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Heterodimeric Interaction between Retinoid X Receptor α and Orphan Nuclear Receptor OR1 Reveals Dimerization-Induced Activation as a Novel Mechanism of Nuclear Receptor Activation

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OR1 is a member of the steroid/thyroid hormone nuclear receptor superfamily which has been described to mediate transcriptional responses to retinoids and oxysterols. On a DR4 response element, an OR1 heterodimer with the nuclear receptor retinoid X receptor α (RXRα) has been described to convey transcriptional activation in both the absence and presence of the RXR ligand 9-*cis* retinoic acid, the mechanisms of which have remained unclear. Here, we dissect the effects of RXRα and OR1 ligand-binding domain interaction on transcriptional regulation and the role of the respective carboxy-terminal activation domains (AF-2s) in the absence and presence of the RXR ligand, employing chimeras of the nuclear receptors containing the heterologous GAL4 DNA-binding domain as well as natural receptors. The results show that the interaction of the RXR and OR1 ligand-binding domains unleashes a transcription activation potential that is mainly dependent on the AF-2 of OR1, indicating that interaction with RXR activates OR1. This defines dimerization-induced activation as a novel function of heterodimeric interaction and mechanism of receptor activation not previously described for nuclear receptors. Moreover, we present evidence that activation of OR1 occurs by a conformational change induced upon heterodimerization with RXR.

Members of the steroid/thyroid hormone nuclear receptor superfamily are complex regulators of transcription that are themselves regulated in a number of different ways. Their designation as receptors goes back to the identification of the first family members which, in a search for the conveyors of specific hormone-dependent activities, were isolated on the basis of their hormone binding properties (19). However, a shift in techniques from biochemical to molecular genetic approaches has led to the identification of a large number of so-called orphan receptors for which potential ligands remain to be found (24, 46, 47). The members of the steroid receptor branch of the superfamily usually bind to DNA as homodimers on palindromic response elements, but the situation in the thyroid hormone/retinoid acid receptor (TR/RAR) branch of the family is much more complex. While some members of this branch can bind to DNA as monomers or homodimers, a large number of them exert their physiological function as heterodimers with the retinoid X receptor (RXR), adding further degrees of regulative complexity with respect to specific DNA interaction and relative orientation, heterodimeric interaction, and receptor-specific activation. The typical response element for nuclear receptor heterodimers consists of a direct repeat of a hexanucleotide sequence motif (consensus AGGTCA), with the spacing between the repeats playing an important role in recognition specificity.

Members of the nuclear receptor superfamily typically display a characteristic domain structure. A well-conserved DNA-binding domain (DBD) is preceded by a highly variable amino terminus that often contains an activation function. Carboxy terminally linked to the DBD by a flexible so-called hinge region is a moderately conserved domain which possesses both ligand-binding and dimerization functions, which have been shown to be structurally overlapping.

Prominent among the mechanisms that regulate the activities of nuclear receptor superfamily members is ligand binding. In the TR/RAR branch it is assumed to relieve a repression function of the ligand-binding domain (LBD) and/or reveal an activation domain at the very carboxy terminus of the LBD, commonly designated AF-2 (or τ4) (3, 4, 18), which has therefore been described as a ligand-dependent activation domain. Based on structural analysis, ligand-binding is assumed to alter the conformation of the LBD, bringing the AF-2 in a position where it can form part of an activating surface (49, 52, 59).

Recently, it has been demonstrated that the transregulation properties of nuclear receptor heterodimers are not simply determined by the individual receptors but strongly depend on the complex properties of the specific DNA-bound heterodimer. Thus, the heterodimeric interaction itself as well as the activation status of the heterodimerization partner can affect ligand responsiveness (13, 14, 20, 32, 61), which in turn can be dependent on the relative orientation of the receptors (32) and the exact structure of the DNA element bound (35, 60).

The nuclear orphan receptor OR1 (57) (also described as UR [56], NER [55], or RIP15 [54]) and its close relative RLD-1/LXR (2, 60) have previously been reported to act as helpers (57) of RXR in a heterodimer mainly on a DR4-type response element (direct repeat of AGGTCA spaced by 4 nucleotides), conferring constitutive transcriptional activation and/or enhanced response to the RXR ligand 9-*cis* retinoic acid (9cRA). However, since transactivation by the individual receptors could not be tested in that system, it remained unclear if a mechanism other than recruitment of RXR or OR1/RLD-1 to the promoter region was responsible for the observed constitutive transactivation. We therefore decided to analyze the

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object of our studies, the nuclear orphan receptor OR1, as a chimeric protein containing the heterologous GAL4 DNA-binding domain that allows its targeting to DNA independent of RXR. In transient-transfection experiments, we then examined the properties of this GAL4-OR1 chimera in the absence or presence of cotransfected RXR as well as in comparison to other nuclear receptors and the role of its putative AF-2 domain in transactivation.

When targeted to DNA by the GAL4-DBD, the LBD of neither OR1 nor RXR conferred transcriptional activation higher than GAL4-DBD background level. However, the combination of the two LBDs led to the formation of a transactivating heterodimeric complex. Moreover, experiments employing carboxy-terminal deletion variants of OR1 and RXR, respectively, revealed that the AF-2 of OR1 only is essential for the activity of the complex, while deletion of the AF-2 of RXR had only a minor effect apart from abolition of 9cRA inducibility of the complex. The same difference was observed when the natural receptors were used on a DR4 response element. We conclude that constitutive transcriptional activation by the OR1/RXR complex requires a modulatory heterodimeric interaction, leading to dimerization-induced activation of OR1, the occurrence of which might be response element dependent. We propose that OR1/RXR heterodimeric interaction defines a novel mechanism of nuclear receptor activation and suggest that activation occurs by a change in LBD conformation similar to that otherwise obtained by ligand binding.

MATERIALS AND METHODS

Plasmids. GAL4-DBD/receptor-LBD fusions were constructed by PCR-based amplification of the respective fragments and introduction of convenient restriction sites and subsequent assembly; all PCR-derived sequence parts were either replaced by template material or confirmed by sequencing. The GAL4-DBD was amino terminally joined to a sequence including the hemagglutinin (HA) epitope (MSSPTS*YPYDVPDYA*SLTSEL); the individually expressed GAL4-DBD terminated in the sequence ML. No additional amino acids were introduced at the DBD/LBD junction for GAL4-OR1 or GAL4-RLD. For GAL4-RXR, a proline residue was inserted, and 4 amino acids (PEFH) were inserted for GAL4-TR. The GAL4-COUP junction contains a polylinker encoding 17 additional amino acids. Stop codons were included after positions 457 (RXR), 439 (OR1), and 438 (RLD-1) for generation of the corresponding AF-2 mutations. OR1-DL and ORΔ-DL consisted of the above-mentioned HA epitope-containing sequence fused amino terminally to amino acids 76 to 446 and amino acids 76 to 417 of OR1, respectively. The sequence MSSPTSGMP preceded amino acids 203 to 467 and amino acids 203 to 457 of rat RXRα (rRXRα) for RXR-L and RXRΔ-L, respectively. N-GAL4-RXR was generated from GAL4-RXR by inserting amino acids 2 to 139 of rRXRα into the *Hpa*I/*Spe*I sites (amino acid sequence LTS) preceding the GAL4-DBD. UASx4-TK-LUC has been described previously (20).

