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Mouse Retinoid X Receptor Contains a Separable Ligand-Binding and Transactivation Domain in Its E Region

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Steroid, thyroid, and retinoid hormones exert their biological functions by interacting with their cognate nuclear receptors. Upon binding receptors, hormones induce a protease-resistant structural change in the receptor ligand-binding domain and subsequently activate the receptors. Utilizing partial proteolysis, we have been able to delineate a region in the mouse retinoid X receptor β (mRXRβ) required for ligand binding. A separable activation domain within the mRXRβ E region has been identified. The activation domain, which is 21 amino acids in length, is located at the extreme C terminus of mRXRβ. This domain is not required for ligand binding since removal of this sequence neither eliminates the ligand-induced, protease-resistant conformational change nor alters the ligand-enhanced DNA binding. Furthermore, deletion of this activation domain converts the receptor into a transcriptional silencer. Finally, a further truncation of 9 amino acids (for a total of 30 amino acids) from the C terminus results in a mutant which does not undergo the protease-resistant conformational change and cannot bind DNA as a homodimer. Nevertheless, this mutant is still able to form a heterodimer with the thyroid hormone receptor. Therefore, homodimerization and heterodimerization can be distinguished by this nine-amino-acid sequence.

Steroid and thyroid hormone receptors are nuclear receptors which are ligand-activated transcription factors (12, 38). They play a key role in many biological functions such as homeostasis, reproduction, development, and differentiation. They link extracellular or intracellular signals directly to transcriptional responses. Nuclear receptors regulate gene expression by binding to specific response elements in the promoter region of target genes. On the basis of structural homologies, this family of nuclear receptors has been classically divided into two general subfamilies (6, 39). One subfamily includes receptors for steroid hormones (glucocorticoids, progestins, androgens, estrogens, and mineralocorticoids), and the other consists of the thyroxine receptor (TR), retinoic acid receptor (RAR), retinoid X receptor (RXR), and vitamin D_3 receptor. The receptors in the latter subfamily can be functionally distinguished from the former ones in the following aspects: (i) they heterodimerize with a common coregulator, RXR (23, 28, 32, 51, 52); (ii) they do not associate with heat shock proteins and can bind to DNA in the absence of hormones (9, 17, 43); and (iii) they all bind to various arrays of the PuGGTCA core motif (34, 49). Nevertheless, both of these groups of nuclear receptors require their cognate ligands for activation of target genes.

It is a general belief that transcription factors have a modular structure (15). Likewise, nuclear receptors are all composed of at least four domains which are differentially conserved among members within each subfamily. The A-B domain, which has been implicated in gene activation and promoter selection, and the hinge domain (D) are highly variable and poorly conserved. The DNA-binding domain (C), which consists of two zinc modules, is the most conserved region among all the receptors. The C-terminal E domain is the largest and has a complex structure. It mediates ligand binding, dimerization, repression, and transactivation.

These domains of nuclear receptors can usually act as functional units independent of their natural structural context. Intramolecular and intermolecular domain swapping experiments have been carried out without affecting the function of individual domains (18, 40). Such an approach led to the identification of the DNA-binding domain (DBD) and activation domains τ1 and τ2 of glucocorticoid receptors (20). One of the routine procedures to identify a functional domain within a receptor is to generate receptor fusions with a heterologous DBD such as the yeast transcription factor Gal4 (Gal4-DBD) (3, 20, 46). Specifically, the entire C termini of various receptors have been fused to the Gal4-DBD, resulting in hormone-dependent transcription factors that bind to a Gal4 binding site (5, 50). Utilizing Gal4-receptor fusions, we recently have identified three functional activation domains within human TRβ (4).

Despite the knowledge that nuclear receptors are composed of functional domains, it is often difficult to precisely localize them. The problem is more obvious with the ligand-binding domain (LBD) because the conservation among receptors in this region is relatively low. Recently, using protease digestion as a probe of conformation, we have demonstrated that upon binding to a hormone, a receptor undergoes a ligand-induced conformational change that produces a distinct proteolytic cleavage pattern (1, 30). The observed structural change centers upon the LBD and is independent of other domains of the receptor (30). This conformational change renders the LBD more resistant to protease digestion. This conclusion was further confirmed by other recent publications (21, 27, 48).

RXR is rather unique among the nuclear receptors. While RXR homodimers appear to bind and function on specific RXR response elements (53), RXR can also act as a heterodimeric coregulator with other members of the subfamily, including TR, RAR, and the vitamin D_3 and peroxisome pro-

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liferator-activated receptors (22, 24). The heterodimers bind DNA elements more efficiently than do the homodimers of TR or RAR and result in increased transcriptional activities. Moreover, while TR and RAR can actively repress basal promoter activity (3, 13), RXR has no detectable silencing function. Thus, we believe there must be some structural characteristics that distinguish RXR from the other nuclear receptors.

In this report, utilizing partial proteolysis, we have defined a 200-amino-acid region in the putative E domain that was able to bind 9-*cis*-retinoic acid (9-*cis*-RA) and attain a protease-resistant conformation. However, this region alone does not have ligand-dependent transactivation activity. Consequently, we found a stretch of 21 amino acids at the C terminus of RXRβ which acts as a separable functional activation domain. This region is conserved in other members of the nuclear receptor superfamily and has been implicated in receptor AF-2 function (10, 41). Deletion of this region did not abolish the receptor ligand-binding ability but, interestingly, converted RXRβ into a transcriptional repressor. Thus, we have been able to functionally separate the ligand-binding function from the ligand-dependent activation domain of RXRβ.

