Characterization of the Repressor Function of the Nuclear Orphan Receptor Retinoid Receptor-related Testis-associated Receptor/Germ Cell Nuclear Factor*

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Retinoid receptor-related testis-associated receptor (RTR)/germ cell nuclear factor is a nuclear orphan receptor that plays an important role in the control of gene expression during early embryonic development and gametogenesis. It has been shown to repress transcriptional activation. In this study, we further characterize this repressor function. We demonstrate that RTR can suppress the transcriptional activation induced by the estrogen receptor related-receptor al through its response element. The latter is at least in part due to competition for binding to the same response element. In addition, RTR inhibits basal transcriptional activation, indicating that it functions as an active repressor. Mammalian two-hybrid analyses showed that RTR interacts with the co-repressor nuclear co-repressor (N-CoR) but is unable to interact with the co-repressor SMRT or RIP140. Pull-down analyses with glutathione S-transferase-RTR fusion protein demonstrated that RTR physically interacts with N-CoR in vitro, suggesting a potential role for N-CoR in the transcriptional repression by RTR. To identify the regions in RTR essential for the binding of RTR to N-CoR, the effect of various deletion and point mutations on this interaction was examined. This analysis revealed that this interaction requires the hinge domain, helix 3 as well as the helix 12 region of RTR. The residues Ser246-Tyr247 in the hinge domain, Lys318 in helix 3, and Lys498-Thr496 in helix 12 are identified as being critical in this interaction. Our results demonstrate that RTR can function as an active transcriptional repressor and that this repression can be mediated through interactions with the corepressor N-CoR. We show that this interaction exhibits several characteristics unique to RTR. Through its repressor function, RTR can suppress the induction of transcriptional activation by other nuclear receptors. These repressor activities may provide important mechanisms by which RTR regulates gene expression during development and gametogenesis.

The nuclear receptor superfamily constitutes a group of ligand-dependent transcriptional factors and a large number of orphan receptors whose ligands have not yet been identified (1–3). Nuclear receptors share a common modular structure composed of several domains that have functions in DNA binding, ligand binding, nuclear localization, dimerization, repression, and transactivation (4, 5). Typically, ligand binding induces a conformational change in the receptor causing dissociation of bound corepressors, such as nuclear co-repressor (N-CoR)6 or silencing mediator for retinoid and thyroid hormone receptors (SMRT), and the recruitment of co-activators (4, 6). The latter leads to histone acetylation, changes in chromatin conformation, and subsequently to transactivation of target genes and changes in the biological functions of cells.

The orphan nuclear receptor, retinoid receptor-related testis-associated receptor (RTR), also named germ cell nuclear factor (NR6A1; Receptor Nomenclature Committee), has been cloned from mouse (7, 8), human (9–12), zebrafish (13), and Xenopus laevis (14). Sequence comparison has shown that RTR is highly conserved between species, suggesting functional conservation during evolution. RTR has an important role in embryonic development as well as in the adult. It is expressed in embryonic stem cells and differentially regulated during retinoid-induced differentiation of embryonal carcinoma and embryonic stem cells (9, 15, 16). During embryonic development, expression of RTR mRNA has been observed during several stages of neuronal development (14, 17, 18). Both RTR mRNA and protein have been detected in the placenta where its expression is restricted to trophoblasts (19).2 The importance of RTR in development was further demonstrated by targeted disruption of the RTR gene (20). Mouse RTR−/− embryos died between days 10.5 and 11.5 of development and displayed open neural tubes while the critical link between chorion and allantasis was not formed. In the adult, RTR expression is much more restricted, and RTR is most abundant in ovary and testis. In the ovary, it was found to be expressed in maturing oocytes before the first meiotic division (7). In the testis, RTR mRNA is differentially regulated during spermatogenesis and present in postmeiotic cells particularly in round spermatids (7, 8, 21–23). These observations suggest a specific role for this receptor in the control of gene expression at this distinct stage of spermatogenesis. Protamine 1 and 2, which are induced in round spermatids, have been identified as potential target genes for RTR regulation (22, 24).

RTR displays the common modular structure characteristic for nuclear receptors, but its helix 12 region is unusual in that it does not contain the consensus AF2 sequence ΦX(E/D)ΦΦ.

The abbreviations used are: N-CoR, nuclear co-repressor; RTR, retinoid receptor-related testis-associated receptor; DBD, DNA-binding domain; LBD, ligand binding domain; ERR, estrogen receptor-related receptor; SMRT, silencing mediator for retinoid and thyroid hormone receptors; RE, response element; GST, glutathione sulfotransferase; PCR, polymerase chain reaction; CAT, chloramphenicol acetytransferase; UAS, upstream activating sequence; CHO, Chinese hamster ovary; EMSA, electromophoretic mobility shift assay; RIP140, receptor-interacting protein 140.