Wild-type rRXRα was expressed from a *Pst*I fragment (21) in pCMV5; the pCMV5-OR1 clone contains a modified OR1 open reading frame which has an HA epitope encoding insertion in the *Spe*I site near the start codon. AF-2 deletions are as described above for GAL4 fusion constructs. The 2xDR4-TK-LUC reporter contains an insertion of two DR4 motifs (GGGTCACGAAAGGTCA) into the *Hin*dIII site of UASx4-TK-LUC, thus replacing the UAS elements.

In in vitro translation, proteins were expressed from the modified vector pGEM-3Z. OR1 was as described above; RXR was also tagged by a sequence including the HA epitope (SSPTS*YPYDVPDYA*SLTS) inserted after the start codon. The OR1-LBD construct encoded amino acids 144 to 446 of OR1 preceded by 6 amino acids (MSSPTS).

Transfection and preparation of extracts. COS7 cells were split onto 3-cm-diameter dishes to achieve approximate confluency at the time of harvest. Dulbecco's modified Eagle medium (with sodium pyruvate and 1 g of glucose per liter) containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in 5% CO_2 was used. When used, serum substitute (SRC 3000; Tissue Culture Services, Botolph Claydon, Buckingham, United Kingdom) was also at 10%. After 24 h, cells were transfected with DOTAP {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate} (Boehringer Mannheim) according to the instructions provided by the supplier; transfection mixes contained 1.2 μg of corresponding constructs in expression vector pCMV-5 and 3 μg of the respective TK-LUC reporter. Later (after 16 h), the medium was exchanged for fresh medium with or without inducers. 9cRA was used at 0.1 μM, triiodothyronine (T3) was used at a final concentration of 1 μM, and corresponding controls received equal amounts of solvent. Cells were harvested at 22 h after transfection by removal of the culture media and resuspension in 40 mM Tris–10 mM EDTA–150 mM NaCl at pH 7.9. One third of the cell harvest was spun down at 4,000 rpm in an Eppendorf centrifuge, the cell pellet was lysed in 100 μl of lysis buffer (25 mM Tris-acetate, 2 mM dithiothreitol, 1.5 mM EDTA, 10% glycerol, 1% Triton X-100), and luciferase activity was determined by standard procedures. The remaining cells were washed once in phosphate-buffered saline (10 mM phosphate buffer, 137 mM NaCl, 2.7 mM KCl at pH 7.4). To prepare whole cell extracts, cells were spun down, the supernatant was removed, and the cell pellet was frozen in liquid nitrogen. After thawing, cells were resuspended in 30 μl of whole-cell extract buffer (10 mM HEPES [pH 7.9], 400 mM NaCl, 0.1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, antipain [5 μg/ml], and 0.5 μg each of pepstatin, leupeptin, and aprotinin per ml, which were added just before use).

TABLE 1. Percent sequence identity within groups of paralogs of nuclear receptors in distinct protein regions

Region	% Sequence identity within group (paralogs):				
	rOR1 and rRLD1	mRXR (α, β, and γ)	mRAR (α1, β1, and γ1)	rTR (α1 and β1)	mPPAR (α, β, and γ)
Full length	61	63–68	69–78	75	53–60
DBD	71	91–96	94–96	87	83–86
Hinge[a]	58	50–69	70–76	74	44–54
LBD[a]	79	87–92	74–83	87	69–71
AF-2[b]	100	86–94	77–97	100	65–77

[a] LBD defined as starting with the Ti domain (37).
[b] As described in reference 4.

Electrophoretic mobility shift assay. The oligonucleotide probe (AGCTTACTAGTTCGGAGGACAGTCCTCCGTCTAGAGCT/CTAGACGGAGGACTGTCCTCCGAACTAGTA for the GAL4 chimeras; TCGATCAGGTCATTTCAGGTCAGAG/TCGACTCTGACCTGAAATGACCTGA for TRα+RXRα) was labelled with DNA polymerase Klenow fragment and [α-^{32}P]dCTP and purified on Sephadex G-50 Nick columns (Pharmacia Biotech). Binding mixes contained 2 μl of in vitro-translated protein (TNTT7 coupled transcription-translation system; Promega) (the heterodimerization of the GAL4 chimeras required cotranslation) or 3 μl of whole-cell extract as well as the following additives (final concentrations are given): 50 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.5), bovine serum albumin (200 μg/ml), 0.2 M spermidine, 8% glycerol, poly(dI-dC) · poly(dI-dC) (100 μg/ml), and salmon sperm DNA (75 μg/ml); additionally, 20 μM $ZnCl_2$ was used for UAS binding and 1 mM EDTA, 1 mM dithiothreitol, and 0.01% Triton X-100 were used for DR4 binding. Ligands or solvent were added where indicated (1/100 volume 9cRA in dimethyl sulfoxide to yield 100 μM, 1/40 volume T3 in phosphate-buffered saline to yield 25 μM). After a 20-min preincubation on ice, approximately 50 fmol (100,000 cpm) of labelled probe was added. Gels (4% polyacrylamide) were prepared and run in 0.25× TBE (22.5 mM Tris-borate, 0.5 mM EDTA).

Limited protease digestion. For in vitro translation, the Promega TNT T7 Quick coupled transcription-translation system was employed. Reticulocyte lysate containing ^{35}S-labelled OR1 was combined with either unprogrammed lysate or lysate containing excess amounts of unlabelled RXR and preincubated for 10 min at room temperature. To 5-μl aliquots of these mixtures, 1-μl aliquots of water or different amounts of trypsin in water were added. After 10 min at room temperature, proteolysis was stopped by the addition of an equal volume of 2× sample buffer containing 0.2 M EDTA. After boiling for 5 min, samples were run on a sodium dodecyl sulfate–12.5% polyacrylamide gel. The dried gels were autoradiographed.

RESULTS

The nuclear receptors OR1 and RLD-1 are paralogs and possess an AF-2 motif. Amino acid sequence alignment of rat OR1 with other members of the steroid/nuclear receptor family illustrates that OR1 is most closely related to the recently described RLD-1/LXR from rat and human (2, 60), respectively. Similarities are approximately equally strong as within the nuclear receptor paralog groups of, e.g., peroxisome proliferator-activated receptors (PPARs) and RXRs (Table 1), indicating that even OR1 and RLD-1 can be regarded as paralogs (subtypes). Interestingly, in contrast to these other groups of paralogs, differences between OR1 and RLD-1/LXR are strikingly high in the DBD region, suggesting that functional

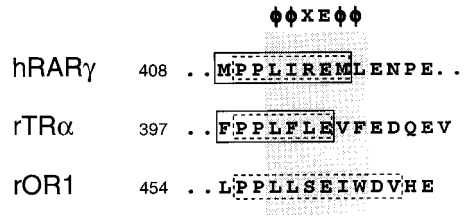

FIG. 1. Predicted and experimentally determined formation of an α-helical structure in the region surrounding the AF-2 motif (16) (displayed on top and shaded grey). Φ, hydrophobic amino acid residue. Closed boxes enclose regions forming α-helical structures as determined by crystal structure analysis (52, 59). Dotted boxes enclose regions forming α-helical regions according to the Chou-Fasman algorithm as determined by the computer program Protean (DNASTAR). Numbers indicate the sequence positions of the first amino acids displayed; dots indicate continuations of the sequences.

differences can be expected mainly with respect to specific receptor-DNA interaction. Also, apart from a short region directly N terminal to the core DBD, their amino termini display no significant sequence similarity, which is common among the nonsteroid branch of the nuclear receptor superfamily.