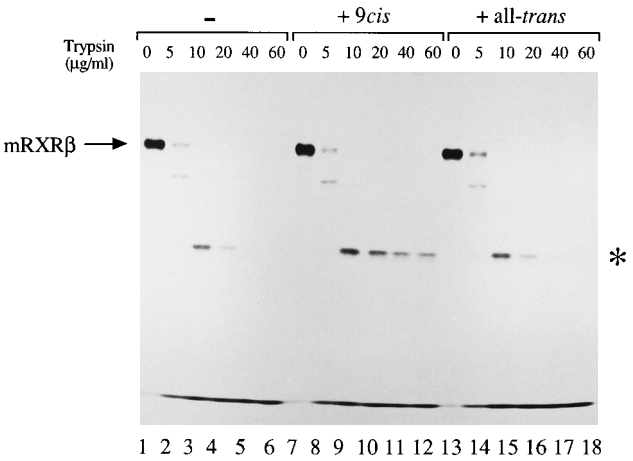

FIG. 1. 9-*cis*-RA alters the kinetics of proteolysis of in vitro-translated mRXRβ. ^{35}S-labeled mRXRβ was treated with 100 nM 9-*cis*-RA (lanes 7 to 12), 100 nM all-*trans* RA (lanes 13 to 18), or carrier control (lanes 1 to 6) prior to digestion with the indicated levels of trypsin. Digestion products were analyzed by SDS-PAGE. Resistant fragments (asterisk) and the undigested proteins (arrow) are indicated as shown.

MATERIALS AND METHODS

Plasmids. pT7βRXR has been described before (30). All RXR mutants for in vitro transcription and translation were constructed in pT7βSal (36). pT7βRXR 16-148, pT7βRXRΔ16-227, pT7βRXRΔ16-263, pT7βRXRΔc2, and pT7βRXR Δc3 were generated by inserting the *Eco*NI-*Eco*RI fragments of pBS-H2R IIBPΔN1, pBS-H2RIIBPΔN2, pBS-H2RIIBPΔN3, pBS-H2RIIBPΔc2, and pBS-H2RIIBPΔc3, respectively, into the *Hin*dII-*Eco*RI site of pT7βSal. The pBS constructs have been described previously (19, 32). pT7βRXRΔc4 was generated by introducing the *Hin*dIII-*Mae*II fragment of pT7βRXR into the *Hin*dIII-*Acc*I site of pT7βSal. pT7βRXR156, pT7βRXR173, and pT7βRXR189 were generated by isolating the *Hin*dII-*Eco*RI, *Bsu*36I-*Eco*RI, and *Ear*I-*Eco*RI fragments from pT7βRXR and subcloning them into the *Hin*dII-*Eco*RI, *Acc*I-*Eco*RI, and *Hin*dII-*Eco*RI sites of pT7βSal, respectively. The expression vectors for Gal94-RXR156, Gal94-RXRΔc2, Gal94-RXRΔc3, and Gal94-RXRΔc4 fusion proteins were created by subcloning *Hin*dII-*Eco*RI fragments of the corresponding pT7βSal constructs into the *Acc*I site of pABGal94 (4). Likewise, pABGal94-RXR173 and pABGal94-RXR189 were subcloned by inserting the *Bsu*36I-*Eco*RI and *Nco*I-*Nco*I fragments, respectively, of the cognate pT7βSal constructs into the *Sma*I site of pABGal94. *Bgl*II-*Eco*RI fragments of pT7βRXRΔ16-227 and pT7βRXRΔ16-263 were subcloned into the *Pvu*II site of pABGal94 to produce pABGal94-RXR227 and pABGal94-RXR263, respectively. Expression vectors for RXR (pCNXRXRβ) and RXRΔc2 (pCNXRXRβΔc2) were generated by inserting the *Nco*I fragments of pExpressRXRβ and pExpressRXRβΔc2 into the *Xho*I site of the pCNX2 expression vector (35). pExpress constructs were described previously (37). If necessary, overhangs were blunt-ended with Klenow enzyme. To generate pABGal94-C21, oligonucleotides (MT672 and MT673) corresponding to the last 21 amino acids of mouse RXRβ (mRXRβ) with a *Bam*HI overhang at the 3′ end were synthesized and subcloned into the *Pvu*II-*Bam*HI site of pABGal94. The MT672 sequence is 5′-CGGATTGGCGACAC CCCCATTGACACCTTCCTCATGGAGATGCTTGAGGCTCCCCACCAG CTGGCCG-3′, and the MT673 sequence is 5′-GATCCGGCCAGCTGGTGGG GAGCCTCAAGCATCTCCATGAGGAAGGTGTCAATGGGGGTGTCGC CAATCCG-3′. The reading frames of all expression vectors were verified by sequencing. 17mer tkCATΔH/N and (17mer)×2 tkCATΔH/N were described previously (3). CRBPII×2 tkLuc was described previously (33).

Gel mobility shift assays. In vitro-translated RXR or its mutants were utilized in DNA binding assays in the presence or absence of 9-*cis*-RA. The experiments were performed as described previously except that the protein was preincubated with 10^{-7} M 9-*cis*-RA for 15 min at room temperature where specified (30).

In vitro transcription and translation and partial proteolytic analysis. [^{35}S] methionine-labeled protein was synthesized with a Promega translation kit according to the manufacturer instructions, and the protease digestion assay was carried out as described previously (30).

Transient transfection and transactivation assay. CV1, Lmtk$^-$, and P19 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% charcoal-stripped fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. For the transfection assay with Gal-receptor chimeras, 5 μg of reporter (17mer [or ×2] tkCATΔH/N) and 2 μg of expression vector were used per 100-mm dish. A total of 10^{-7} M 9-*cis*-RA was added after glycerol shock. Transfections were carried out as described previously (8). The chloramphenicol acetyltransferase (CAT) activity was determined as previously described (44). Transfection assays in P19 cells were done as described previously, except that 300 ng of CRBPII×2 tkLuc, 200 ng of pCH110 (Pharmacia), 20 to 40 ng of expression vectors, and 1 μM 9-*cis*-RA were used (29). Luciferase activity was determined as described previously (45).

RESULTS

9-*cis*-RA alters the kinetics of RXR proteolytic digestion. Partial protease digestion has been used widely to identify structural domains within proteins. Recently, we have shown that it also can be used to detect ligand-induced conformational changes within steroid hormone receptors (1, 30). This conformational change renders the LBD resistant to protease digestion. Our results convincingly indicate that the protease-resistant fragment contains the region sufficient for receptor ligand binding. Thus, we felt this method could be used to identify the LBD of RXR. In vitro-synthesized, [^{35}S]methionine-labeled mRXRβ was digested with increasing concentrations of trypsin. The products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). Although a weak protease-resistant fragment was generated from the aporeceptor, it was clear that the resistant fragment was enhanced upon addition of 9-*cis*-RA (Fig. 1; compare lanes 7 to 12 with 1 to 6). This was not due to nonspecific effects of retinoids, since all-*trans*-RA did not affect the kinetics of proteolysis (Fig. 1; compare lanes 13 to 18 with 1 to 6). Similar patterns were observed when chymotrypsin or V8 protease was used (data not shown). Since there is a strong correlation of ligand binding and protease resistance, the protease-resistant fragment was used to define the boundaries of the LBD of RXR.

