Howard Hughes Medical Institute

Published as: *Nature*. 1992 January 30; 355(6359): 446–449.

Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signalling

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Cellular responsiveness to retinoic acid and its metabolites is conferred through two structurally and pharmacologically distinct[1] families of receptors: the retinoic acid receptors (RAR)[2,3] and the retinoid X receptors (RXR)[1]. Here we report that the transcriptional activity of RAR and RXR can be reciprocally modulated by direct interactions between the two proteins. RAR and RXR have a high degree of cooperativity in binding to target DNA, consistent with previous reports indicating that the binding of either RAR or RXR to their cognate response elements is enhanced by factors present in nuclear extracts[4,5]. RXR also interacts directly with and enhances the binding of nuclear receptors conferring responsiveness to vitamin D_3 and thyroid hormone T_3; the DNA- binding activities of these receptors are also stimulated by the presence of nuclear extracts[6–9]. Together these data indicate that RXR has a central role in multiple hormonal signalling pathways.

RXR, but not RAR, can activate gene expression through the RXR response element found in the promoter of the cellular retinol binding protein type II (CRBPII-RXRE)[10]. Unexpectedly, RAR represses RXR-mediated activation through the CRBPII-RXRE (ref. 10 and Fig. l*a*). By contrast, transfection of expression plasmids for either RAR or RXR resulted in an induction of expression from a reporter construct driven by two copies of an RAR response element (RARE)[11] (Fig. 1*b*, RAR or RXR); cotransfection of expression plasmids for both receptors together yielded an enhanced level of expression relative to transfection of either receptor expression plasmid alone (Fig. 1*b*, RAR+RXR). Together, these data provide evidence for a functional interaction between the two retinoid responsive

Similar experiments in which RAR was replaced with radiolabelled glucocorticoid receptor (GR) failed to reveal RXR-GR interactions, demonstrating the specificity of the RAR-RXR interaction (Fig. *2a*, lanes 5 and 6). Consistent with our transfection data indicating the importance of the C terminus of RAR in mediating RAR-RXR interactions, a truncated RAR protein (amino acids 155–462), lacking the amino terminus and DNA binding domain of the protein, was also efficiently coprecipitated with RXR (Fig. 2*a*, lanes 3 and 4). Thus, the C terminus of RAR, containing the dimerization domain, is sufficient for forming a stable solution complex with RXR.

These results led us to examine the properties of the RAR-RXR complex when associated with DNA. Gel mobility shift experiments were done using *in vitro* synthesized RAR and RXR and a radiolabelled oligonucleotide encoding the CRBPII-RXRE. RAR synthesized *in vitro* bound with very low affinity to the CRBPII-RXRE (Fig. 2*b*, lane 3). But the affinity of

binding of RAR to CRBPII-RXRE was greatly stimulated by the addition of *in vitro* synthesized RXR (Fig. 2*b*, lane 4). *In vitro* synthesized RXR alone had no detectable binding activity (Fig. 2*b*, lane 2). Inclusion of polyclonal antisera prepared against either RAR or RXR in the reaction resulted in complexes with reduced mobility (Fig. 2*b*, lanes 5 and 6), indicating that both RAR and RXR were present in the complex. Thus, the RAR-RXR complex is capable of interacting with the CRBPII- RXRE with an affinity much higher than either receptor alone.

The specificity of the RAR-RXR interaction with DNA was next examined using unlabelled oligonucleotides as competitor. Oligonucleotides containing the CRBPII-RXRE competed efficiently for RAR-RXR complex binding at a 10-fold molar excess (Fig. 2*c*, lane 2), whereas oligonucleotides containing an unrelated glucocorticoid response element (GRE) failed to compete when used at a 40-fold molar excess relative to the radiolabelled CRBPII-RXRE (Fig. 2*c*, lane 7). Oligonucleotides containing the RARE of the RARβ promoter (βRARE)[15,16] also competed efficiently for RAR-RXR binding to the CRBPII- RXRE (Fig. 2*c*, lanes 4 and 5).