Many nuclear receptors have been described to contain a characteristic motif at the carboxy terminal end of the E domain, referred to as AF-2, which has been described to be critical for ligand-dependent transactivation (4, 18) and by structural analysis has been shown to adopt a helical conformation (6, 52, 59). The OR1/RLD-1 amino acid sequence contains a typical AF-2 core motif (16), and an appropriate helical structure is predicted by a corresponding computer analysis (Fig. 1).

OR1 does not possess a constitutive activation function. OR1 and RLD-1/LXR have previously been shown to heterodimerize with RXR on a DR4 element, leading to transcriptional activation that under some conditions could be enhanced by 9cRA (2, 54, 56, 57, 60). However, it could not be concluded if transactivation by OR1 was merely due to recruitment of RXR to the DNA and possible displacement of other nuclear receptor complexes or if a constitutive activation function of OR1 itself or its mode of interaction with RXR contributed to the transactivation function. Moreover, it remained unclear why inducibility of the OR1/RXR heterodimer was observed in some experiments but not in others, although for LXR this was shown to be at least in part dependent on the response element used (60). Therefore, chimeric expression constructs were made that code for the heterologous DBD of the yeast GAL4 protein (amino acids 1 to 147 [7]) fused to the complete hinge and LBD region of different nuclear receptors (rOR1, rRLD-1, rRXRα, human [hTRα1], hARP-1/COUP-TFII [cf. Fig. 2B]).

Constructs were transiently transfected into COS7 cells together with a reporter containing multiple GAL4-response (UAS) elements, and reporter activity was determined. In parallel, specific GAL4 response element-binding activity of whole-cell extracts of transfected cells was assayed as a control for expression levels of the chimeric proteins. Intolerably large differences in expression levels were observed in initial experiments (data not shown) but were overcome for the critical experiments by reducing the incubation time after transfection to 22 h (Fig. 2A). By using a UASx4-TK-LUC reporter, GAL4-RXRα and GAL4-TRα were shown to be inducible by their respective ligands, whereas unliganded GAL4-TRα or GAL4-RXRα, GAL4-COUP-TFII, and GAL4-OR1 and GAL4-RLD-1 were transcriptionally inactive or yielded a weak repression effect (Fig. 2B). Hence, the LBD of neither RXRα, OR1, nor RLD-1 displayed any intrinsic transactivation potential on its own. An OR1-GAL4 chimeric construct including the complete amino terminus of OR1 was equally inactive (data not shown).

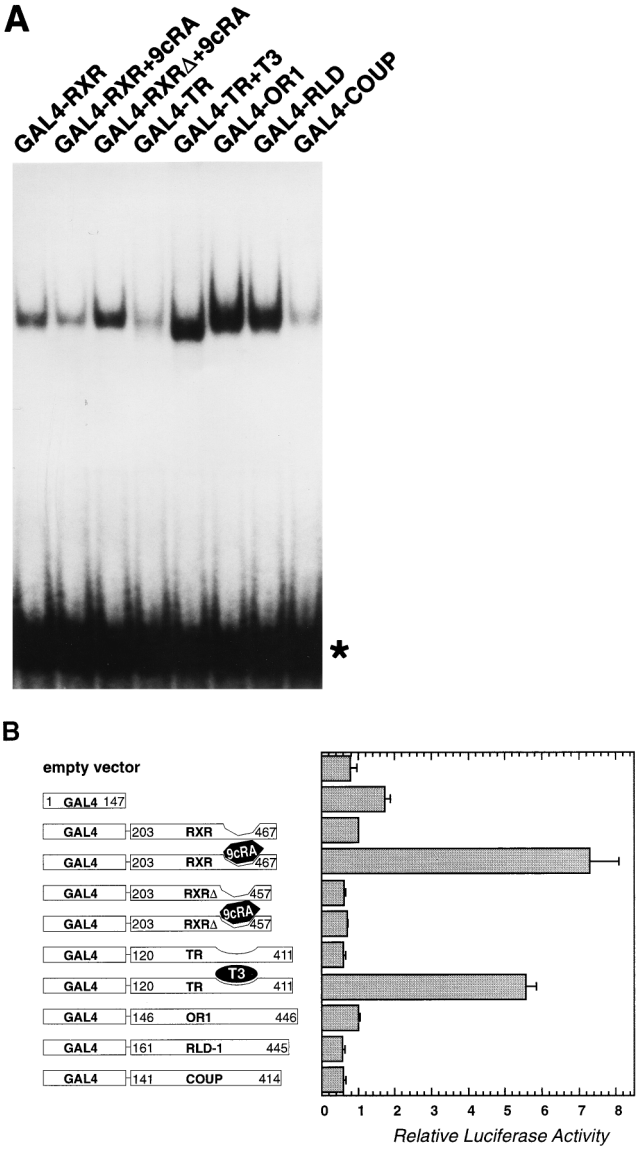

FIG. 2. OR1 does not transactivate constitutively. COS7 cells were transfected with plasmids encoding fusion proteins between GAL4-DBD and different nuclear receptor LBDs along with the reporter GAL4x4-TK-LUC and cultured in the absence or presence of the ligands indicated until 22 h after transfection. (A) Electrophoretic mobility shift assay performed with whole-cell extracts of transfected cells on a probe containing the GAL4 response element. The free probe is indicated by an asterisk. The additions of ligands listed above the lanes refer to culture conditions. (B) Luciferase activity of transfected cells. Relative luciferase activities are given as means from at least three different experiments; bars represent standard errors. Activities were standardized for that obtained for GAL4-RXR, which was arbitrarily set at 1. Delineating amino acid positions of the fused protein parts are indicated.