A protease-resistant fragment is located in the C-terminal half of RXR. To precisely localize the region of RXR that harbors the protease resistance, we constructed a series of deletion mutants (Fig. 2). Mutants were examined by trypsin digestion, and the results were summarized in Fig. 2. Analogous to other receptors that we have tested, the resistant fragment localized to the C-terminal half of RXR. Removal of the N-terminal sequence of RXR resulted in a receptor (i.e., RXRΔ16-148) which exhibited the same protease-resistant fragment as contained in the wild-type receptor (Fig. 3a). When we compared the trypsin digestion patterns of RXR156 and RXR173 with that of RXRΔ16-148, we found that the addition of 9-*cis*-RA altered the kinetics of the protease diges-

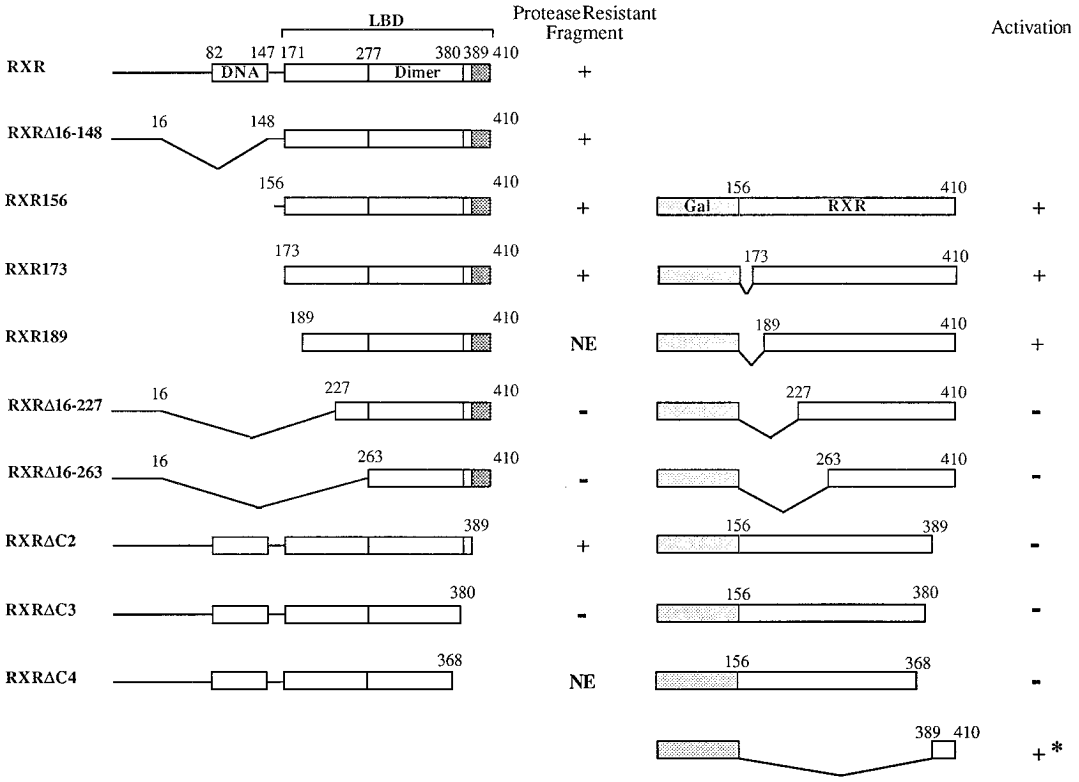

FIG. 2. A schematic representation of various deletion mutants of RXR and Gal94-RXR fusions. Full-length RXR in our constructs consists of 448 amino acids. With reference to other steroid receptors, the sequences from amino acids 1 to 81, 82 to 146, 147 to 170, and 171 to 410 are designated as domains A-B, C, D, and E, respectively (31). The plus sign indicates that the protein retained the protease-resistant fragment (left panel) and the hormone-dependent activation of the corresponding Gal fusion (right panel). The asterisk indicates that the fusion was active in the absence of the ligand. NE, not examined. The names of the expression plasmids were derived according to those of the deletion mutants.

tion of these mutants (data not shown) and rendered all three mutants resistant to trypsin digestion (Fig. 3a). Since the protease-resistant fragment of RXR173 was slightly smaller than the resistant fragment of RXRΔ16-148, we deduced that the N-terminal boundary of the resistant fragment of the full-length receptor was located at one of the three putative trypsin cleavage sites in this region (amino acid 158, 160, or 162). Although the protease-resistant fragment is sufficient for ligand binding, the minimal domain required for ligand binding may be smaller. A further deletion from the N-terminal end (RXR16-227) destroyed the protease resistance (Fig. 3b; compare lanes 3 and 4 to 11 and 12). Thus, these results suggested that the N-terminal border of the RXR LBD lies between amino acids 211 and 265. These data also imply that the extreme N-terminal sequence of the E domain is not important for RXR ligand binding.

The C-terminal truncation mutant RXRΔc2, but not RXRΔc3, can undergo the hormone-induced conformational change. In order to define the C-terminal boundary of the RXR LBD, we constructed RXR C-terminal truncation mutants and tested them in the proteolytic digestion assay. Removal of 21 amino acids from the C terminus (RXRΔc2) did not affect the ligand-induced, protease-resistant conformation, suggesting that these 21 amino acids are not required for hormone binding (Fig. 4a). Similar protease-resistant conformations were also detected with chymotrypsin and V8 protease (data not shown). Since the protease-resistant fragment from RXRΔc2 was clearly smaller than that from wild-type RXR (Fig. 4a; compare lanes 1 to 4 with 9 to 12), the protease-resistant fragment of the full-length receptor must encompass the entire sequence downstream of amino acids 158 to 162. Interestingly, another mutant (RXRΔc3) bearing a further nine-amino-acid deletion from the C terminus yielded no detectable protease-resistant fragment even in the presence of a high concentration of 9-*cis*-RA (Fig. 4a). The ability of RXRΔc2 to retain the protease-resistant fragment in the presence of the ligand was intriguing because removal of the similar region in other receptors, i.e., TR and RAR, destroys the ligand-binding ability of these receptors as well as the protease resistance (30).