2 D. Mehta and A. M. Jetten, unpublished observations.
Repressor Function of RTR/Germ Cell Nuclear Factor

...containing the ERR1 (ERRα1) likely through competition for the same response element. These results indicate that RTR may regulate biological processes by interfering with the transcriptional activation by other nuclear receptors. In addition, we show that RTR can function as an active repressor. To repress transcription RTR must communicate with the basal transcription apparatus either directly or indirectly via interaction with protein intermediates. We demonstrate that RTR is able to interact with the co-repressor N-CoR (30, 31) but not with the co-repressor SMRT (32) or RIP-140 (33, 34), suggesting a potential role for N-CoR in the transcriptional repression by RTR. The nature of the interaction between nuclear receptors and N-CoR has been reported to differ substantially between receptors (30, 35–37). To identify the regions and residues in RTR critical in the binding of RTR to N-CoR, we examined the effect of various deletion and point mutations on this interaction. Our study revealed that the hinge domain, helix 3, and the helix 12 region of RTR each are essential in the binding of RTR to N-CoR and demonstrates that the interaction of RTR with N-CoR exhibits several unique characteristics. We believe that these repressor activities will provide important mechanisms by which RTR controls biological processes during development and gametogenesis.

EXPERIMENTAL PROCEDURES

Plasmids—The vector pSG5-VP16 containing the VP16 activation domain was obtained from Dr. J. Lehmann (Tularik Inc., San Francisco, CA). The expression plasmids pZeovSV-RTR encoding full-length mRTR and pSG5-VP16RTR encoding the VP16 (activation domain) fused to the full-length mRTR were described previously (22). The Gal4N-RIPI3N4 expression plasmid encoding ID-I and ID-II of RIPI3/NCr was kindly provided from Dr. D. Moore (Baylor College of Medicine, Houston, TX) (31). The expression plasmid pcDNA3.1c-N-CoR encoding RG was kindly provided by Dr. M. Privalsky (University of California Davies) (38, 39). The plasmid expression plasmid pDNA3.1-ERR1 programmed reticulocyte lysate system from Promega. The GST and RTR-RIP13/NCr were used in binding buffer and then boiled in 30 μl of 2× SDS-polyacrylamide gel electrophoresis loading buffer. Solubilized proteins were separated by 8% SDS-polyacrylamide gel electrophoresis, and the radioactivity was visualized by autoradiography.

Electrophoretic Mobility Shift Assays—Double-stranded ERR1-RE (5'-GACACCTTCAGGTCTACCTG-3') oligonucleotides were end-labeled with [γ-32P]ATP by T4 polynucleotide kinase (Promega). RTR and ERR1 proteins were synthesized from pcdNA3.1-RTR and pcdNA3.1-ERR1 expression plasmids using the TNT-dependent reticulocyte lysate system (Promega). EMSA was performed as described previously (41) with some modifications. Briefly, 2–6 μl of RTR or ERR1 programmed reticulocyte lysate were incubated on ice in reaction buffer (20 mM Tris-HCl, pH 7.9, 100 mM KCl, 0.1% Nonidet P-40, 10% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.5% nonfat dry milk) with the [35S]methionine-labeled N-CoR. After 1 h of incubation at room temperature, the beads were washed five times in binding buffer and then boiled in 30 μl of 2× SDS-polyacrylamide gel electrophoresis loading buffer. Solubilized proteins were separated by 8% SDS-polyacrylamide gel electrophoresis, and the radioactivity was visualized by autoradiography.

Site-directed Mutagenesis—Point mutations in the hinge domain, helix 3, and C terminal of RTR were introduced using a QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer’s protocol. The pSG5-VP16RTR plasmid was used as parental DNA template. Two oligonucleotide primers were synthesized that are complementary to opposite strands of the vector and contain the desired mutation(s). The oligonucleotide primers were used during PCR, and the PCR product was sequenced by the Sequencing Facility at the National Institutes of Health. The PCR product was used to create ERR1-RE/CAT containing the ERR1 response element TCAAGGTCA were kindly provided by Dr. C. Teng (NIAMS, National Institutes of Health) (38, 39).