GAL4 chimeras of RXR- and OR1-LBDs reveal dimerization-induced activation. As a next step, we decided to test the transactivation potential of a GAL4-OR1/GAL4-RXR chimeric heterodimer in comparison to other heterodimeric combinations. Electrophoretic mobility shift analysis using whole-

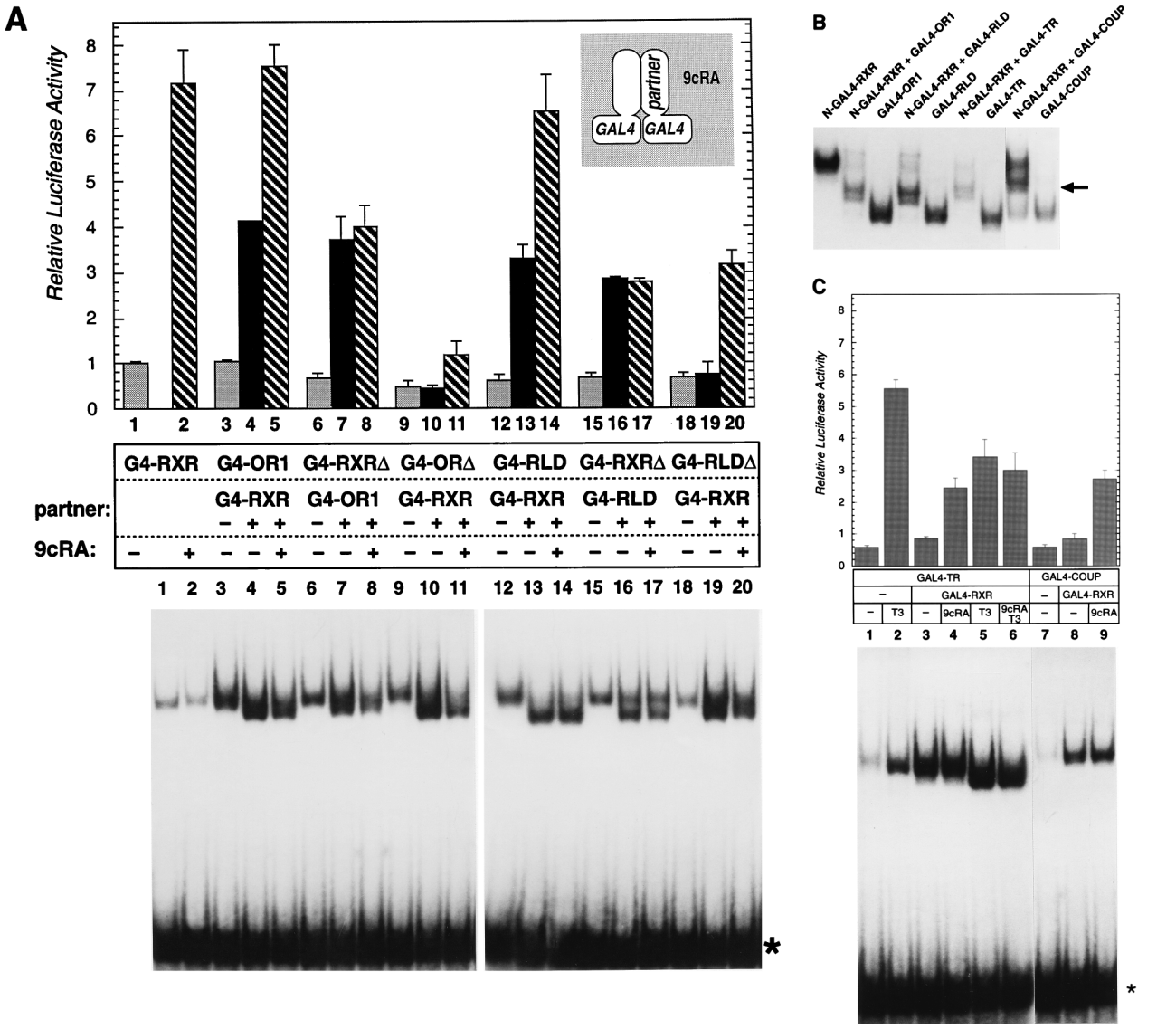

FIG. 3. Effect of coexpression of GAL4 chimeric proteins. Transfections, electrophoretic mobility shift assays from whole-cell extracts, and evaluations of luciferase activities were performed as described in the legend to Fig. 2. (A) GAL4 (G4) chimeras of RXR- and OR1-LBDs reveal dimerization-induced activation. The upper panel shows luciferase activity obtained from cells transfected with combinations of GAL4 chimeric constructs. Bars represent standard errors. The inset gives a schematic display of the experimental setup. The lower panel shows electrophoretic mobility shift assays from whole-cell extracts of a representative experiment. An asterisk marks the free probe. Although the expression of GAL4-RXR in the experiment shown is comparatively low, control experiments show that the dimerization-induced activation inferred is not an effect of increased expression levels upon cotransfection (data not shown). (B) GAL4-OR1, GAL4-RLD-1, GAL4-TR, and GAL4-COUP form heterodimers with N-GAL4-RXR as demonstrated by the formation of a band of intermediate mobility (arrow). Electrophoretic mobility shift assays were performed with in vitro-translated protein. The double band observed for combinations of N-GAL4-RXR is due to an efficient internal translation start site of that construct. (C) Dimerization-induced activation with GAL4-RXR is not observed for TRα and COUP chimeras. The upper and lower panels are as described for panel A above.

cell extracts of transfected cells (Fig. 3A, lower panel) demonstrated comparable expression levels for the different combinations of chimeric constructs. For all chimeras, heterodimer formation with GAL4-RXR was confirmed in a separate experiment employing in vitro-translated GAL4 chimeras of altered lengths (Fig. 3B). For OR1, RLD-1, and TR, heterodimers with RXR were formed in clear preference over homodimers.

Intriguingly, in the transactivation assay, heterodimers of GAL4-RXR with GAL4-OR1 or GAL4-RLD gave rise to transcriptional activation independent of added ligand (Fig. 3A, upper panel, lanes 4 and 13). This was also observed when cultivation media were prepared with serum supplement, indicating that it was not a component specifically present in fetal calf serum that acted as an activator (data not shown). In accordance with the results observed for the wild-type proteins (57, 60), a superinduction was obtained by addition of the RXR ligand 9cRA (lanes 5 and 14). In clear contrast (Fig. 3C, upper panel, lane 3), the GAL4-RXR/GAL4-TR heterodimer was transcriptionally inactive. Transcription by the complex could be activated by addition of T3 and/or 9cRA, with no synergism observed when both ligands were added (lanes 4 to 6). As expected (15), cotransfection of GAL4-RXR with GAL4-COUP did not result in increased transactivation (lane

