The Nuclear Corepressors Recognize Distinct Nuclear Receptor Complexes

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The thyroid hormone receptor (TR) and retinoic acid receptor (RAR) isoforms have the capacity to silence gene expression in the absence of their ligands on target response elements. This active repression is mediated by the ability of the corepressors, nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT), to recruit a complex containing histone deacetylase activity. Interestingly, NCoR and SMRT share significant differences in their two nuclear receptor-interacting domains (IDs), suggesting that they may recruit receptors with different affinities. In addition, the role of the receptor complex bound to a response element has not been fully evaluated in its ability to recruit separate corepressors. We demonstrate in this report that the proximal ID in NCoR and SMRT, which share only 23% homology, allows preferential recognition of nuclear receptors, such that TR prefers to recruit NCoR, and RAR prefers to recruit SMRT, to DNA response elements. However, mutations in the TR found in the syndromes of resistance to thyroid hormone can change the corepressor recruited by changing the complex (homodimer or heterodimer) formed on the TRE. These results demonstrate that the corepressor complex recruited can be both nuclear receptor- and receptor complex-specific. (Molecular Endocrinology 14: 900–914, 2000)

INTRODUCTION

The thyroid hormone (TR) and retinoic acid receptor (RAR) isoforms are members of the nuclear receptor superfamily (1). Unlike the majority of the members of this family, the TR and RAR possess ligand-independent activity that leads to the silencing of positively regulated target genes. This silencing activity has been shown to be due to the recruitment of at least two nuclear corepressor proteins, nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) (2–7), which, in turn, recruit a multiprotein complex with histone deacetylase activity that appears to modify chromatin to prevent transcription (8–10). In the presence of their cognate ligands, the TR and RAR isoforms release the nuclear corepressors and recruit members of the coactivator family, which include the p160 family members [steroid receptor coactivator-1 (SRC-1), TIF II, and ACTR], CREB-binding protein (CBP) and p300, pCAF (11–18) (reviewed in Ref. 19), and other coactivators such as p120 (20). Unlike the nuclear corepressors, the coactivator complex possesses histone acetyl transferase activity, which allows for transcriptional activation.

NCoR and SMRT are modular proteins (see Fig. 1) that contain at least three repressing domains in their N termini, and two domains that mediate interactions with the TR and RAR isoforms in their C termini (21–24). In addition to mediating interactions with mSin3, Sun-CoR (25), and other members of the corepressor complex, the central domains of NCoR and SMRT also appear to mediate interactions with the AML-ETO product, which may prevent normal differentiation and lead to acute myelogenous leukemia in patients with this chromosomal translocation (26). In addition, the extreme amino-terminal domain of NCoR appears to be important in the regulation of the mature protein through its interaction with mSIAH2, which allows for proteolytic degradation of NCoR (27).

Both NCoR and SMRT contain two C-terminal interacting domains that mediate interactions with both the TR isoforms and the RAR isoforms. While similar in structure, the more proximal of the interacting domains (ID 2) shares only 23% amino acid homology, while the more distal interacting domains (ID 1) are 53% homologous. Consistent with these differences in structure are results from several different groups, including our own, which suggest that NCoR and SMRT interact differently with nuclear receptors and that specificity may exist in the recruitment of nuclear
corepressors by nuclear complexes. For example, it has been demonstrated by Zamir et al. (22) that the orphan receptor RevErb can interact only with NCoR on its DNA response element and is unable to recruit SMRT. We have demonstrated that the TRβ1 isoform preferentially recruits NCoR rather than SMRT to a DR + 4 response element (24). In addition, using glutathione-S-transferase (GST) pull-down assays and the mammalian two-hybrid system, Wong and Privalsky (28) have shown that separate RAR isoforms can recruit SMRT with different affinities. Taken together, these data indicate that the polypeptides present in the interacting domains of NCoR and SMRT allow for specific interactions with nuclear receptors that may allow for separate biological actions in vivo.