Gal4(DBD)SMRT751–1291 and Gal4(DBD)SMRT1292–1495 were kindly provided by Dr. M. Privalov (University of California Davies) (40). Gal4(DBD)RIPI40 was obtained from Dr. S. Kurabayashi (NIAMS, National Institutes of Health). The pG-Coomassie blue R110-LUC reporter plasmids containing five copies of the GAL4 upstream activating sequence (UAS) were purchased from CLONTECH and Stratagene, respectively. The anti-VP16 antibody and pCMVβ reporter vector expressing β-galactosidase were purchased from CLONTECH.

Cell Culture—Chinese hamster ovary (CHO) and CV-1 cells were obtained from American Type Culture Collection and routinely maintained in Ham’s F-12 and Dulbecco’s modified Eagle’s medium, respectively, supplemented with 10% fetal bovine serum.
ERRα1 have been shown to be co-expressed in several cell types, including trophoblasts, embryonic stem cells, and embryonal carcinoma cells (9, 19), we analyzed whether RTR would interfere with ERRα1-induced transcriptional activation. To analyze this, we examined the effect of increasing levels of RTR expression on the transcriptional activation of a CAT reporter by ERRα1 through a natural ERRα1-RE (39). CHO cells were co-transfected with an ERRα1 expression plasmid and an (ERRα1-RE)-CAT reporter gene construct in the presence or absence of the expression plasmid pZeoSV-RTR. As shown in Fig. 1A, ERRα1 caused a 2.5-fold increase in transcriptional activation through ERRα1-RE, whereas RTR strongly inhibited this activation in a dose-dependent manner. Increasing amounts of RTR reduced transactivation to a level severalfold lower than that of the basal (minus ERRα1) transactivation. A similar repression could be observed when the pZeoSV-RTR expression plasmid was co-transfected into CHO cells together with a CAT reporter plasmid under the control of three consecutive RTR-REs (Fig. 1B). These observations indicate that RTR acts as a repressor and can interfere with the transcriptional activation induced by ERRα1 and likely other nuclear receptors able to bind to these REs.

Cross-talk between nuclear receptors can occur at different levels of transcriptional control, including competition for the same heterodimerization partners (42), for binding to the same response elements (43), or for shared co-repressors and co-activators (4, 6). Because of the sequence similarities between ERRα1-RE and the consensus RTR-RE, we determined whether the repression by RTR could be due to competition between the two receptors for binding to the same RE. Electrophoretic mobility shift assays demonstrated that both ERRα1 and RTR were able to bind to ERRα1-RE (Fig. 1C). The RTR- and ERRα1-oligonucleotide complexes migrated at different positions. EMSA using a combination of RTR and ERRα1 showed only two complexes that migrated at the same position as the RTR- and ERRα1-nucleotide complexes. Mammalian two-hybrid analyses using pSG5-VP16RTR and Gal4(DBD)-ERRα1 indicated that ERRα1 and RTR did not interact with each other (not shown). These observations are consistent with the concept that ERRα1 and RTR do not form a heterodimer. Our results further suggest the repression of ERRα1-induced transactivation by RTR is at least in part due to competition for the same binding site.

**Repression of Basal Transcriptional Activation by RTR—** Repression of basal transactivation by RTR could be demonstrated in several ways. As shown in Fig. 2A, Gal4(DBD) cotransfected with (UAS) 5CAT into either CHO or CV-1 cells exhibited basal transcriptional activity while Gal4(DBD)-RTR 149–495, containing the hinge domain and the ligand binding domain of RTR fused to Gal4(DBD), showed a dramatically (about 9-fold) reduced transactivation activity compared with

![Fig. 1. Suppression of ERRα1-RE- and RTR-RE-dependent transactivation by RTR. A, CHO cells were cotransfected with the reporter plasmids (ERRα1-RE)-CAT (0.5 μg) and β-actin-LUC (0.1 μg), the expression plasmid ERRα1 (0.5 μg), and increasing amounts of RTR expression plasmid pZeoSV-RTR (0.1, 0.3, or 0.5 μg). B, cells were cotransfected with the reporter plasmids (RTR-RE) 3-CAT (0.5 μg) and β-actin-LUC (0.1 μg) and ERRα1 (0.1 μg) and increasing amounts of pZeoSV-RTR (0.05, 0.15, 0.3 or 0.5 μg) as indicated. After 48 h cells were collected and assayed for CAT protein levels and luciferase activity. The relative level of CAT protein was calculated and plotted. C, analysis of RTR and ERRα1 binding to ERRα1-RE by EMSA. RTR and ERRα1 proteins were obtained by *in vitro* translation, and their binding to 32P-labeled ERRα1-RE was examined by EMSA as described under "Experimental Procedures." The following lysates were used in EMSA: lane 1, 4 μl of unprogrammed lysate; lanes 2 and 3, 3 and 6 μl of ERRα1 programmed lysate, respectively; lanes 4 and 5, 2 and 4 μl of RTR programmed lysate, respectively; lane 6, 3 μl of ERRα1 plus 2 μl of RTR lysate; lane 7, 6 μl ERRα1 plus 4 μl RTR lysate. RTR-oligonucleotide and ERRα1-oligonucleotide complexes migrated at different positions as indicated on the right.
Gal4(DBD). These results suggest that this region of RTR harbors an active transcriptional repressor function that inhibits basal transcription, likely through the interaction with co-repressors.