To verify that RXRΔc2 could bind 9-*cis*-RA, we employed a gel shift assay. It has been shown previously that 9-*cis*-RA can induce RXR to bind as a homodimer to its response element with a higher affinity than that seen in the absence of the ligand. If RXRΔc2 is able to bind 9-*cis*-RA, we should be able to detect stronger DNA binding in the presence of the ligand. As expected, RXRΔc2 displayed a higher DNA-binding affinity in the presence of the ligand (Fig. 4b). In contrast, we did not detect any DNA binding by RXRΔc3 even though twofold more RXRΔc3 was used in the binding assay (Fig. 4b). This observation was surprising since both RXRΔc2 and RXRΔc3 still contained the intact C-terminal heptad repeats and were able to heterodimerize with TR as efficiently as the wild-type RXR (Fig. 4c).

These data indicate that the boundaries of the RXR LBD are located between amino acids 173 and 227 at the N terminus and 380 and 389 at the C terminus. We conclude that the extreme C-terminal end of the E region is not important for

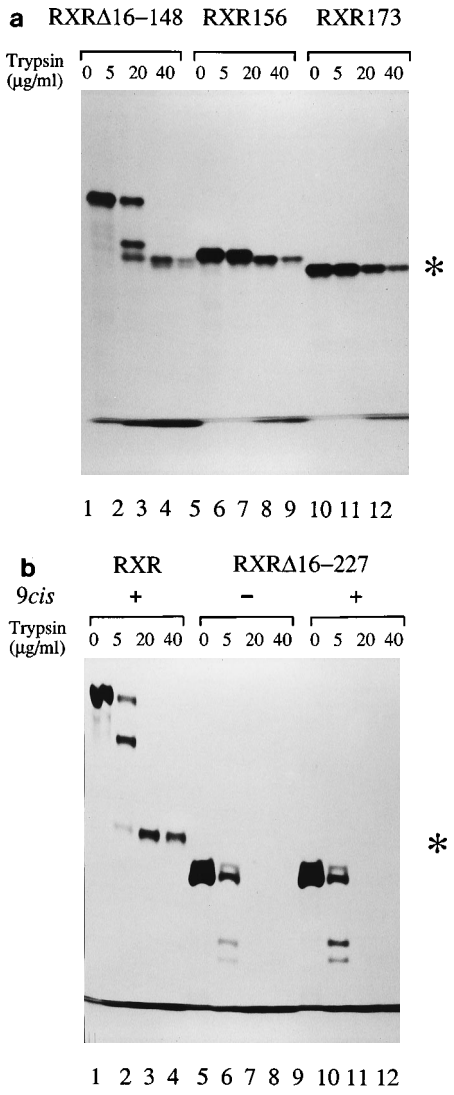

FIG. 3. Protease-resistant fragments of RXR encompass the entire C terminus of the receptor. (a) RXRΔ16-148, RXR156, and RXR173 were all treated with 100 nM 9-*cis*-RA and digested as described in the legend to Fig. 1. The asterisk indicates the resistant fragments. (b) Details are similar to those for panel a, except that RXR and RXRΔ16-227 were used in the analysis.

ligand binding. Moreover, we presented evidence that different regions in the receptor are involved in receptor homodimerization and heterodimerization functions.

A C-terminal truncation converts RXR to a transcriptional repressor. In addition to hormone binding, the LBD contains regions important for dimerization and transactivation. Since proteolytic analysis has enabled us to dissect the minimal region of RXR for ligand binding, it was of interest to determine whether this region is sufficient for transactivation. Various fragments of the C terminus of RXR were then fused to the yeast Gal4-DBD (amino acids 1 to 94) (Fig. 2). The Gal4-DBD contains dimerization and nuclear localization functions, thus eliminating interpretation problems with receptor mutations affecting these functions. The Gal4 receptor deletion mutants were cotransfected into CV1 cells together with a reporter containing a single Gal4 binding site upstream of the tk promoter CAT reporter. As expected, fusion of the complete C terminus of mRXRβ to the Gal4-DBD (Gal94-RXR156) results in a protein that activated reporter gene expression upon addition of 9-*cis*-RA (Fig. 5). This confirms that the E domain of RXR is a functional and independent transcriptional module.

As expected, Gal94-RXR173 exhibited ligand-dependent activation, whereas Gal94-RXR227 and Gal94-RXRΔc3 could not activate transcription (Fig. 5a). In fact, as summarized in Fig. 2, there was a close correlation between the transactivation activity of a fusion protein and hormone-binding ability, as determined by the protease resistance of the receptor fragment. The only exception, however, was the RXRΔc2 mutant; Gal94-RXRΔc2 had no hormone-dependent transactivational activity (Fig. 5a), even if higher concentrations of hormone (10^{-5} M) were used (data not shown). Since removal of 21 amino acids from the receptor C terminus did not hamper the ligand-binding ability, the inability of Gal94-RXRΔc2 to transactivate suggested that the 21 C-terminal amino acids must be important for transactivation. Therefore, we were able to separate the LBD from the ligand-dependent transactivation.

Interestingly, on closer examination, we found that the CAT activity derived from Gal94-RXRΔc2 and Gal94-RXRΔc3 in the absence of the ligand was actually lower than the reporter construct transfected with a control vector containing only the Gal4-DBD (Gal94). In order to confirm this repression, we used tkCAT linked to two copies of the Gal4 binding site as a reporter to increase the silencing effect. As shown in Fig. 5b, Gal94-RXRΔc2 and Gal94-RXRΔc3 actively silenced the basal promoter activity about fivefold. The full-length construct, Gal94-RXR156, on the other hand, had no effect (Fig. 5b). The repression activity was abolished when 42 amino acids were truncated from the C terminus of RXR (Gal94-RXRΔc4). These results suggest that the 21 C-terminal amino acids of the receptor suppress an intrinsic repression activity within the receptor and that deletion of this sequence converts the receptor to a negative regulator.