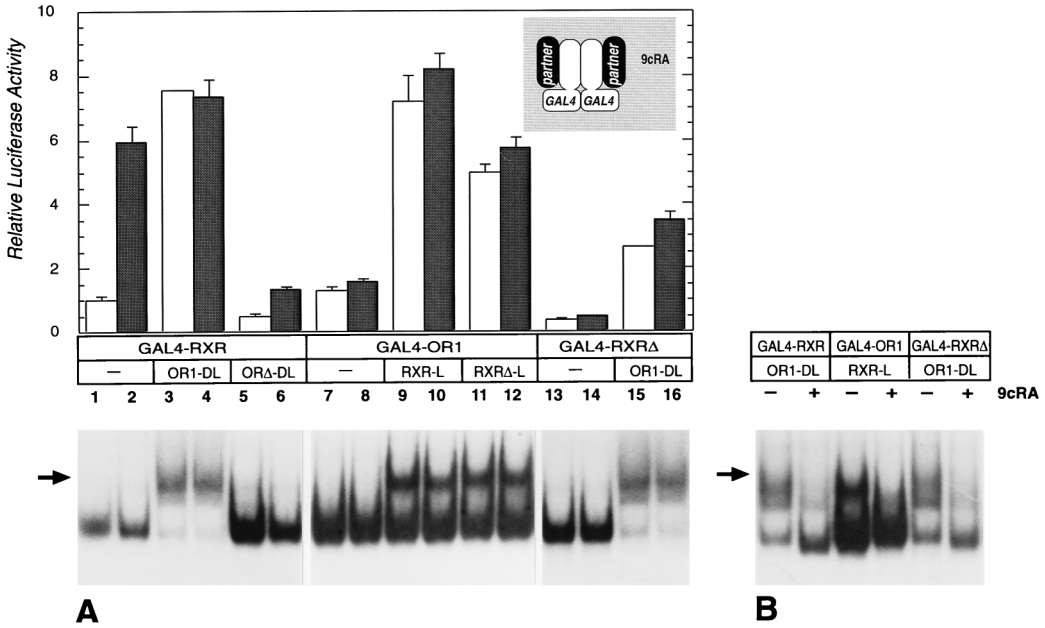

FIG. 4. Dimerization-induced activation by higher-order RXR/OR1 chimeric complexes (arrow). Transfections, electrophoretic mobility shift assays from whole-cell extracts, and evaluations of luciferase activities were performed as described in the legend to Fig. 2. (A) Coexpression of an RXR- or OR1-GAL4 chimera with the corresponding partner LBD confers transcriptional activation. The upper and lower panels are as described in the legend to Fig. 3A. Bar color indicates the addition of vehicle (dimethyl sulfoxide [white bars] or 9cRA [black bars]) during culture. Activities were standardized for that obtained for GAL4-RXR/OR1-DL coexpression. The inset gives a schematic display of the experimental setup. (B) The higher-order OR1/RXR chimeric complexes observed in electrophoretic mobility shift assays are destabilized by addition of 9cRA but not vehicle alone (−).

8). For the COUP and TR chimeras, expression levels were somewhat uneven (Fig. 3C, lower panel) but expression was increased for those samples from which lack of dimerization-induced activation was concluded.

The experiments described above demonstrate that heterodimeric interaction of the RXR-LBD with the LBD of OR1 or its paralog RLD-1 unleashes a transactivation potential which is not obtained by recruiting the individual LBDs to DNA. The effect is observed for RXR interaction with OR1 or RLD-1 but not TRα or COUP-TFII. Since no evidence for formation of GAL4-RXR homodimers was found in electrophoretic mobility shift analysis (Fig. 3A, lower panel, lane 5), the fact that the OR1/RXR complex can be superinduced by 9cRA suggests that dimerization-induced activation and ligand activation of the complex are not mutually exclusive.

The OR1-LBD is the transcriptionally active component of the unliganded complex. Given that a transcriptional activation function was gained by heterodimeric interaction of OR1 and RXR, we were interested in understanding which of the two receptors was activated by dimerization. Since the carboxy-terminal AF-2 motif of nuclear receptors has been reported to be an important structural feature of nuclear receptor transcriptional activity, short carboxy-terminal deletion mutants impairing the AF-2 of the OR1/RXR GAL4 chimera were made and the transactivation potential of the corresponding complexes were tested in the transient-transfection assay. A 10-amino-acid carboxy-terminal deletion in GAL4-RXR AF-2 (GAL4-RXRΔ) did not reduce constitutive transactivation by the heterodimeric complexes significantly (Fig. 3A, upper panel, compare lanes 7 and 4), while it completely abolished 9cRA inducibility (compare lanes 8 and 5). Previous reports (43) and electrophoretic mobility shift analysis (see Fig. 6B) argue that the truncated RXR-LBD can still bind 9cRA. Thus, the results indicate that the 9cRA signal can be mediated by the activation function of RXR only and not by that of OR1. In striking contrast, a corresponding 7-amino-acid deletion in the AF-2 of GAL4-OR1 (GAL4-ORΔ) led to a complex which was even less active than the GAL4-RXR homodimer and barely inducible by 9cRA (Fig. 3A, lanes 10 and 11), pointing at a repression effect of truncated OR1 on RXR. In conclusion, dimerization-induced activation of the GAL4 chimeric OR1/RXR complex is independent of the carboxy-terminal transactivation function AF-2 of RXR, while the AF-2 of OR1 is indispensable for the activity of the complex. This identifies the OR1-LBD as the transcriptionally active component of the complex in the absence of added ligand.

Almost identical results were found in the corresponding experiments performed for RLD-1 (Fig. 3A, lanes 15 to 20). However, a difference was found in that upon truncation of the AF-2 of RLD-1, clear 9cRA inducibility of the GAL4-RXR/GAL4-RLD complex was still observed (lane 20). This might reflect a functional difference between the two paralogous receptors but is probably due to formation of homodimers of excess GAL4-RXR which retain 9cRA inducibility.

Dimerization-induced activation of the OR1/RXR LBD complex can be observed in different heterodimeric configurations. Even though the strong preference for heterodimer formation of the chimeric proteins in the experiments described above can be taken as an argument that a functional and therefore supposedly rather natural heterodimeric interaction between OR1 and RXR is achieved, the fact that the GAL4 response element is a palindromic element whereas the known response elements for OR1/RXR heterodimers are arranged as direct repeats was still a caveat. We therefore tested the effect of the free RXR and OR1 LBDs on their respective GAL4-DBD-bound partners. Strikingly, cotransfection of an OR1 variant comprising the DBD and full LBD region (OR1-DL) with GAL4-RXR resulted in the formation of a higher-order complex (Fig. 4A, lower panel, lane 3) which was strongly transcriptionally active in the absence of added ligand (upper

panel, lane 3). In the reverse situation (lane 9), cotransfection of GAL4-OR1 with the LBD of RXR (RXR-L) resulted in an equally strong increase in transcriptional activation. Again, deletion of the AF-2 of OR1 has a strong repressive effect on the complex (construct ORΔ-DL, lanes 5 and 6), abolishing both dimerization-induced activation and 9cRA inducibility of the DNA-bound GAL4-RXR, thus supporting the result from the previous experiment that the OR1 AF-2 is a region essential for an active OR1/RXR complex.

Thus, the results confirmed the dimerization-induced activation effects obtained with the GAL4 chimera alone, but some differences were observed. As a first difference, neither the GAL4-RXR/OR1-DL nor the GAL4-OR1/RXR-L complex was 9cRA inducible (Fig. 4A, lanes 4 and 10), which might be due to the fact that the addition of 9cRA favors the formation of RXR-LBD homodimers over dimerization-activated RXR/OR1 complexes (62) and/or to a specific destabilization of OR1/RXR interaction by 9cRA. Indeed, addition of 9cRA to the electrophoretic mobility shift assay destabilized the higher-order complexes (Fig. 4B), but the transfection data do not support this notion. A significant negative effect of 9cRA on heterodimer formation would be expected to result in a relatively stronger decrease in transactivation by GAL4-OR1/RXR-L as compared to the reverse complex, since in the latter case loss of dimerization-induced activation cannot be compensated for by 9cRA-induced activation by RXR. Also, it should lead to the release of repression by OR1-DLΔ of GAL4-RXR. Neither is observed.