If the repression by RTR is mediated through binding of co-factor proteins (such as co-repressors) that mediate the interactions of RTR with the basic transcriptional machinery, increasing concentrations of RTR would lead to squelching of that repression. To test this concept, Gal4(DBD)-RTR149–495 was co-transfected with (UAS)5-CAT into CHo cells along with increasing amounts of pZeoSV-RTR expression plasmid. As shown in Fig. 2B, overexpression of RTR, which by itself did not affect basal promoter activity, can squelch the transcriptional repression by Gal4(DBD)RTR in a dose-dependent manner, presumably by competing for the limiting amounts of co-repressor(s) in the cell. These results suggest that interactions with co-repressors play a pivotal role in RTR-mediated transcriptional repression.

Analysis of the Interaction of RTR with Different Co-repressors—Repression of transcription by nuclear receptors involves interaction of the receptor with specific co-factors. N-CoR and SMRT are two co-repressors that have been reported to interact with several different nuclear receptors. RIP140 has been reported to function as a co-repressor as well as co-activator (33, 34). RIP140 can bind several different nuclear receptors and may repress transcription by competing with co-activators for binding to ligand-bound receptors (33). To determine whether any of these co-repressors could be involved in the transcriptional repression by RTR, we examined by mammalian two-hybrid analysis the interaction of RTR with these co-repressors. CHO cells were co-transfected with (UAS)5-LUC reporter plasmid and either Gal4(DBD)-N-CoR, Gal4(DBD)-SMRT, or Gal4(DBD)-RIP-140 in the presence or absence of pSG5-VP16RTR DNA (Fig. 3). These results showed that N-CoR but not SMRT or RIP140 was able to interact with RTR under the conditions tested, suggesting that the repression by RTR could be mediated through interactions with the co-repressor N-CoR. RTR was unable to bind the co-activators SRC-1 and CBP (not shown).

To analyze the interaction between RTR and N-CoR in more detail, its dependence on the VP16-RTR concentration as well the ability of RTR to squelch this interaction were examined (Fig. 4). CHO cells co-transfected with Gal4(DBD)-N-CoR and (UAS)5-LUC reported plasmid either pSG5-VP16RTR in the presence or absence of pSG5-VP16RTR DNA as indicated in Fig. 3. These results showed that N-CoR but not SMRT or RIP-140 was able to interact with RTR under the conditions tested, suggesting that the repression by RTR could be mediated through interactions with the co-repressor N-CoR. RTR was unable to bind the co-activators SRC-1 and CBP (not shown).
Repressor Function of RTR/Germ Cell Nuclear Factor

A

![Graph](image1)

**Fig. 5.** RTR interacts with N-CoR in GST pull-down assays. GST and GST-RTR fusion protein were bound to glutathione-Sepharose 4B beads. After extensive washing, labeled bound proteins were separated by SDS-electrophoresis and visualized by autoradiography. The input represents 20% of the radiolabeled protein used in the binding assay.

B

![Graph](image2)

**Fig. 4.** Characterization of the interaction between RTR and N-CoR by mammalian two-hybrid analysis. A, CHO cells were cotransfected with (UAS)_{5}-CAT (0.5 μg) and β-actin-LUC (0.1 μg), the expression vector Gal4(DBD)-N-CoR (0.5 μg) and increasing amounts of pSG5-VP16RTR expression plasmid (0.025, 0.05, 0.075, 0.1, 0.2, or 0.3 μg). After 48 h, cells were collected and assayed for CAT protein levels and luciferase activity.

control levels. These results further establish that RTR but not VP16 interacts efficiently with N-CoR. As shown in Fig. 4B, co-transfection with increasing concentrations of pZeoSV-RTR plasmid inhibited the transcriptional activation by VP16RTR. This squelching is could be due to competition between RTR and VP16RTR for binding to N-CoR or other nuclear proteins.

