, the heterodimerization and the binding of the RARα/RXRα heterodimer to βRARE induce no detectable conformational changes in RARα or RXRα.

All of the agonist- as well as antagonist-induced fragments resistant to proteolytic degradation contain the RARα region E, the LBD (Fig. 5C). Furthermore, the fragments generated by complexation of RARα with agonists are much more resistant to digestion and thus resist higher protease concentrations, as high as 500 μg/ml, than do those generated by complexation with antagonists (data not shown). These findings indicate that agonist binding to RARα most probably induces a more compact folding of the chains of the LBD

than that induced by the antagonists. The existence of two autonomous transcriptional activation functions (AFs) was identified within the RARs and RXRs (2, 33, 34). The ligand-dependent transactivation function AF-2 present in the DE regions synergizes effectively with the ligand-independent transactivation function AF-1, which is located in the AB regions. It is very likely that binding of RA to RARα activates AF-2 via the induced conformational change within the LBD. The activation of AF-2 may be necessary for the interaction of the DNA-bound RARα/RXRα heterodimers with transcriptional intermediary factors, which could be necessary to induce the AF effects on the basic transcriptional activity (15).

In all cases, one of the protease cleavage sites is located in region F and the other one is located in region D or at the beginning of region E (Fig. 5C). An exception is the RA-induced 41-kDa fragment obtained with elastase digestion. Interestingly, all of the resistant fragments induced by agonists contain RARα region D or a part of it, whereas all of the antagonist-induced fragments do not contain region D. Therefore, the suggested agonist-induced conformational change in RARα appears to implicate structural alterations in region D, which could be necessary for the activation of AF-2. The different antagonist-induced conformational change seems not to involve region D, which may be the reason for the inactivity of antagonist-complexed RARα. In the case of RARα digestion with elastase, RA treatment resulted not only in resistant fragments of approximately 30 kDa but also in a resistant 41-kDa fragment containing the complete regions B, C, D, and E (Fig. 4A and Fig. 5C). The diminished proteolysis of the 41-kDa fragment induced by RA could be an indication that agonist-induced conformational changes occur not only in regions E and D but also within regions C and B. This structural alteration could influence further transcriptional regulation functions located in these regions.

In the present communication, we show that RA treatment of RARβ and RARγ leads to a trypsin digestion pattern qualitatively similar to that observed with RARα. As expected from the low binding affinity, even with very high concentrations of Ro 41-5253, only a partial induction of a RARβ or RARγ digestion pattern similar to that induced by antagonist treatment of RARα could be seen (Table 1 and Fig. 6A). The observed concentration dependency of the RA- and Ro 41-5253-induced effects on the RARβ and RARγ digestion patterns (data not shown) is in close correlation with the RARβ and RARγ binding data. All our results, taken together, suggest that binding of RA and Ro 41-5253 induces conformational changes within RARβ and RARγ, which are probably similar to that induced in RARα.

We could show that RXRα probably also undergoes a structural alteration after binding its natural ligand 9-*cis* RA. This is suggested by the appearance of a chymotrypsin-resistant 23-kDa fragment of RXRα in the presence of 9-*cis* RA (Fig. 6B). In contrast to the RARs, a trypsin-resistant 32-kDa fragment of RXRα was observed also in the absence of 9-*cis* RA (Fig. 6C). We can speculate that this greater resistance to proteolysis could lead to an intracellular half-life of RXRα longer than those of the RARs. If so, the concentration of the RARs may be more sensitively controlled than that of the more general coregulator RXRα.

Proteolytic fragmentation patterns similar to those of the RARs were observed when the VDR was digested with trypsin. Complexation with 1,25-dihydroxyvitamin D_3 resulted in a greatly enhanced resistance to proteolysis of a 33-kDa protein fragment (Fig. 6D). The molecular weight is approximately the same as that of the resistant fragments observed with trypsin digestion of RA-complexed RARs, suggesting some analogies between the agonist-induced conformational changes of the RARs and the VDR.

Limited proteolytic digestions carried out with the glucocorticoid receptor (41) and the progesterone receptor (1) also resulted in the proteolytic resistance of protein fragments when the receptors were treated with natural or synthetic agonists. Progesterone antagonists were shown to lead to a proteolytic fragmentation pattern different from that resulting from the effect of progesterone (1). The molecular weights of the occurring resistant fragments are remarkably similar to those found in the present study for RARs, RXRα, and the VDR. Furthermore, Allan et al. (1) show that the observed resistant fragments contain the LBD of the progesterone receptor and conclude that ligand-induced conformational changes within the receptor are responsible for the resistance to proteolytic degradation. For RARα we could show good arguments that the resistant fragments contain the LBD of the receptor, and we assume that this is also the case for RARβ, RARγ, RXRα, and the VDR. Therefore, it seems to be very likely that ligand-induced conformational changes within the LBD, resulting in a more compact folding, are a common mechanism of hormone action within the nuclear steroid-thyroid-vitamin D_3-retinoid receptor superfamily. Our results further suggest that conformational changes within regions D and E play an important role both in the activation of RARα by agonists and in their neutralization by antagonists. The conformational change induced by RA in RARα seems not to be necessary to stimulate the dimerization with RXRs or the binding of the heterodimer to the response element, but it is most probably required for the subsequent transcriptional activation of target genes. The antagonists compete with agonists for binding to RARα and appear to induce a different structural alteration. The resulting RARα conformation seems to allow RARα/RXRα binding to DNA but not the subsequent transactivation of target genes.

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