As a second difference, the activity of the GAL4-OR1/RXR-LBD complex was somewhat reduced upon deletion of the AF-2 of RXR (Fig. 4A, lane 11), an effect which was not seen when both receptor LBDs were targeted to the DNA directly as GAL4 chimeras (cf. Fig. 3A). Possibly, deletion of the AF-2 of RXR destabilizes OR1/RXR interaction and thereby diminishes the number of heterodimerization-activated complexes. However, electrophoretic mobility shift data indicate that this is not the case (Fig. 4A, lanes 9 to 12).

Corresponding cotransfection experiments were performed for GAL4 chimeras of TRα and COUP-TFII, but again no transcriptional activation was observed for these combinations (data not shown).

Constitutive transactivation from a DR4 response element by the heterodimeric complex requires the AF-2 of OR1 but not of RXRα. To confirm the role of the respective AF-2 domains of OR1 and RXRα in transactivation from a response element recognized by the heterodimer of wild-type receptors, we performed transient-transfection studies in COS7 cells employing a 2xDR4-TK-LUC reporter (Fig. 5). Transfection of RXR or RXRΔ did not result in any significant change in reporter activity as compared to empty vector, while transfection of OR1 yielded a weak activation, presumably together with endogenous RXR. However, a threefold constitutive activation over vector control was obtained when OR1 and RXR were cotransfected. This activation could also be observed when the AF-2 of RXR was deleted (RXRΔ), while deletion of the AF-2 of OR1 abolishes constitutive activation by the heterodimer and additionally reduced background 9cRA inducibility, which can be presumed to be due to endogenous receptors. Thus, the results are fully concordant with the results obtained from experiments with GAL4 chimeric receptors showing that the AF-2 of OR1 but not of RXR is essential for dimerization-induced activation by OR1/RXR heterodimers.

Transactivating GAL4 chimeric receptor heterodimers show altered mobility in electrophoretic mobility shift analysis. An interesting observation was made in electrophoretic mobility shift assays that were used to verify equal expression levels of

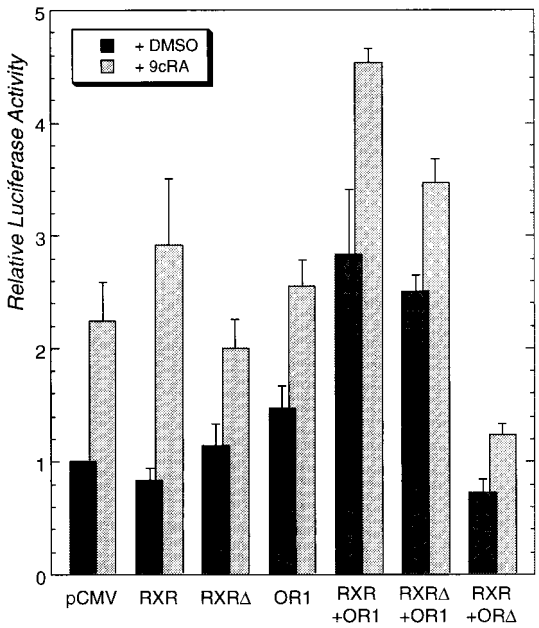

FIG. 5. Role of the AF-2 of OR1 and RXR, respectively, in transcriptional activation from a DR4-containing reporter. COS7 cells were transfected with plasmids encoding wild-type OR1 or RXR or deletion variants thereof along with the reporter 2xDR4-tkLUC and grown in the absence or presence of 9cRA for 6 h prior to harvest at 22 h after transfection. Relative luciferase activities from transfected cells are given as means from three independent experiments and are standardized for the activity obtained with empty vector (pCMV); bars represent standard errors. DMSO, dimethyl sulfoxide.

the chimeric receptors, which was analyzed in more detail by using in vitro-translated protein. The GAL4-RXR/GAL4-OR1 heterodimer displayed a higher mobility than the corresponding homodimers in electrophoretic mobility shifts employing whole-cell extracts from transfected cells (Fig. 3A, lower panel) or in vitro-translated protein (Fig. 6A). The same was observed for GAL4-RXR/GAL4-RLD and to a lesser extent for TR but not for ARP heterodimers (Fig. 6A and Fig. 3A and C, lower panels). With GAL4-TR, GAL4-RXR, and GAL4-RXRΔ (indicating that it can still bind ligand), down-shift effects were specifically observed when the respective ligands were added (Fig. 6B). Also, when the TR ligand T3 was added to the GAL4-RXR/GAL4-TR complex, a clear increase in mobility was observed (cf. also Fig. 3C). Such an effect was also seen for a wild-type hTRα/rRXRα heterodimer on a DR4 (Fig. 6C). Since the occurrence of changes in electrophoretic mobility was receptor specific and correlated with the addition of respective ligands, a feasible explanation for higher mobility is a conformational change within the LBD into a more compact structure induced by ligand binding or heterodimerization (44).

It should be noted that the AF-2 deletion variants employed in this study all showed a slightly decreased mobility in the native electrophoretic mobility shift assay compared to the respective full-length constructs, despite the lower molecular weight of the former, which can be taken as an indication that deletion of the AF-2s might affect the packing of the characteristic α-helical sandwich structure of the nuclear receptor LBDs. However, the presence of neither AF-2 is necessary for induction of the conformational change in the OR1/RXR complex (Fig. 3A).

Dimerization with RXR results in increased protease resistance of an OR1 fragment. For several nuclear receptors, it has

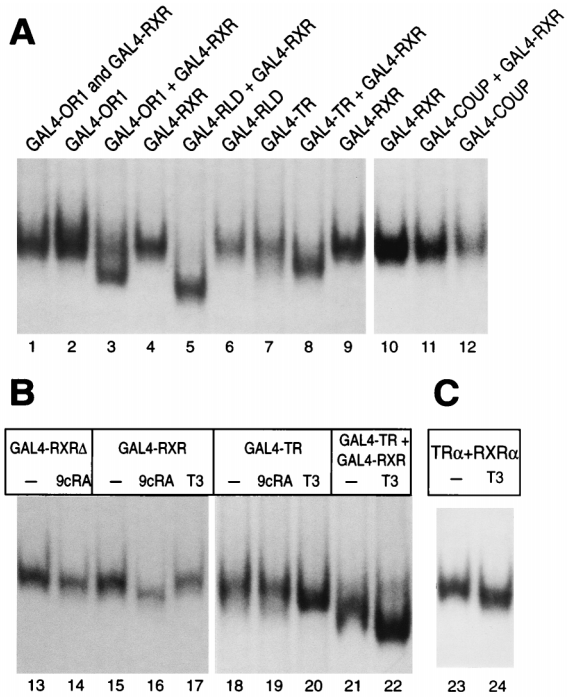

FIG. 6. Effects of dimerization and ligand addition on the electrophoretic mobility of receptor complexes. Electrophoretic mobility shift assays were performed with in vitro-translated protein; gels were run 1.7 times longer than usual to increase resolution. Ligands or vehicles (−) were added as indicated. (A) OR1-, RLD-, and TR- but not COUP-GAL4 chimeras display increased mobility upon heterodimerization with GAL4-RXR. Constructs that were cotranslated are connected by a plus sign. (B) Ligands of RXR and TR specifically increase the mobilities of corresponding DNA-bound GAL4 chimeras. Note that negative "smile" effects (e.g., in lane 21) are caused by the lateral diffusion of added ligand. (C) The mobility of a complex of wild-type RXRα/TRα on DR4 is increased by the addition of T3.

been shown that binding of the respective ligand not only results in increased electrophoretic mobility but by induction of a conformational change also renders the LBD of the protein less susceptible to proteases (1, 5, 17, 30, 42, 44). We therefore tested if a similar effect could be obtained upon heterodimerization of OR1 with RXR. When subjected to limited trypsin digestion (Fig. 7), a fragment with an apparent molecular weight of 33,000 indeed turned out to be more resistant to proteolysis in the presence of RXRα. The size of this fragment coincides with that of a recombinant protein representing the full OR1 LBD (data not shown). For hTRβ and hRARα tested using the same protease, heterodimerization with RXR has been reported not to be sufficient to produce this effect (44).