Since RXRΔc2 can still form homo- or heterodimers, one would expect, given its intrinsic repression function, that this mutant may function in a dominant negative fashion when coexpressed with other receptors into cells. In order to examine the repression function in the context of the wild-type receptor and a natural response element, RXR and RXRΔc2 were cotransfected with CRBPII×2 tkLuc into P19 cells. As predicted, cotransfection of increasing amounts of RXRΔc2 gradually diminished the ligand-induced activation of wild-type RXR on a reporter driven by a natural RXRE (Fig. 5c; compare results with and without cotransfection with RXRβΔc2). For unknown reasons, the effects of RXRΔc2 on RAR- and TR-mediated transactivation were far more complicated. Although it can potentiate T3 responsiveness on the myosin heavy-chain (MHC) TRE (29), RXRΔc2 blocks RAR- and TR-dependent activation of reporter genes driven by βRARE and ME TRE, respectively (unpublished data). Nevertheless, the fact that RXRΔc2 can function as a powerful dominant negative receptor substantiates the identification of the masked intrinsic repression function of RXR and strongly suggests that the C-terminal amino acids play an important role in the transcriptional activation function.

A conserved stretch in the last 21 amino acids of RXR is a functional activation domain. The results presented above suggested that the 21 C-terminal amino acids of RXR are important for transactivation. Alignment of these 21 amino acids with the C-terminal ends of other members of the TR-RAR nuclear hormone receptor subfamily revealed a high homology within the sequence (Fig. 6a). Computer modeling of these 21 amino acids predicted an amphipathic helical structure (Fig. 6b). This is reminiscent of the C-terminal regions of TR and

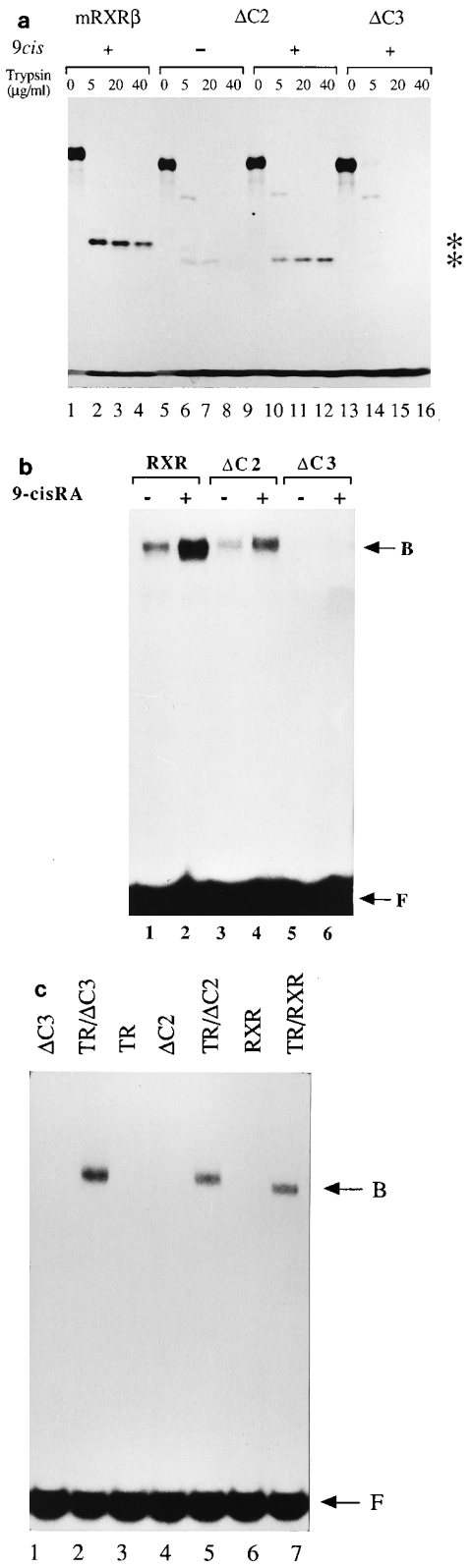

FIG. 4. RXRΔc2 but not RXRΔc3 can still bind the ligand. (a) mRXRβ, RXRΔc2 (Δc2), and RXRΔc3 (Δc3) were treated and digested as described in the legend to Fig. 1. Equal volumes of translation product for each protein were used since the translation efficiencies for RXR, RXRΔc2, and RXRΔc3 were similar. The asterisks indicate the resistant fragments. (b) Addition of 9-*cis*-RA enhanced the binding affinity of in vitro-translated RXR and RXRΔc2 to a DR1 element. One microliter of RXR or RXRΔc2 was used in total 10-μl gel shift reactions, whereas 3 μl of RXRΔc3 was added. A total of 100 nM 9-*cis*-RA was included as indicated. B and F indicate the DNA-protein complex and the free probe, respectively. (c) Both RXRΔc2 and RXRΔc3 can heterodimerize with TR. One microliter of TR and/or 0.5 μl of RXR, RXRΔc2, and RXRΔc3 were incubated with ^{32}P-labeled MHC TRE elements in a gel shift assay. DR1 and MHC TRE oligonucleotides were described previously (8). B and F indicate the DNA-protein complex and the free probe, respectively.

the receptors for estrogens and glucocorticoids, which are also capable of forming a putative amphipathic α-helix (10, 41). Substitution of one of the conserved hydrophobic residues with a proline residue destroys the helical structure as well as the ligand-dependent transactivation of TR (41). Consequently, this putative α-helical region, known as AF-2, was implicated in the C-terminal ligand-dependent activation function. In order to prove directly that these 21 amino acids can serve as a transactivation domain, the region was fused to the Gal4-DBD. Again, cotransfection experiments were performed in CV1 cells and mouse fibroblast Lmtk$^-$ cells. Gal94-C21 led to a protein that was active in both cell lines (Fig. 6c). These results suggest that although the last 21 amino acids are functional alone, the sequence is subject to hormonal regulation if embedded in its natural context of the RXR LBD. We believe it corresponds to the activation domain referred to as AF-2 in RARα or τ4 in TRβ (4, 47). Complete removal of this activation domain from RXR does not abolish the ligand-binding function of RXR. Thus, RXR differs from RAR and TR in that the AF-2 and τ4 activation functions are not separable from the ligand-binding function (4, 47). Removal of AF-2 or a portion of τ4 eliminates 9-*cis*-RA or T3 binding to RARα and TRβ, respectively.