The interactions between the corepressors and the TR isoforms are also influenced by complex formation. We and others have demonstrated that the TRβ1 isoform recruits NCoR preferentially as a homodimer on DNA and that the addition of retinoid X receptor (RXR) causes a diminution in corepressor binding. Indeed, NCoR appears to stabilize the homodimer complex in solution where it normally does not form (24). In contrast, work using the mammalian two-hybrid system and the TRα1 ligand-binding domain (LBD) has demonstrated that RXR can enhance interactions with nuclear corepressors (29). However, this study investigated TR-corepressor interactions in the absence of an underlying thyroid hormone response element, and did not utilize full-length TRs. To address these issues, we studied TRβ1 and RARα and examined their ability to recruit either NCoR or SMRT to their cognate response elements. In addition, we examined the ability of RXR to influence corepressor recruitment in the context of specific nuclear receptors. By using a TR mutant that is defective in its ability to homodimerize, we demonstrate that the TR complex present on a native response element determines the nature of the corepressor recruited. Our data demonstrate that the polypeptides that represent the corepressor interacting domains appear to recognize both specific nuclear receptors and the complexes that they form.

RESULTS

The Proximal Corepressor Interacting Domains Allow for Specific Interactions with TRs and RARs

To delineate the specificity of NCoR and SMRT for the nuclear hormone receptors, TR and RAR, we constructed plasmids that express either both receptor-interacting domains as GST fusions, or the individual interacting domains as GST fusion proteins (Fig. 2). We have shown previously using in vitro translated (IVT) proteins that the TRβ1 isoform prefers NCoR over SMRT (24). Similar data are seen in electrophoretic mobility shift assay (EMSA) using the GST fusion proteins representing both interacting domains of NCoR and SMRT on a DR + 4 element (Fig. 3A; compare, for example, lanes 3 and 5, or 7 and 9). Both NCoR and SMRT bind TRβ1 on this thyroid hormone response element (TRE), but NCoR is bound significantly more avidly than SMRT. This preference is seen over a wide range of amount of GST protein used, although it is seen most dramatically with lower amounts of protein (20–100 ng). The shifts associated with corepressor binding result in a decreased amount of remaining TRβ1 homodimer (compare lane 1 with lanes 3, 7, and...
11). The addition of RXR causes a loss of corepressor binding (lanes 4, 6, 8, 10, 12, and 14). There is no further supershift in the presence of RXR (see especially lanes 4 and 8), consistent with specific binding to the TR homodimer. These data suggest that the TRβ1 homodimer binds corepressors more avidly than does the TRβ1-RXR heterodimer. In contrast, on a DR15 element (Figure 3B), which is a retinoic acid response element, RARα binds corepressor solely when heterodimerized to RXR (compare lanes 9 and 10, and 13 and 14). Furthermore, the RARα/RXR heterodimer binds SMRT, but does not interact well with NCoR (compare, for example, lanes 7–8 with 9–10). The shifts associated with SMRT bind-

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**Fig. 2.** NCoR and SMRT-Interacting Domain Constructs

A, Schematic illustration of GST-corepressor (GST-CoR) constructs. The amino acid sequences of the interacting domains are indicated. The locations of the GST-CoR constructs are indicated by bars, and their specific amino acid sequences are identified in Materials and Methods. In particular, the amino acid sequences of the individual interacting domain constructs are as follows: GST-N1 (aa 2239–2300); GST-N2 (aa 2063–2142); GST-S1 (aa 2267–2307); GST-S2 (aa 2098–2266). B, SDS-PAGE of GST-CoR constructs. After analysis by SDS-PAGE, protein quantification was performed by Bradford assay, so that equivalent amounts of protein constructs could be used in each EMSA. 1, ladder; 2, GST alone; 3, GST-N1; 4, GST-N2; 5, GST-S1; 6, GST-S2; 7, GST-N2S1; 8, GST-S2N1; 9, GST-NCoR; 10, GST-SMRT.
Fig. 3. TRβ and RARα Show Distinct Preferences for the Nuclear Corepressors