**RTR Interacts with N-CoR in Vitro**—The interaction between RTR and N-CoR was further examined in vitro by GST pull-down analysis. GST-RTR_{149–495} fusion protein and GST were immobilized on glutathione-Sepharose beads and then incubated with [35S]methionine-labeled N-CoR. After extensive washing, labeled bound proteins were separated by SDS-electrophoresis and visualized by autoradiography. Fig. 5 shows that [35S]labeled N-CoR was able to bind to GST-RTR but not to GST alone, suggesting that this binding is specific for RTR. This observation demonstrates that N-CoR is able to interact with RTR in vitro and supports the results obtained by two-hybrid analysis.

The C-terminal Helix 12 of RTR Is Essential for the Interaction with N-CoR—To identify the domains within RTR that play a role in the interaction with N-CoR, the effect of a series of RTR truncations and point mutations on the interaction with N-CoR was examined by mammalian two-hybrid analysis. In addition, we wanted to examine the role of the unique helix 12 of RTR in this interaction. For this purpose CHO cells were co-transfected with Gal4(DBD)-N-CoR, (UAS)_{5}-CAT, and different pSG5-VP16RTR deletion mutants. We first examined the effect of several C-terminal RTR deletion mutants on the interaction with N-CoR (Fig. 6A). RTR_{1–149}, containing only the N terminus and the DBD, and RTR_{1–268}, which also includes the hinge domain, did not induce reporter activity, indicating that these regions are not sufficient to promote interaction with N-CoR. All the smaller C-terminal deletions, even the deletion of the last 9 amino acids, abolished the interaction of RTR with N-CoR. These results indicate that the C terminus of RTR is essential in RTR/N-CoR interactions.

The helix 12 region constitutes the very C-terminal end of RTR (from Lys^{482} through Glu^{495}; Fig. 6) (7, 8, 26). The amino acid sequence of helix 12 of RTR (Fig. 6B) is unique in that it does not contain the nuclear receptor AF-2 consensus sequence (25). The importance of helix 12 region in nuclear receptor/N-CoR interactions has been reported to be very much dependent on the type of receptor (30, 35–37). To further analyze the role of helix 12 in the interaction of RTR with N-CoR, several point mutations were introduced within this region, and their effects on the interaction of RTR with N-CoR examined in mammalian two-hybrid assays. The mutations introduced into the C terminus are shown in Fig. 6B. Only the RTR mutant containing the double mutation K^{489}A,T^{490}A showed a greatly diminished ability to induce reporter activity in two-hybrid analysis, indicating the critical role of these amino acids in RTR/N-CoR interactions.

The Hinge Domain of RTR Is Essential for the Interaction with N-CoR—We next examined the effect of several N-terminal deletions and the role of the hinge region and LBD on the interaction of RTR with N-CoR. In RTR, the hinge domain stretches from Glu^{419} to Leu^{269}, and the LBD stretches from Ser^{269} to the C terminus. As demonstrated in Fig. 7A, only the pSG5-VP16RTR deletion construct encoding RTR_{149–495}, containing the hinge and LBD domain, induced reporter gene activity to levels similar to that of full-length RTR, suggesting that this region interacts strongly with N-CoR. Further deletion up to Ser^{212} caused a 50–60% decrease in reporter activity, whereas deletion up to Tyr^{240} did not cause any additional
decrease. Transcriptional activation was almost totally abolished with RTR\textsuperscript{269–495}, which lacks the whole hinge domain. The results suggest that two regions in the hinge domain of RTR, one from Gly\textsuperscript{149} to Leu\textsuperscript{211} and the other from Tyr\textsuperscript{240} to Leu\textsuperscript{268}, influence its interaction with N-CoR. In agreement with the results obtained in Fig. 6, RTR\textsuperscript{149–268}, containing the hinge domain only, did not promote interaction with N-CoR significantly. The results from Figs. 5 and 6 indicate that both the hinge domain and the helix 12 region at the C terminus of RTR are required for the interaction with N-CoR.

To analyze the importance of the RTR hinge domain in N-CoR binding further, we examined the effect of several point mutations within this region on RTR/N-CoR interaction. Fig. 7B demonstrates that the double mutation S246G,Y247G almost totally abolished the interaction of RTR with N-CoR, whereas the mutations L254A,P255A and S265A,Y266A had little effect on this interaction. These results further support the critical role of this part of the hinge domain in the interaction with N-CoR.