DISCUSSION

The C terminus of a nuclear receptor, which encompasses the D and E-F regions, has a complex structure with multiple functions. Since the D region is highly variable in length and poorly conserved in sequence, it has been very difficult to separate the D region from the E region. Taking advantage of the protease-resistant conformation change in the LBD upon ligand binding, we have been able to localize a region essential for ligand binding (30). Using limited proteolysis, we defined a 200-amino-acid region in the E region of RXR that is essential for hormone binding (Fig. 2).

It has been suggested that, at least for the TR-RAR subfamily, both the N- and C-terminal ends of the E domain are necessary for ligand binding (14, 16). We have demonstrated previously that the removal of eight amino acids from the extreme C terminus of the human TRβ E region or part of the D region of human RARα eliminates the ligand-dependent conformational change in TR and RAR (30). In the "regulatory zipper" model presented by Forman and Samuels (14), the central part of the E region is devoted to dimerization and contains a so-called τi domain implicated in the ligand-dependent transactivation function. For RXR, however, this is not the case. Our studies clearly show that the truncation of 18 (RXR189) and 21 (RXRΔc2) amino acids from the N and C termini of RXR's E region, respectively, did not abolish the ligand-binding ability of the receptor. RXR is more distinct from other members of the subfamily when compared with RARα. For RARα, the determinant for high-affinity 9-*cis*-RA binding resides in a stretch of 15 amino acids (corresponding to amino acids 393 to 408 of mRXRβ) (47). Deletion of this region from RARα completely destroys the 9-*cis*-RA-induced conformational change and its ligand binding. Thus it has been

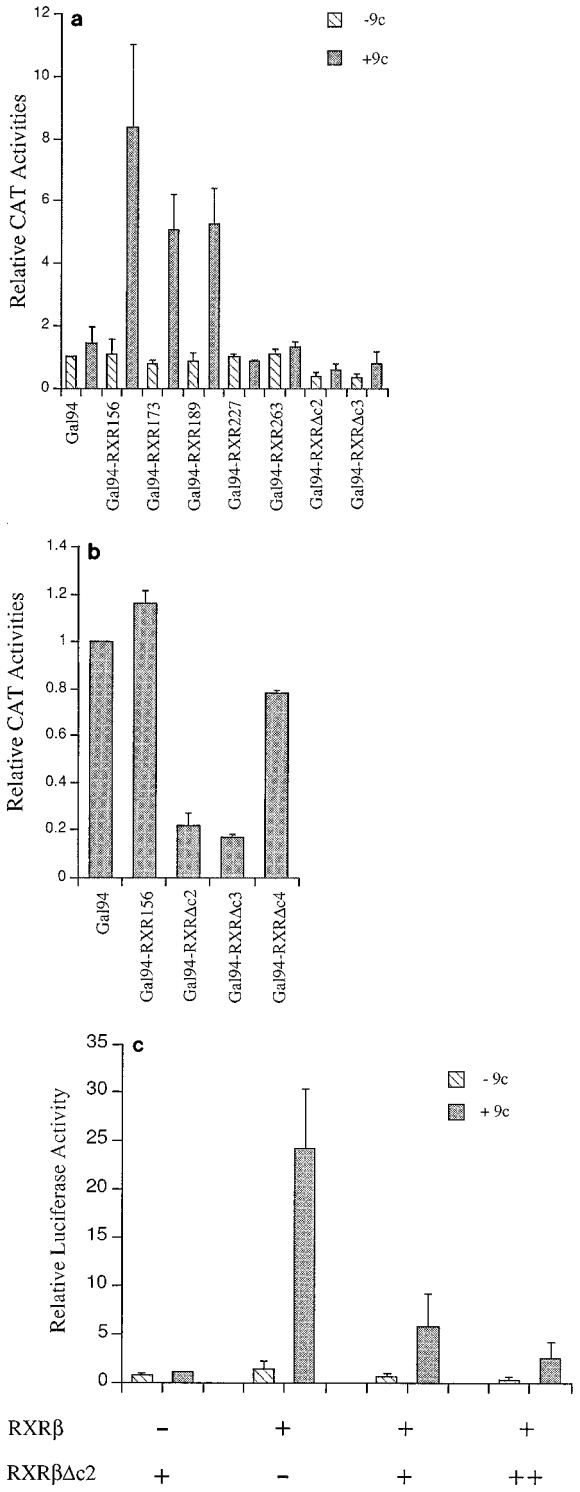

FIG. 5. Transcriptional activation and repression by RXR mutants. (a) Comparison of 9-*cis*-RA induction of transactivation of 17mer-tkCAT by RXR deletion mutants in CV1 cells. Transfections were performed with equal amounts of expression vectors and reporters. After transfection, the cells were incubated in the absence (−) or presence (+) of 9-*cis*-RA (10^{-7} M) for 36 to 42 h. Cell extracts were subsequently prepared and assayed for protein concentration and CAT activity. CAT activities of all mutants were normalized to that of Gal94. Error bars indicate standard deviations. (b) Gal94-RXRΔc2 and Gal94-RXRΔc3 can actively repress basal promoter activity. The reporter 17mer ×2 tkCAT was cotransfected with the receptor deletion mutants. Conditions were the same as those described for panel a. CAT activity of Gal94 served as basal promoter activity. Results shown are a summary of at least two experiments done in duplicate. Error bars indicate standard deviations. (c) RXRβΔc2 can reduce the 9-*cis*-RA-dependent activation of wild-type RXR. Undifferentiated P19 cells were transfected with CRBPII×2 tkLuc, pCH110, and expression vectors for mRXRβ and/or mRXRβΔc2 (20 ng for each +). Cells were incubated with 1 μM 9-*cis*-RA for 24 h before being harvested in lysis buffer. Transfection efficiency was normalized against control βGal activities. The relative luciferase activity of each assay was normalized to that of control pCNX2 vector in the absence of the ligand. Results shown are a summary of three independent experiments. Error bars indicate standard deviations.

suggested that the conserved amino acid sequence within this region, Asp-Met-Leu-Asp, may be important for 9-*cis*-RA binding (47). Our results show that, at least for mRXRβ, we can remove the Asp-Met-Leu-Asp residues (amino acids 401 to 404) by truncating up to amino acid 389 without abolishing 9-*cis*-RA binding by the receptor. Thus, we believe there is a separate determinant(s) in the RXR LBD that directs 9-*cis*-RA binding.