To investigate further this interaction of the RAR-RXR complex with the βRARE, oligonucleotides containing the βRARE were labelled and used as probe in a gel mobility shift assay. As in the case of the CRBPII-RXRE, both *in vitro* synthesized RAR and RXR were required for high-affinity DNA-protein interactions with the βRARE (Fig. 2*d*, lanes 2–4). Similar results indicating a requirement for the presence of both RAR and RXR for formation of a high-affinity DNA-protein complex on the βRARE were obtained using whole-cell extracts prepared from COS cells which had been transfected with either RAR, RXR or RAR and RXR (Fig. 2*e*). Taken together, these results demonstrate that RXR dramatically stimulates the binding affinity of RAR to a strong RARE and that the RAR-RXR complex is likely to be present *in vivo.*

A functional relationship among the vitamin D receptor (VDR), thyroid hormone receptor (TR) and RAR has recently been described in which these receptors bind and activate through tandem repeats of consensus AGGTCA spaced by 3, 4 and 5 nucleotides, respectively (3–4-5 rule)[11]. Like the RAR, accessory factors present in nuclear extracts are necessary for high-affinity binding of the TR and VDR to their cognate response elements[6–9]. The relatively high degree of amino-acid conservation in the C termini of these nuclear receptors suggested that RXR might functionally interact with TR and VDR. Indeed, in immunoprecipitation experiments, *in vitro* synthesized TR and VDR coprecipitate with bacterially expressed RXR (Fig. 3*a*, lanes 1–4). The interactions of these receptors with RXR were also manifest at the level of DNA binding: *in vitro* synthesized RXR greatly stimulates TR and VDR binding to the Moloney leukaemia virus long terminal repeat thyroid hormone response element (MLV-LTRTRE) and osteopontin vitamin D response element (VDRE), respectively (Fig. 3*b*, lanes 1–8). The ability of RXR to stimulate the binding of nuclear receptors was not a general phenomenon, however, as RXR failed to increase GR binding to a GRE (Fig. 3*b*, compare lanes 11 and 12). Taken together, these data strongly suggest a central role for RXR in modulating the hormonal responses conferred through the RAR, TR and VDR.

The formation of RXR complexes with RAR, VDR and TR in which the complex displays new DNA-binding properties relative to the individual homodimers is reminiscent of interactions reported between the Jun and Fos families of proteins[17] as well as between members of the HLH family of transcription factors such as MyoD and E12/47 (ref. 18). Through the formation of heterodimers, small families of structurally related proteins can yield large numbers of transcription factors with distinct functional properties. Two additional isoforms of RXR (RXRβ and RXRγ) have been recently identified (ref. 19, and D.J.M. and R.E., unpublished observations). Thus, the interaction of multiple RXR isoforms with additional nuclear receptors responsive to a diverse array of ligands is likely to have a critical role creating the high degree of diversity and specificity necessary to regulate the battery of hormone responsive genes.

Why the VDR, TR and RAR interact with a common partner is not yet clear, particularly as vitamin D, and thyroid hormone actions are not apparently retinoic acid-dependent. Further adding to the puzzle is the observation that RXR can activate through the CRBPII-RXRE in the absence of VDR, TR and RAR[10], suggesting a role for other nuclear factors in this process. It is clear that characterization of the RXR family, its patterns of expression, and the nature of the RXR ligand will be essential to better understanding the complex molecular nature of hormonal signalling.

ACKNOWLEDGEMENTS.

We thank P. Rangarajan for RAR mutant expression constructs RS-Δ81–153 and RS-Δ203–360, R. Heyman for RAR mutant expression construct RS-185*, J. Dyck for antisera prepared against RARa and RXRa and H. Sucov, M. McKeown and T. Perlmann for discussions and critically reading the manuscript and E. Stevens for manuscript preparation. S.A.K. is a Fellow of the Jane Coffin Childs Memorial Fund for Medical Research. K.U. and D.J.M. are Research Associates and R.M.E. is an Investigator of the Howard Hughes Medical Institute at the Salk Institute for Biological Studies. This work was supported by the NIH and the Mathers Foundation.