Importance of Helix 3 in RTR to N-CoR Binding—The region in the LBD containing helices 3–5 is moderately conserved among nuclear receptors (26, 44, 45). Recently, this region has been demonstrated to form the binding surface for the LA\textsuperscript{D}LL motif in co-activators (46) and is in some receptors also involved in co-repressor binding. In RTR, helices 3–5 constitutes the region between Phe\textsuperscript{299} and Val\textsuperscript{350}. Fig. 8 shows that the mutation K318A in helix 3 greatly diminished transactivation in two-hybrid analysis, whereas I317A reduced transactivation by about 50%. Several mutations in helix 4 had little effect on the interaction of RTR with N-CoR. These results demonstrate that residue Lys\textsuperscript{318}, and to a certain extent Ile\textsuperscript{314}, in helix 3 of RTR are critical in N-CoR binding.

DISCUSSION

It is clear from previous observations and from the present study that RTR can function as an active repressor of transcription. In this study, we characterized in more detail this repressor function and identified several regions in RTR that are critical in the interaction with the co-repressor N-CoR. Our results demonstrate that this interaction exhibits differences as well as similarities with those reported for other receptors (30, 35–37).

Regulation of gene expression by nuclear receptors is complicated by the co-existence of multiple nuclear receptor signaling pathways that can interfere with each other. Cross-talk can involve any step in the mechanism by which the nuclear receptors regulate gene transcription, including competition for the same heterodimerization partners, co-repressors or co-activators, or competition for the same REs. A number of nuclear receptors, including ERRs and SF-1, bind REs that are very similar to RTR-REs (7, 22, 26, 27, 47, 48). Moreover, some of these receptors have been demonstrated to be co-expressed in several cell types. For example, embryonal carcinoma and embryonic stem cells express RTR, SF-1, and ERRs (7, 8, 21, 47, 48). RTR and ERR\textsubscript{a} have also been shown to be co-expressed in trophoblasts (19, 49, 50).

Differences in the affinity for the respective DNA element, the expression level of the receptors, and the presence of ligand are contributing factors for the degree of cross-talk between receptors. In this study, we show that RTR and ERR\textsubscript{a} can bind the same response element and that RTR can suppress the transcriptional activation mediated by ERR\textsubscript{a} by competing for binding to the same site. This antagonism could be relevant to the control of gene expression in several cell systems. Previously, we reported that RTR expression is down-regulated during retinoid-induced differentiation in embryonal carcinoma F9 cells (9); this decrease in RTR expression could relieve the repression of SF-1- and
ERR-dependent transactivation of common target genes. Recent studies have identified protamine 1 and 2 as putative target genes for RTR (22, 24). RTR and ERRs could positively and/or negatively control the transcription of these genes. Therefore, cross-talk between RTR and other nuclear receptor signaling pathways may play an important role in the control of gene expression during development and gametogenesis.

We demonstrated that RTR could repress the basal transcriptional activation. The repression of basal transcription could be reversed by increased expression of RTR and is likely due to squelching of the limiting amounts of co-repressor activity in the cell. The observations indicate that RTR can function as an active suppressor of gene transcription.

To repress transcription, nuclear receptors communicate with the basic transcription apparatus indirectly via interaction with protein intermediates, including co-repressors and deacetylases (4, 6). RTR repressor activity likely involves interactions of RTR with various co-repressors, some of which may be highly specific for RTR. Three-hybrid analysis demonstrated that RTR is able to interact with the co-repressor N-CoR but not with SMRT or RIP-140. The interaction with N-CoR was confirmed by pull-down analysis and indicates that these two proteins physically interact with each other. These results suggest a potential role for N-CoR in the transcriptional repression by RTR.

Previous studies have implicated a number of different regions in nuclear receptors in co-repressor interactions and shown similarities and important differences in the way nuclear receptors interact with N-CoR. Our study shows that the interaction of RTR with N-CoR has several unique characteristics. To determine which regions in RTR are critical in the interaction between RTR and N-CoR, we introduced a number of different deletions and point mutations in RTR and determined their effect on RTR/N-CoR interactions by two-hybrid analysis. This analysis identified several subdomains in RTR that are essential in RTR/N-CoR interactions. Although the modular structure of RTR is similar to that of other nuclear receptors, RTR has several unique features. Structural analysis has indicated that the hinge domain of RTR stretches from Gly to Leu, whereas its putative LBD starts at Ile with helix 1 and ends right at the C terminus with helix 12. Crystallographic analysis of the ligand binding domain of RTR has indicated that the C terminus (from Ly through Val) constitutes the H12 region. The sequence of H12 is unusual in that it does not contain the AP2 consensus sequence . Based on these observations it has been suggested that RTR may have a mode of action that is different from that of other receptors (51, 52). Our results indicate that deletion of H12 or the introduction of specific point mutations abolish the interaction with N-CoR, suggesting that H12 is essential for and regulates N-CoR binding. Although several studies have indicated that the conformation of the H12 regulates the association of co-repressors and co-activators with nuclear receptors, the nature of this control can differ substantially between receptors (53–55). The H12 in retinoid X receptor has been found to sterically hinder co-repressor binding (37), whereas deletion of H12 converts apo-retinoid X receptor from a weak repressor to a strong repressor. In the case of estrogen receptor, only antagonist-bound receptor represses transcription and is able to interact with N-CoR (36). Antagonist binding likely causes a shift in the position of H12 of estrogen receptor, thereby exposing the co-repressor binding surface. Deletion of H12 in the TR and RAR receptors enables co-repressors, including N-CoR, to bind even in the presence of