The fact that the 21 extreme C-terminal amino acids were not essential for ligand binding does not suggest that they are the F region. Although the exact function of the F region is not clear, deletion of the F region from estrogen receptor does not affect any known function of the receptor (25). Removal of these 21 amino acids from RXR, however, abolished the ligand-dependent transactivation. In fact, when fused to the Gal4-DBD, these 21 amino acids functioned as an activation domain. These results are consistent with previous findings which suggest that similar regions in TR and RAR also act as activation domains (τ4 and AF-2, respectively) (4, 47). The difference between the 21 amino acids in RXR and those in TR or RAR is that τ4 and AF-2 are not separable from the receptor ligand-binding function. Nonetheless, it is feasible that this region is important for ligand-dependent activation in many other nuclear receptors because similar regions of other receptors share significant conservation in the primary sequence.

Although deletion of the last 21 amino acids (Gal94-RXRΔc2) eliminated ligand-dependent activation of RXR, it is quite possible that RXR has other ligand-dependent activation domains. In recent studies with human TRβ, three activation domains have been identified in the TR LBD (4). Similarly, a small deletion into the τ4 region (homologous to the 21 amino acids in RXR) renders the thyroid hormone receptor completely inactive. It is likely that this 21-amino-acid sequence may serve as an interaction site for an important cellular factor(s), which, when deleted, renders the receptor inactive.

Unlike other members in the subfamily (i.e., TR and RAR), full-length RXR has not been reported to contain a silencing function on any known RXRE. Thus, it was interesting to discover that Gal4-RXRΔc2 and Gal4-RXRΔc3 were active transcriptional repressors and that RXRΔc2 can function in a dominant negative fashion. Recent studies suggest that (i) the repression function of a nuclear receptor (i.e., TR or RAR) is mediated though an interaction with a corepressor(s) and the general transcriptional factor TFIIB, and (ii) hormone-dependent derepression involves the release of these interactions (2, 4). We also suggest that at least the C-terminal activation domain of TR is required for the hormone-dependent release of a corepressor(s) since a small truncation of this activation domain results in a constitutive silencer which still retains the hormone-binding ability (4). Since RXR displays significant homology with TR and RAR, it is likely that RXR could have a similar domain(s) required for these interactions. Considering that the C-terminal activation domain is essential for hormone-dependent release of repression, we believe this is what