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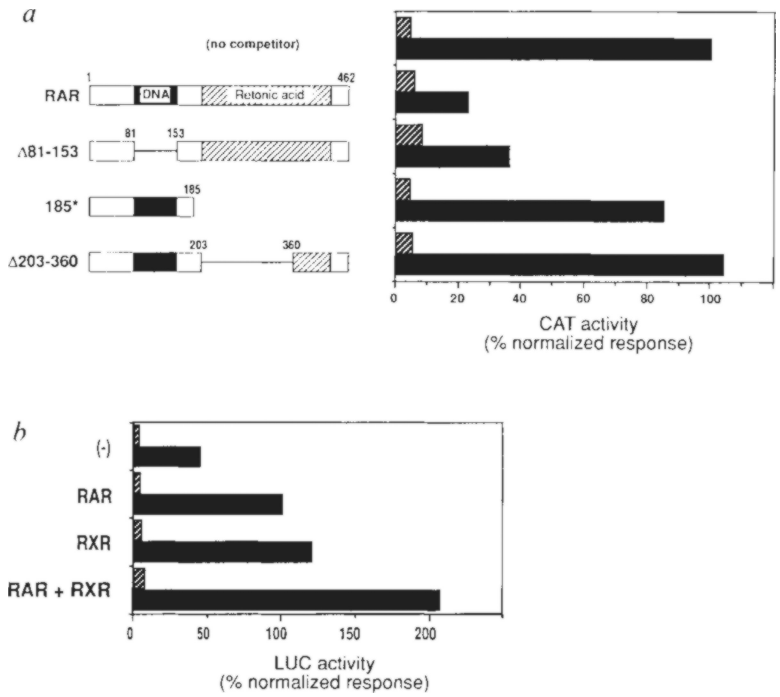

FIG. 1.

a, the C terminus of RAR is required for suppression of RXR-dependent transactivation through the CRBPII-RXRE. CV-1 cells were cotransfected in duplicate with the reporter construct ΔSV-CRBPII-CAT, expression plasmid RS-hRXRa, and the control expression vector RS-LUC (no competitor) or the expression plasmids RS-hRARa, RS-Δ81–153, RS-185* and RS-ΔA203–360. Cells (right-hand figure) were then exposed to either ethanol (cross-hatched) or 10 μM retinoic acid (filled bars). CAT (chloramphenicol acetyltransferase) activity Is presented as per cent conversion where retinoic acid-induced activation in the presence of RXR is arbitrarily set at 100%. *b*, RXR enhances RAR-dependent transactivation through an RARE. CV-1 cells were cotransfected in duplicate with the reporter construct tk-DR5.2-LUC, containing two copies of the DR-5 RARE upstream of the thymidine kinase promoter, and expression plasmids RS-CAT (−), RS-RARα, and/or RS-RXRα as Indicated. Cells were then exposed to either ethanol or 10 μM retinoic acid (cross-hatched and filled bars respectively). Luciferase activity is presented as per cent normalized response where retinoic acid-induced activation in the presence of RAR is arbitrarily set at 100%.

METHODS. CV-1 monkey kidney cell culture, transfections, CAT and luciferase assays were done as previously described[10,11,20]. Transfection was on 10-cm plates and, for experiments using CAT, included 1 μg of RS-receptor or RS-LUC, 0.5 μg RS-hRXRα, 1 μg ΔSV-CRBPII-CAT reporter, 5 μg RAS-*β*- galactosidase reporter and 7.5 μg pGEM4 carrier DNA. Transfections using luciferase included 50 ng RS-RARα and/or 100 ng RXRα (the total amount of RS-expression plasmid was maintained constant in each transfection through the addition of RS-CAT), 0.5 μg tk-DR5.2-LUC reporter, 5 μg RAS-/3- galactosidase reporter and 8.5 μg pGEM4 carrier DNA.