A, Gel mobility shift assays were carried out using 4 μl IVT TRβ1; 2 μl IVT RXR; 20 ng, 100 ng, or 1 μg GST-CoR complex (as noted); and DR+4 radiolabeled probe. B, Gel mobility shift assays were carried out using 4 μl IVT RARα; 2 μl IVT RXR; 20 ng, 1 μg, or 2 μg GST-CoR complex (as noted); and DR+5 radiolabeled probe. When no RXR was used, an equal amount of unprogrammed (UP) reticulocyte lysate was added in its place. CoR shift indicates mobility shift(s) caused by the binding of indicated corepressor interacting domain constructs.
ing result in a decreased amount of remaining RARα/ RXR heterodimer (compare lane 2 with lanes 10 and 14). In contrast to TRβ, when 20 ng (lanes 3–6) or 100 ng (data not shown) of GST protein are used, no specific binding is detected, and it is only at higher amounts (lanes 7–14) that the preferential binding of the RAR/RXR heterodimer to SMRT is identified. However, when identical amounts of protein are used (e.g. 1 μg), the RAR-RXR heterodimer prefers to interact with SMRT, while the TRβ1 homodimer prefers to interact with NCoR.

To delineate which of the interacting domains (IDs) mediates this specificity, we next performed EMSA with TRβ1 or RARα using bacterially expressed GST proteins containing individual interacting domains (Fig. 4, A and B). As Fig. 3 had demonstrated that differences in corepressor binding to TRβ1 are most apparent with 20 ng of GST fusion protein (lanes 3–6), this amount of protein was used in Fig. 4A; similar data were obtained when 1 μg of GST fusion proteins was used (data not shown). In contrast, since binding to RAR could only be clearly detected using higher amounts of GST fusion proteins, 1 μg of GST protein was used in the EMSA in Fig. 4B.

As is demonstrated in Fig. 4A, the proximal ID 2 allows for specificity in that N2 strongly binds the TRβ1 homodimer (lane 5) while S2 does not bind the TRβ1 isomorph well (lane 7) on the DR4+4 element. In contrast, on the retinoic acid response element (RARE) (Fig. 4B), RAR interacts well with S2 (lanes 7–8) and does not bind N2 well (lanes 5–6). These equivalent domains share only 23% homology at the amino acid level, suggesting that specificity is encoded for within the ID 2 polypeptide. With the amount of GST protein used in each of these experiments, neither ID 1 from NCoR or SMRT was able to bind to either receptor complex on DNA response elements. However, binding of N1 to the TR homodimer could be seen when greater amounts of protein (1 μg) were used. In addition, N1 binding was enhanced by sequences 3’ to its defined boundary (N1’, see Fig. 2), but never approached the strength of N2 binding. Interestingly, the individual interacting domains (for example N2 and S2) bound optimally to TR and RAR in the absence of RXR (compare Fig. 4A, lanes 5 and 6, and Fig. 4B, lanes 7 and 8). Although the RAR homodimer is not believed to exist in vivo, these data suggest that under certain circumstances it might be stabilized by interacting proteins. Importantly, however, in the context of both interacting domains together, RAR binds corepressors solely as a heterodimer with RXR (Fig. 3B, lanes 10 and 14).