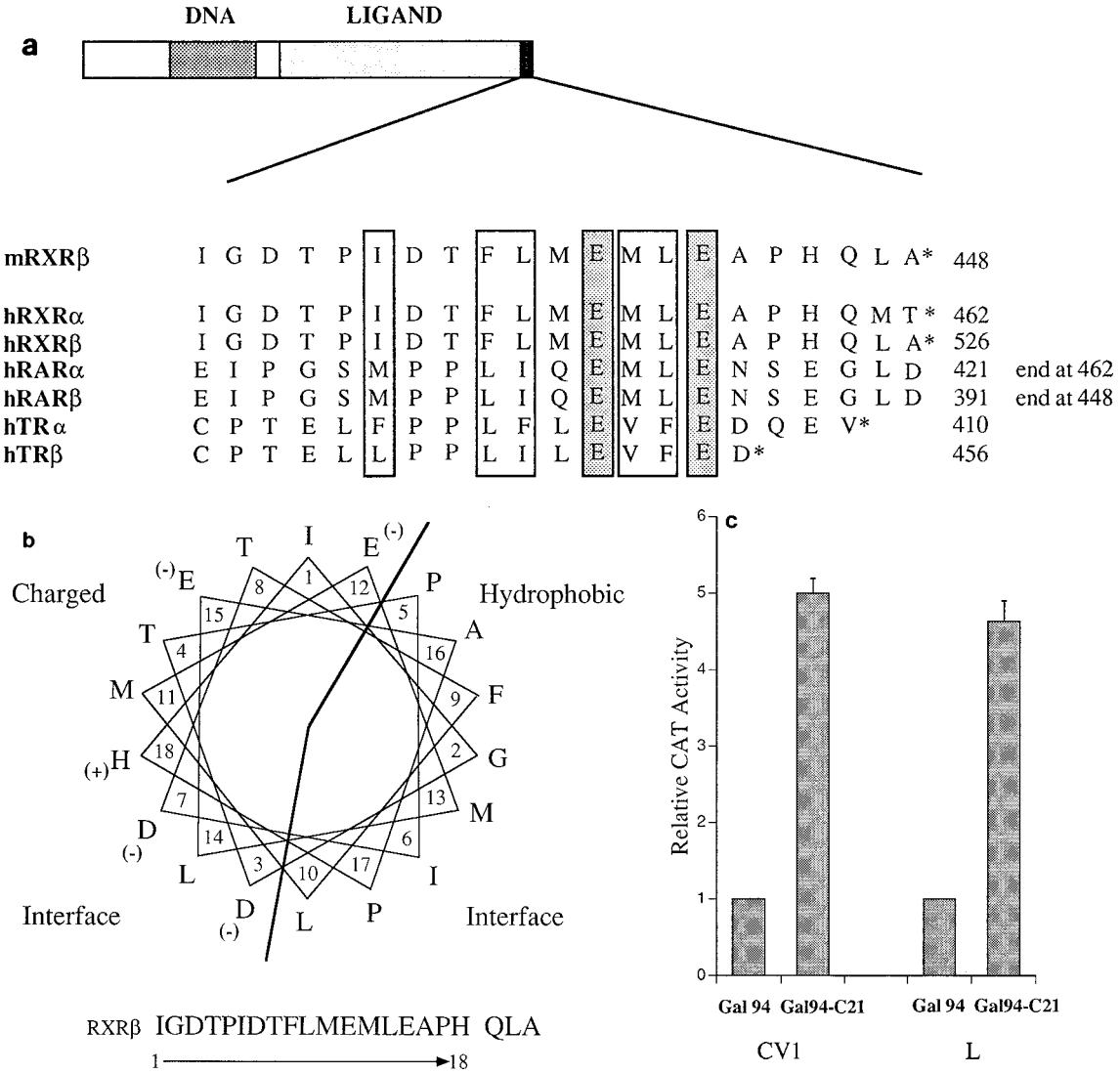

FIG. 6. The 21 amino acids of the RXR C terminus are a functional activation domain. (a) Sequence comparison of 21 RXR C-terminal amino acids with similar regions of other receptors in the subfamily. Numbers indicate the positions of amino acids. Open boxes outline the conserved hydrophobic amino acids, and shaded boxes indicate the identical acidic amino acids. *, end of receptor. (b) Computer modeling demonstrates that the 21-amino-acid region of RXR can form an amphipathic helical structure. The Genetics Computer Group (University of Wisconsin) program was used to illustrate the helical-wheel model of this region. Numbers and (+) and (−) indicate the position and the net charge of each amino acid, respectively. (c) Gal94-C21 is transcriptionally active in both CV1 and Lmtk$^-$ (L) cells. The Gal94-C21 expression vector (2 μg) was cotransfected with 5 μg of 17mer tkCAT reporter into the cells. Conditions were the same as those described in the legend to Fig. 5a. Results shown are a summary of three experiments done in duplicate. Error bars indicate standard deviations.

distinguishes RXR from TR and RAR. Since the sequence of the activation domain is involved in ligand binding for TR and RAR, it is possible that this domain may not be available to release repression prior to binding a hormone. For RXR, however, the C-terminal activation domain is functionally and structurally separable from ligand binding, and it could be available to prevent the interaction of RXR with a corepressor(s) involved in transcriptional silencing. Deletion of this region (RXRΔc2 and RXRΔc3), therefore, could permit the receptor to interact with factors which facilitate repression. However, it remains to be determined if the intrinsic repression function of RXR can also be unmasked upon binding to certain DNA response elements, as many recent studies have indicated that DNA response elements have profound influences on receptor-mediated transactivation (7, 11, 29, 42).

We noted that a further truncation of 9 amino acids (RXRΔc3) beyond the 21-amino-acid activation domain abrogated the ligand-induced, protease-resistant conformation. Very likely, the truncation interfered with the ligand-binding pocket of the receptor. Moreover, this nine-amino-acid region is important for receptor homodimerization, as we could not detect any high-affinity binding of homodimers of RXRΔc3 to DNA. In contrast, RXRΔc3 could still form heterodimers with TR or RAR as efficiently as the full-length RXR. Thus, using this mutant, we could preferentially inhibit RXR homodimer formation without impairing its function as a heterodimer coregulator with other nuclear receptors.

It is noteworthy that although the Gal4-receptor fusion system diminishes the interferences of the endogenous receptors, it could not eliminate the potential for homo- and heterodimerization between Gal4-receptor fusions and endogeneous receptors, especially in the presence of the ligand (53).

Since such dimers are very unlikely to bind to the 17mer DNA element (4, 28), potential dimerization between Gal4-receptor chimeras and endogenous receptors will not affect our conclusions about the repression function of Gal4-RXR mutants. Nevertheless, the ability of endogenous receptors to form non-DNA-binding dimers with the Gal4-receptor fusions in the presence of the ligand could explain the ligand-induced partial release of the silencing function of Gal4-RXR chimeras (Fig. 5a). We also wish to point out that using a heterologous DBD (Gal4) and response element (17mer) may overlook the important contribution of the hormone response element in the receptor activation of target genes (26, 42).

During the review process for this paper, we learned that Zhang et al. identified an almost identical region in human RXRα that determines the homo- and heterodimeric formation (54). In terms of dimerization and DNA binding, their mutants ΔRXR2, ΔRXR3, and ΔRXR4 (which have deletions of 19, 29, and 49 amino acids from the C terminus, respectively) function essentially the same as our RXRΔc2, RXRΔc3, and RXRΔc4, respectively (which have deletions of 21, 30, and 42 amino acids from the C terminus, respectively). Sequence comparison indicates that the regions separating the homo- and heterodimerization are identical. In a manner similar to that of their human RXRα counterparts, RXRΔc2 and RXRΔc3 could also potentiate the T3 responsiveness of the MHC TRE in cotransfection assays (29, 54). However, the scenario is more complicated with these RXRβ deletion mutants, since we could show that their exact function as heterodimeric partners is at least partially mediated by the specific sequence of a response element (29). Moreover, because of the intrinsic repression function of RXRβ, RXRΔc2 can even function as a powerful dominant negative receptor on certain response elements when cotransfected with RXR, TR, RAR, and the vitamin D_3 receptor (unpublished data). Thus, although ΔRXR2 of human RXRα and RXRΔc2 of mRXRβ share an identity in their E regions of more than 90%, they may function differently as heterodimeric partners on certain response elements. This is reminiscent of the recent studies by Saatcioglu et al., in which they have identified a novel response element that can discriminate the function of TR isoforms (42).

In summary, we have delineated the boundaries of the LBD and have subsequently isolated and characterized an activation domain of mRXRβ. These two regions can be physically separated without destroying either of their functions. Thus we have functionally dissected the ligand-binding and transactivation functions of the nuclear receptor RXRβ. Removal of this activation domain, however, converts the receptor into a repressor. We also observed that the deletion of 30 amino acids from the extreme C terminus impaired RXRβ homodimer formation and DNA binding but left intact the capacity for RXRβ heterodimerization with other receptors.

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