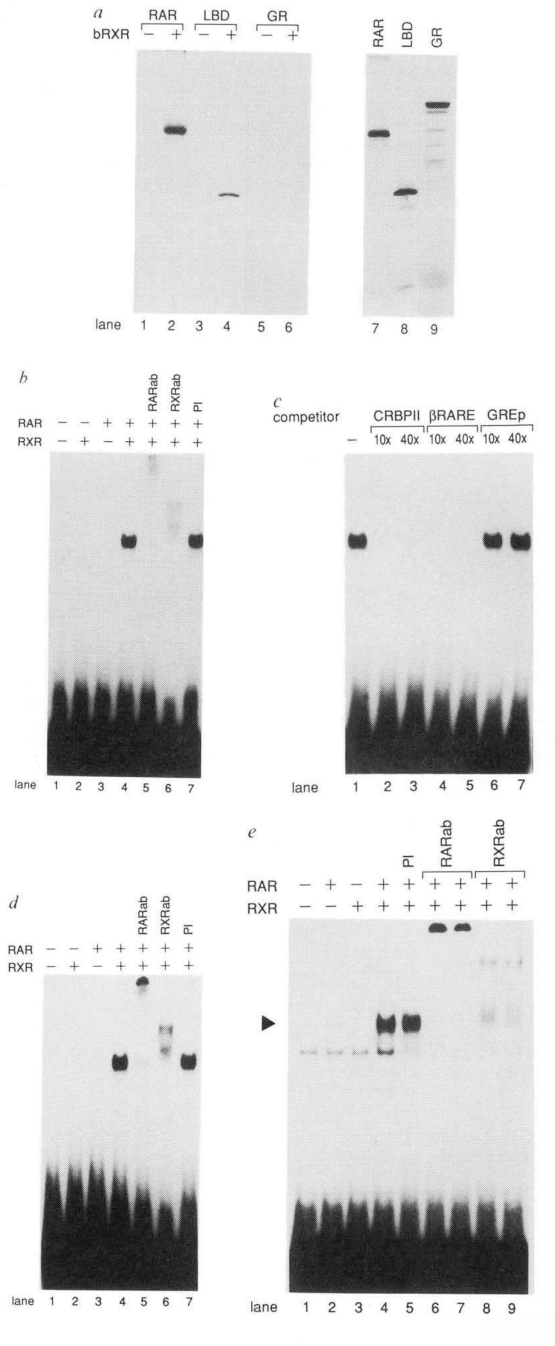

FIG. 2.

Direct Interactions between RAR and RXR in the absence or presence of DNA. *a*, RAR and RXR form a complex In solution. Immunoprecipltation reactions were done using *in vitro* synthesized, ^{35}S-methionlne-labelled RARα (lanes 1 and 2), the ligand binding domain of RARα (LBD) (amino acids 155–462) (lanes 3 and 4), or the GR (glucocorticoid receptor) (lanes 5 and 6) In either the absence (lanes 1,3,5) or presence (lanes 2,4,6) of bacterlally expressed RXR (bRXR). Polyclonal antisera prepared against RXRa was used, *in vitro* synthesized RAR, LBD, and GR proteins not subjected to Immunoprecipitatlon are shown in