DISCUSSION

Heterodimeric complexes of nuclear orphan receptor OR1 and its partner RXR on DR4s have previously been reported to display constitutive transcriptional activation (56, 57). In this work, we have tested the effect of heterodimeric interaction between the nuclear orphan receptor OR1 and its heterodimerization partner RXR on transcriptional activation separate from the recruitment event to the promoter region by employing a GAL4 chimeric system in which targeting of the receptor LBDs to DNA is provided by the heterologous GAL4-DBD and therefore is independent of heterodimerization. We show that while chimeric GAL4-OR1 is transcriptionally silent in the absence of RXR, heterodimerization between the LBDs of RXR and OR1 releases a transactivation potential which is strictly dependent on the AF-2 of OR1. We conclude that constitutive transcriptional activation by the OR1/RXR complex is not obtained by simple recruitment of the receptors to the DNA but requires a modulatory heterodimeric interaction leading to dimerization-induced activation of OR1.

Dimerization-induced activation was initially observed when both OR1 and RXR were targeted to the DNA as heterodimerization-independent GAL4-DBD chimeras arranged on the GAL4 palindromic response element, while the previously reported response mediated by wild-type OR1 occurs as an RXR heterodimer on a direct repeat element. However, it could be demonstrated that, when the OR1 and RXR GAL4 chimeras were coexpressed and the resulting complex was analyzed in an electrophoretic mobility shift experiment, heterodimer formation was strongly preferred over homodimerization, which suggests that, owing to the flexibility of their hinge region, the two LBDs can arrange themselves so as to find their natural heterodimerization interface. Indeed, this kind of rotational flexibility has been ascribed to the hinge region of nuclear receptors before (23, 34, 50). Moreover, the same effect of activation by an OR1/RXR complex was also observed when only one receptor was DNA bound and the corresponding other free LBD was coexpressed, a situation in which heterodimerization was still efficient and possible sterical constraints can be assumed to be quite different.

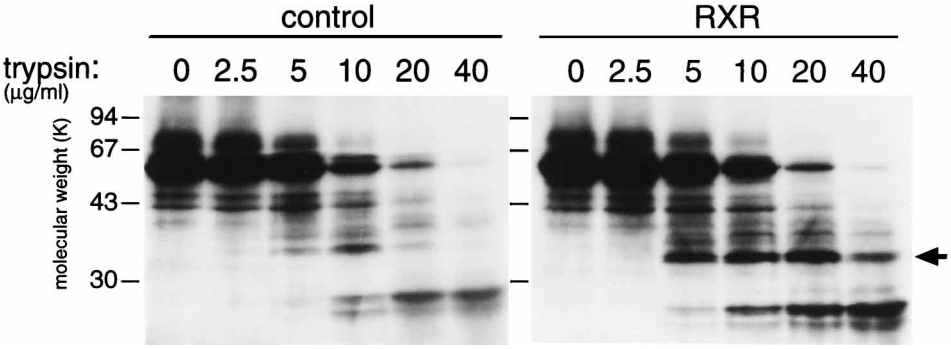

FIG. 7. Heterodimerization effect on limited trypsin digestion of OR1. In vitro-translated, ^{35}S-labelled OR1 was preincubated with either unprogrammed reticulocyte lysate (control) or an excess of in vitro-translated RXR before a 10-min exposure to various concentrations of trypsin. Samples were run on a sodium dodecyl sulfate–12.5% polyacrylamide gel, and the dried gel was autoradiographed. The resistant protein fragment occurring in the presence of RXR is marked by an arrow. A control experiment demonstrated that no significant amount of ^{35}S-labelled RXR is produced during the coincubation period (data not shown). K, thousands.

RXR heterodimerization-induced transcriptional activation was observed for OR1 and its paralog RLD-1 but not for TRα or COUP-TFII. Similar experiments involving one GAL4 chimeric and one free nuclear receptor LBD were performed recently for NURR1, TR, RAR, and VDR (20). In none of these cases was any dimerization-induced activation observed. Thus, among the nuclear receptors tested here and by others, activation by RXR interaction is unique for OR1 and its paralog RLD-1. It will be interesting to test further family members for dimerization-induced activation.

Interaction of the LBD of RXR with that of OR1/RLD-1, but not of the other receptors tested, appeared to induce a conformational change, as judged by electrophoretic mobility shift analysis, that is similar to that otherwise obtained by addition of a corresponding ligand. This notion is supported by the fact that heterodimerization with RXR additionally results in increased protease resistance of an OR1 fragment, which again is reminiscent of the described effect of ligand binding on several other nuclear receptors. Hence, we propose that the likely molecular switch in dimerization-induced activation is a heterodimerization-dependent conformational change within the LBDs of the interacting receptors resulting in a more compact structure that brings the activation domain AF-2 into an intramolecular context appropriate for transcriptional activation. Such a mechanism would be closely related if not mechanistically identical to that described for ligand activation. Comparative structural analysis of crystals of monomeric and heterodimeric OR1 or RLD-1 will have to be carried out to test this hypothesis.

The concept of dimerization-induced activation does not exclude the possibility that the RXR heterodimeric complex is additionally regulated by specific ligands of OR1/UR or RLD-1/LXR. Such ligands could stabilize the complex or modulate its precise conformation and transcriptional properties. Indeed, oxysterols have recently been reported to act as activators for LXR and an OR1 homolog (27, 41). Activation by oxysterols (but not 9cRA [60]) was also found for the corresponding GAL4 chimeras, indicating that the activated unit is the receptor LBD itself and not a complex with RXR. Therefore, dimerization-induced activation and activation by oxysterols appear to occur as two independent events.

As confirmed exemplarily for one DR4 element recognized by the wild-type receptors, the constitutive (heterodimerization-induced) transcriptional activity of the OR1/RXR complex described here is not dependent on the AF-2 of RXR, which is in contrast to reports on similar experiments performed for RAR, TR, and VDR in the presence of their respective ligands (53). Most importantly, the activation potential of the OR1 heterodimeric complex is dependent on the carboxy-terminal transcriptional activation domain AF-2 of OR1, identifying the OR1-LBD as the transcriptionally active component of the unliganded complex and challenging the idea of the AF-2 as a generally ligand-dependent activation domain. As expected, induction of the OR1 heterodimeric complex by 9cRA requires the AF-2 of RXR and can thus not be mediated via OR1.