3 Z. Yan and A. M. Jetten, manuscript in preparation.
Repressor Function of RTR/Germ Cell Nuclear Factor

In summary, we demonstrated that RTR functions as an active repressor of gene expression and can inhibit transcriptional activation mediated by other nuclear receptors. Our results suggest a potential role for N-CoR in the transcriptional repression by RTR. Deletion and point mutation analysis identified three RTR subdomains, a specific region in the hinge domain, helix 3, and the helix 12 region, that either provide an interaction interface for some co-repressors and may function as an interaction surface important in the binding of R-CoR or controls indirectly the interaction with N-CoR. Our study shows that this interaction exhibits several characteristics unique to RTR. These repressor activities may provide important mechanisms by which RTR regulates gene expression during development and spermatogenesis.

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REFERENCES

1. Laudet, V. (1997) J. Mol. Endocrinol. 19, 207–226
2. Gigoure, V. (1999) Endocr. Rev. 20, 689–725
3. Willy, P. J., and Mangelsdorf, D. J. (1998) in Hormones and Signaling (O'Malley, B. W., ed) Vol. 1, pp. 308–358, Academic Press, San Diego
4. McKenna, N. J., Xu, J., Nawaz, Z., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999) J. Steroid Biochem. Mol. Biol. 69, 3–12
5. Kumar, R., and Thompson, E. B. (1999) Steroids 64, 310–319
6. Xu, L., Glass, C. R., and Rosenfeld, M. G. (1999) Curr. Opin. Genet. Dev. 9, 140–147
7. Chen, F., Coney, A. J., Wang, Y., Law, S. W., and O'Malley, B. W. (1994) Mol. Endocrinol. 8, 1434–1444
8. Hirose, T., O'Brien, D. A., and Jetten, A. M. (1995) Gene (Amst.) 152, 247–251
9. Lei, W., Hirose, T., Zhang, L. X., Adachi, H., Spinella, M. J., Dmitrovsky, E., and Jetten, A. M. (1997) J. Mol. Endocrinol. 18, 167–176
10. Kapelle, M., Krutzschmar, J., Hasemann, M., and Schleuning, W. D. (1997) Biochim. Biophys. Acta 1352, 17–17
11. Susens, U., and Bormgayer, U. (1996) Biochim. Biophys. Acta 1309, 179–182
12. Agoulnik, I. Y., Che, Y., Niederberger, C., Kieback, D. G., and Coney, A. J. (1998) FEBS Lett. 424, 73–78
13. Braat, A. K., Zandbergen, M. A., De Vries, E., Van Der Burg, B., Boger, J., and Gos, H. J. (1999) Mol. Reprod. Dev. 53, 369–375

FIG. 8. Mutations in helix 3 affect the interaction of RTR with N-CoR. Sequence of the region of RTR containing helix 3 and 4 and the point mutations in residues introduced into pSG5-VP16RTR are shown at the top. To examine the effect of these point mutations on RTR/N-CoR interactions CHO cells were cotransfected with (UAS)5-LUC (0.5 μg), Gal4(DBD)N-CoR (0.5 μg), and one of the mutant pSG5-VP16RTR constructs (0.2 μg). After 48 h cells were collected and assayed for luciferase and β-galactosidase activities.

ligand (30). In contrast, H12 has been shown to be essential in chicken ovalbumin upstream promoter-transcription factor (COUP-TF/V-CoR interactions (35, 56). Our results with RTR show that deletion of the H12 or the introduction of the double mutation K489A,T490A almost totally abolished the interaction with N-CoR. The double mutation S246G,Y247G found to also abolish their interaction with N-CoR and SMRT. Whether the H12 of RTR serves as a structural determinant rather than a direct interface (53, 55). Whether the region Tyr240–Leu268 in the hinge domain of RTR greatly diminishes the binding of N-CoR. This lysine is highly conserved among many nuclear receptors and has been shown to play a key role in the recruitment of co-activators and the ligand-dependent activation of receptors (45). Mutation of the homologous lysine in TR and retinoid X receptor has been found to also abolish their interaction with N-CoR and SMRT (54, 58). These observations suggest that in several receptors, and likely RTR as well, helices 3–5 play a key role in the interaction with co-repressors by serving as a binding surface.