lanes 7–9. *b*, RAR and RXR bind cooperatively to the CRBPII-RXRE. Gel mobility shift assays were done using *in vitro* synthesized RAR and/or RXR as indicated and ^{32}P-labelled CRBPII-RXRE oligonucleotide[10]. Polyclonal antisera prepared against either RAR (RARab) (lane 5) or RXR (RXRab) (lane 6) or prelmmune serum (PI) (lane 7) were included in the reactions as indicated, *c*, Binding specificity of the RAR-RXR complex. Gel mobility shift competition reactions were done using ^{32}P-labelled CRBPII-RXRE oligonucleotide and either a 10-fold (10 ×) or 40-fold (40 ×) excess of unlabelled competitor oligonucleotide encoding either the CRBPII-RXRE (lanes 2 and 3), the βRARE (lanes 4 and 5), or a palindromic GRE[20] (lanes 6 and 7). *d*, RAR and RXR bind cooperatively to the βRARE. Gel mobility shift assays were done using *in vitro* synthesized RAR and/or RXR as indicated and ^{32}P-labelled βRARE oligonucleotide. Polyclonal antisera prepared against either RAR (RARab) (lane 5) or RXR (RXRab) (lane 6) or preimmune serum (PI) (lane 7) were Included in the reactions as Indicated, e, RAR and RXR overexpressed in COS cells bind cooperatively to the βRARE. Gel mobility shift assays were done using ^{32}P-labelled βRARE and whole cell extracts prepared from COS cells in which RAR (lane 2), RXR (lane 3) or RAR and RXR (lanes 4–9) were overexpressed. Prelmmune serum (3 μl) (lane 5) or 0.2 μl or 1 μl RAR-specific antiserum (RARab) (lanes 6 and 7, respectively) or 1 μl or 3 μl RXR-specific antiserum (RXRab) (lanes 8 and 9, respectively) were included in the reactions as Indicated. The position of the RAR-RXR-βRARE complex is Indicted by an arrowhead.

METHODS. The LBD expression vector was generated through insertion of an *Xhol-Bam*HI fragment of Δ81–153, including amino acids 155–462 of RARα, Into the pCMX expression vector containing a synthetic translation start site sequence[11,21]. RARα, LBD, and GR RNA was prepared and subsequently translated in rabbit reticulocyte lysates as directed by the supplier (Promega). RXR was expressed in bacteria as a fusion with glutathlone-S-transferase using the pGEX-2T expression vector (Pharmacia) and purified as previously described[10]. Immunoprecipltation reactions (20 μl) included 5 μl ^{35}S-methionine-labelled receptor protein and 150 ng of either purified GST-RXR or GST alone in 20 mM Tris, pH 8.0. Proteins were incubated 20 min on Ice before the addition of 5 μl polyclonal antisera prepared against an RXRα peptide (amino acids 214–229). Antigen-antibody complexes were collected by the addition of Protein A-Sepharose (Pharmacia) and the Immunocomplexes washed three times with 400 μl RIPA buffer (10 mM Tris (pH 8.0), 150 mM NaCI, 1% Triton X-100,1% sodium deoxycholate). Immunopreclpl- tated complexes were resolved by SDS-PAGE on 10% gels which were then fixed In 30% methanol, 10% acetic acid, dried and autoradiographed. Gel mobility shift assays (20 μl) contained 10 mM Tris (pH 8.0), 40 mM KCI, 0.05% Nonidet P-40,6% glycerol, 1 mM DTT, 0.2 pg of poly(dl-dC) and 2.5 μl each of *in vitro* synthesized RAR and RXR proteins. The total amount of reticulocyte lysate was maintained constant in each reaction (5 μl) through the addition of unprogrammed lysate. Where indicated, prelmmune serum or polyclonal rabbit antisera prepared against bacterlally expressed RARa or an RXRa peptide (amino acids 214–229) were included. After a 10 min Incubation on ice 1 ng ^{32}P-labelled oligonucleotide was added and the incubation continued for an additional 10 min. DNA-protein complexes were resolved on a 4% polyacrylamide gel in 0.5× TBE (lx TBE is 90mM Tris, 90mM boric acid, 2 mM EDTA). Gels were dried and autoradiographed at −70°. Gel mobility shift assays using COS cell-expressed receptors were as described[11], using 5 μg whole cell extracts

prepared from COS cells transfected with 10 μg of either pCMX-hRARα, pCMX-hRXRα, or both expression plasmids.