To confirm that indeed the proximal ID 2 was responsible for receptor specificity, we next swapped the distal ID 1 region among the NCoR and SMRT constructs, creating chimeric interacting domains consisting of N2S1 and S2N1 (Fig. 4, C and D). As expected, the TRβ1 isoform homodimer preferred the N2S1 chimera (Fig. 4C, lane 3), while the RAR/RXR heterodimer preferred to interact with the S2N1 chimera (Fig. 4D, lane 6). Note that since the chimeric constructs bound TR and RAR more weakly than did wild-type constructs, 2 μg of chimeric constructs were used in Fig. 4D. However, these data again suggest that the proximal interacting domains mediate the preferential interactions of TR and RAR for NCoR and SMRT, respectively, and that the distal IDs do not exert restrictive properties in this context. Moreover, while the individual S2 domain bound RAR well in the absence of RXR (see above), the addition of either S1 or N1 resulted in enhanced binding to the RAR-RXR heterodimer (see Fig. 3B, lane 10; and Fig. 4D, lane 6). These data suggest that portions of the NCoR and SMRT interacting domains aid in the recognition of receptor complexes (homodimer or heterodimer).

To define the effects of ligand on the interactions between nuclear receptors and the individual corepressor interacting domains, we performed an EMSA with TRβ1 in the presence or absence of T3. As shown in Fig. 5A, TRβ1 prefers to bind NCoR over SMRT (compare lanes 5 and 9). The addition of T3 results in decreased homodimer formation (lanes 1 and 3). In addition, both NCoR and SMRT are released in the presence of T3 (compare lanes 5 and 7, and lanes 9 and 11). As shown in Fig. 5B, TRβ1 binds strongly to N2 (lane 1), and this interaction is disrupted by T3 (lane 3). Finally, TRβ1 binds weakly to S2, and only in the absence of T3 (lanes 5 and 7).

Given the ability of TRβ1 to interact well with NCoR in EMSA, we next asked whether the TRβ1 homodimer recruits a single NCoR, or whether more than one NCoR binds the homodimer (i.e. perhaps each partner independently binds a separate NCoR molecule). To do this, we used the EMSA assay with two different sized proteins, each representing both interacting domains of NCoR as shown in Fig. 5A. GST-NCoR includes NCoR amino acids 2063–2300 downstream of GST. IVT-NCoR consists of NCoR amino acids 1579–2454. If the TRβ1 homodimer bound two NCoR molecules, we would expect a mobility shift between the two individual bands produced (consisting of a TRβ1 homodimer binding one GST-NCoR and one IVT-NCoR). As the TRβ1 homodimer forms separate complexes with each NCoR protein, it appears more likely that the TRβ1 homodimer binds a single NCoR molecule. We next performed a similar experiment using two different sized N2 constructs: GST-N2 (which contains amino acids 2063–2142) and IVT-N2 (which contains amino acids 1579–2211). As shown in Fig. 6B, again no intermediate band is seen, suggesting that each homodimer complex is able to bind a single interacting domain. This is specific to the TR homodimer, as Figs. 3B and 4D above suggest that the presence of a second interacting domain does influence receptor complex recruitment.
Mutant TRs Found in Syndromes of Resistance to Thyroid Hormone Suggest that the TR Complex Also Determines the Corepressor Recruited

The syndromes of resistance to thyroid hormone (RTH) have been linked to mutations in the TRβ gene and are generally inherited in an autosomal dominant fashion (30). Affected patients have elevated free thyroid hormone levels, with a nonsuppressed TSH, and exhibit variable tissue resistance to the actions of thyroid hormone. To determine whether specificity in the context of corepressor recruitment is complex-specific, as well as nuclear hormone receptor-specific, we took advantage of mutations in TRβ found in kindreds with RTH that affect receptor complex formation on thyroid hormone response elements. We first employed a natural mutation of the ninth heptad of the TR, R429Q. This mutation has been shown to be defective in its ability to homodimerize but to still retain its ability to silence gene expression on positive TREs (31, 32). Patients with this mutation clinically have impaired central T3 action, but preserved peripheral T3 action, and exhibit variable tissue resistance to thyroid hormone, or PRTH. We analyzed the ability of R429Q TRβ1 to recruit either NCoR or SMRT using EMSA. As is shown in Fig. 7A, the R429Q mutation in context of the TRβ1 isoform is unable to homodimerize well and does not recruit NCoR either as a homodimer or a heterodimer (lanes 5–6). However, the R429Q-RXR heterodimer is able to recruit the SMRT interacting domains to the DR+4 binding site (lane 8). Of note, the R429Q mutant does not bind either NCoR or SMRT well at low amounts (i.e. 20 ng; lanes 1–4) and required higher amounts of GST corepressor construct than did wild-type TRβ1 to exhibit strong corepressor binding in EMSA. However, these data are consistent with the hypothesis that the structure of the complex influences specific corepressor recruitment.