in regulating RTR activation or RTR repressor activity has yet to be established. Recently, we have identified a novel repressor protein referred to as RAP80 that interacts with RTR in mammalian two-hybrid and pull-down analyses. This protein requires solely the region (Tyr240 to Leu268) in the hinge domain of RTR for its interaction and in contrast to N-CoR does not need H12 for binding. We have found that this protein can block the binding of N-CoR to RTR and competes with N-CoR for binding to this site. Whether this competition is based on steric hindrance or binding to the same sequence. These observations suggest that this hinge region plays an important role in controlling the interaction of RTR with several different co-repressors and may function as an interaction surface for some co-repressors. Neither the Gly149–Leu211 nor the Tyr240–Leu268 region of the hinge domain of RTR have sequence homology with the CoR box, a sequence in the hinge domain of TR and RAR, shown to be involved in N-CoR binding (30). The CoR box appears to function as structural determinant rather than a direct interface (53, 55). Whether the region Tyr240–Leu268 in the hinge domain of RTR interacts directly with N-CoR or controls RTR/N-CoR interactions indirectly has yet to be determined.

Recent studies have demonstrated that helices 3–5 of nuclear receptors form an interaction surface important in the binding of co-activators (53, 57). Mutational analysis in this region of the retinoid X receptor and TR receptors have demonstrated the importance of this region also in the binding of co-repressors (54, 58). The point mutation K318A in helix 3 of RTR greatly diminishes the binding of N-CoR. This lysine is highly conserved among many nuclear receptors and has been shown to play a key role in the recruitment of co-activators and the ligand-dependent activation of receptors (45). Mutation of the homologous lysine in TR and retinoid X receptor has been found to also abolish their interaction with N-CoR and SMRT (54, 58). These observations suggest that in several receptors, and likely RTR as well, helices 3–5 play a key role in the interaction with co-repressors by serving as a binding surface.

In summary, we demonstrated that RTR functions as an active repressor of gene expression and can inhibit transcriptional activation mediated by other nuclear receptors. Our results suggest a potential role for N-CoR in the transcriptional repression by RTR. Deletion and point mutation analysis identified three RTR subdomains, a specific region in the hinge domain, helix 3, and the helix 12 region, that either provide an N-CoR binding surface or control indirectly the interaction with N-CoR. Our study shows that this interaction exhibits several characteristics unique to RTR. These repressor activities may provide important mechanisms by which RTR regulates gene expression during development and spermatogenesis.

1. Laudet, V. (1997) J. Mol. Endocrinol. 19, 207–226
2. Gigoure, V. (1999) Endocr. Rev. 20, 689–725
3. Willy, P. J., and Mangelsdorf, D. J. (1998) in Hormones and Signaling (O'Malley, B. W., ed) Vol. 1, pp. 308–358, Academic Press, San Diego
4. McKenna, N. J., Xu, J., Nawaz, Z., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999) J. Steroid Biochem. Mol. Biol. 69, 3–12
5. Kumar, R., and Thompson, E. B. (1999) Steroids 64, 310–319
6. Xu, L., Glass, C. R., and Rosenfeld, M. G. (1999) Curr. Opin. Genet. Dev. 9, 140–147
7. Chen, F., Coney, A. J., Wang, Y., Law, S. W., and O'Malley, B. W. (1994) Mol. Endocrinol. 8, 1434–1444
8. Hirose, T., O'Brien, D. A., and Jetten, A. M. (1995) Gene (Amst.) 152, 247–251
9. Lei, W., Hirose, T., Zhang, L. X., Adachi, H., Spinella, M. J., Dmitrovsky, E., and Jetten, A. M. (1997) J. Mol. Endocrinol. 18, 167–176
10. Kapelle, M., Krutzschmar, J., Hasemann, M., and Schleuning, W. D. (1997) Biochim. Biophys. Acta 1352, 13–17
11. Susens, U., and Bormgayer, U. (1996) Biochim. Biophys. Acta 1309, 179–182
12. Agoulnik, I. Y., Che, Y., Niederberger, C., Kieback, D. G., and Coney, A. J. (1998) FEBS Lett. 424, 73–78
13. Braat, A. K., Zandbergen, M. A., De Vries, E., Van Der Burg, B., Boger, J., and Gos, H. J. (1999) Mol. Reprod. Dev. 53, 369–375