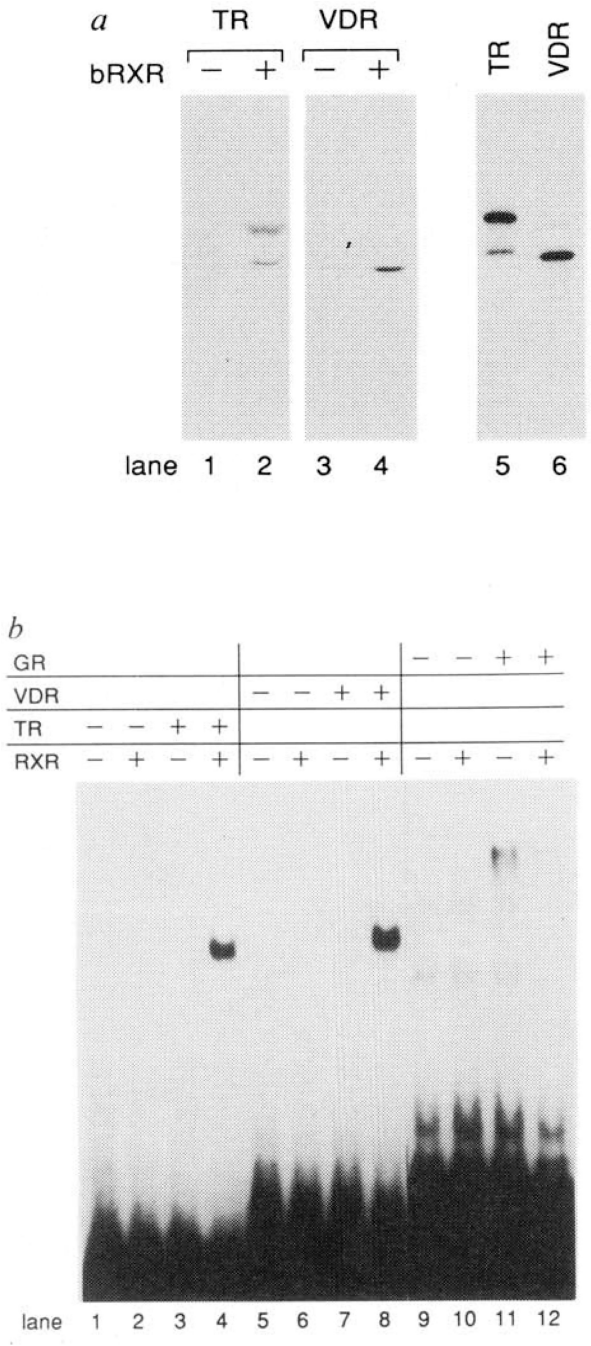

FIG. 3.

Direct interactions between RXR and TR or VDR in the absence or presence of DNA. *a*, RXR forms complexes with either TR or VDR in solution. Immunoprecipitation reactions were done with RXR-specific antiserum and *in vitro* synthesized, ^{35}S-methionine-labelled TRβ (lanes 1 and 2) or VDR (lanes 3 and 4) in either the absence (lanes 1, 3) or presence (lanes 2, 4) of bacterially expressed RXR (bRXR). Vitamin D_3 (1×10^{-7}M) was included in reactions containing the VDR. *In vitro* synthesized TR and VDR proteins not subjected to immunoprecipitation are shown in lanes 5 and 6. *b*, RXR interacts cooperatively with TR

and VDR in DNA binding. Gel mobility shift assays were done using *in vitro* synthesized RXR, TR, VDR and GR as indicated and ^{32}P-labelled oligonucleotides encoding Moloney leukaemia virus LTR TRE[11] (lanes 1–4), the mouse osteopontin VDRE[11] (lanes 5–8), or the palindromic GRE[20] (lanes 9–12).

METHODS. TRβ and VDR RNA was prepared and subsequently translated in rabbit reticulocyte lysates as directed by the supplier (Promega). Immunoprecipitation and gel mobility shift assays are described in Fig. 2 legend.