Our results from experiments with palindromically arranged heterodimeric GAL4 chimeras slightly differed from those employing one DNA-targeted and one free receptor. In the latter case, 9cRA induction of the OR1/RXR complex was not observed, while deletion of the RXR–AF-2 had a notable effect even in the absence of the ligand. One possible explanation for the observed differences is that both a conformational change upon binding of 9cRA by RXR (62) and deletion of the AF-2 of RXR destabilize heterodimerization with OR1, thereby reducing the number of activated complexes, but this argument is not supported by our data. Alternatively, the two LBDs could be arranged in a slightly different way in this configuration, leading to an altered ligand responsiveness. Possibly, even RXR could be in an activated state in the absence of ligand dependent on the specific configuration, making it nonresponsive to ligand but sensitive to deletion of its AF-2.

In fact, the complex situation observed here is reminiscent of the in part seemingly contradictory results previously obtained for the OR1/UR and RLD-1/LXR branch of the nuclear receptor superfamily. Cotransfection of OR1 and RXRα as described in this work or with CHO cells (57) resulted in constitutive activation which was superinducible by 9cRA, while UR/RXRα on a DR4 with different half-site-surrounding sequences in COS-1 cells conferred constitutive but not 9cRA-induced activation (56). LXR in a complex with RXRα has been described to be constitutively active as well as responsive to 9cRA on a specific degenerate DR4 response element (LXRE) but silent on another, although both DNA elements are bound with equal efficiency (60), while for the LXR ortholog RLD-1, constitutive activation but no 9cRA inducibility was observed on yet another DR4 response element when the same cell line was used (2). Detailed comparative studies remain to be performed, and the relatively low sequence similarity between the DBDs of the paralogous receptors is particularly intriguing in this context. Taken together, the available data suggest that transcriptional activation by OR1/ or LXR/RXR heterodimers does not obey a simple on-and-off mechanism with respect to either ligand- or dimerization-induced activation but is rather more complex and dependent on the particular heterodimer-DNA configuration which may be dictated by the response element. Thus, nuclear receptors of the OR1/LXR type appear to modulate signaling by RXR and its activators in a highly differentiated fashion.

Recent results obtained for one of the most well-studied nuclear receptor heterodimers, RXR/RAR, point in a similar direction. Comparison of the effects of different synthetic RXR-ligands suggests that ligand binding can indeed induce various transcriptionally active LBD conformations that differentially interact with other components of the transcriptional machinery (36). Also, the ability of RXR to respond to its ligand can depend on the relative orientation of the receptors, the activation status of RAR, and the particular receptor subtype but also on the exact nature of the response element (20, 32, 35). The last of these explanations is easily envisioned for the cases in which response elements differ in spacing or orientation of the half-sites, but even simple sequence variations of a half-site and/or the surrounding areas could have an impact on the overall structure of the complex by imposing differential conformational constraints. For instance, protein-DNA contacts between amino-terminal nuclear receptor portions or the region immediately carboxy terminal to the DBD and sequence elements outside the half-site (22, 28, 51) could subtly modulate intramolecular and heterodimeric interactions (22, 28, 45, 51).

Several prototype mechanisms of ligand activation of nuclear receptors have recently been distinguished with respect to the role of RXR, its partner, and the corresponding ligands (20, 38, 60). RXR homodimers can bind and be activated by 9cRA, although the physiological relevance of this remains unclear (32). Upon heterodimerization with a (ligand-responsive) partner, RXR can either maintain (Fig. 8A), as in the case of PPAR (31) or OR1/LXR (57, 60), or lose (Fig. 8B) (20, 32) its ability to act as an active, ligand-inducible factor. Conversely, the *Drosophila* ecdysone receptor (EcR) is dependent on heterodimerization with USP/RXR to acquire its ligand-binding property (Fig. 8C) (61). The modulatory effect of li-

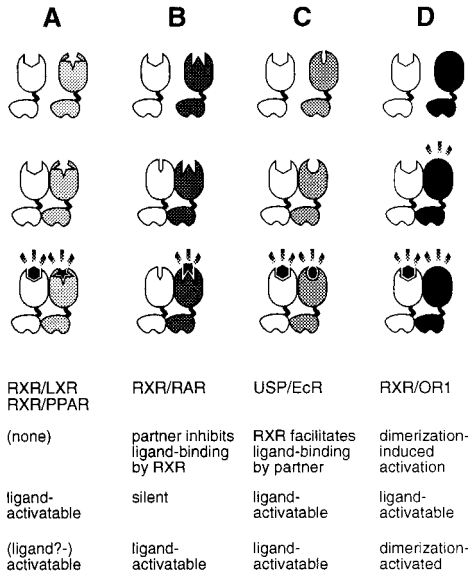

FIG. 8. Extended model of allosteric effects of nuclear receptor dimerization. The upper row illustrates that individual receptors can be able or unable to bind their respective ligands (symbolized by the pocket-like openings). The middle row illustrates that heterodimerization can affect ligand-binding properties (displayed as changes in the pocket-like openings) or lead to receptor activation (indicated by three dashes). The lower row illustrates ligand activation of the heterodimeric complexes. Only selected examples of receptors are given. See the text for details and references.

gand binding is commonly ascribed to a conformational change (1, 49, 58), and the effects of heterodimerization on ligand-binding abilities might in turn be explained by stabilization or induction of a conformation favoring either the liganded or unliganded state (32, 61). Observations of this kind have led to the formulation of an allosteric control model of ligand responsiveness (20). Given that a conformational change of the LBD can be induced by heterodimerization as well as ligand binding, the possible consequences are conceivably overlapping. The work presented here extends the allosteric control model to include dimerization-induced activation (Fig. 8D) as exemplified by RLD-1/ or OR1/RXR heterodimers as a novel type of nuclear receptor activation. In conjunction with the recent identification of activators and potential ligands (27, 41), heterodimers of members of the OR1/LXR branch of the nuclear receptor superfamily with RXR thus appear to be activatable by multiple mechanisms, that is, by specific ligands or activators for either receptor (Fig. 8A) as well as by heterodimerization (Fig. 8D).

A growing number of factors that are thought to mediate transcriptional regulation by nuclear receptors have recently been described (8–11, 25, 26, 29, 39, 40, 48), and models of transcriptional regulation by nuclear receptors are being extended to include the roles of these proteins (see above and references 12 and 33). Briefly, the ligand-dependent intramolecular positioning of the AF-2 is thought to be critical for displacement of corepressors which bind to nuclear receptors in the absence of ligand and for binding of components of the basal transcriptional machinery or coactivators which are recruited to mediate between nuclear receptors and the latter to achieve transcriptional activation. Such models can be applied equally well to conformational changes brought about by heterodimeric interaction alone. Moreover, some interacting proteins might require both partners of a heterodimer for recognition. Future studies will aim at the analysis of differential interaction of monomeric versus heterodimeric OR1 with these coregulators.

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