We next examined another mutant TR found in certain kindreds with RTH, Δ337T. This mutant TR does not bind T3, and patients with this mutation have severe generalized resistance to thyroid hormone (GRTH) (30). In contrast to R429Q TRβ1, the Δ337T TRβ1 mutant homodimerizes well on thyroid hormone response elements. As shown in Fig. 7B, Δ337T TRβ1 binds NCoR more strongly than SMRT (compare lanes 1 and 3). As with wild-type TRβ1, this preference is seen most dramatically at lower amounts of GST fusion proteins used (compare lanes 1–4 with 5–8), although it is also seen at higher amounts (compare lanes 5 and 7). Thus, Δ337T TRβ1 is an example of a TR mutant that exhibits enhanced homodimer formation and preserved corepressor specificity.

SMRT and NCoR Recruit Distinct Nuclear Receptor Complexes in Cells

To complement the EMSA assay and determine whether SMRT and NCoR would prefer to interact with homo- or heterodimers, we employed a two-hybrid assay in mammalian cells. In this system, we fused the interacting domains of NCoR and SMRT to the Gal4-DNA-binding domain and fused full-length nuclear receptors downstream of the VP-16 activation domain. Full-length nuclear hormone receptors were used instead of the corresponding LBDs, as we and others have demonstrated that the amino termini influence complex formation (33, 34). In Fig. 8A, the basal activity of Gal4-NCoR or Gal4-SMRT is set at 1; the interactions between the Gal4-corepressor constructs and the nuclear receptor-VP16 constructs is then expressed as relative luciferase activity. As shown in Figure 8B, the experiments were next done in the presence of cotransfected RXR-VP16 to examine the effect of heterodimerization on corepressor interactions. These data are presented as fold expression in the presence vs. absence of cotransfected RXR-VP16. A ratio greater than 1 implies greater luciferase activity in the presence of RXR.

As shown in Fig. 8A (upper panel), transfection of TRβ1-VP16 with Gal4-NCoR causes approximately 80-fold stimulation over the activity of Gal4-NCoR alone. In contrast, transfection of RARα-VP16 with Gal4-NCoR causes only 25- to 30-fold stimulation over the activity of Gal4-NCoR. Finally, R429Q TRβ1-VP16 only minimally interacts with Gal4-NCoR in the absence of RXR. We next used the identical paradigm with Gal4-SMRT as the bait (Fig. 8A, lower panel). Cotransfection of RARα-VP16 with Gal4-SMRT caused a 40-fold stimulation over the activity of Gal4-SMRT alone. In contrast, TRβ1-VP16 caused only a 6- to 7-fold stimulation in luciferase activity. Again, R429Q TRβ1-VP16 did not interact well in the absence of cotransfected RXR. All of the nuclear receptor-VP16 constructs interact to a similar degree with Gal4-RXR (data not shown), suggesting that they are expressed at similar levels in cells.

These constructs were then cotransfected with RXR-VP16; as shown in Fig. 8B, cotransfection with RXR-VP16 actually decreased the interactions between TRβ1-VP16 and Gal4-NCoR. In contrast, the interactions of R429Q TRβ1-VP16 with Gal4-NCoR was enhanced 18-fold by the cotransfection of RXR. Although RXR-VP16 decreased interactions between RARα-VP16 and Gal4-NCoR (upper panel), cotransfection of RXR-VP16 enhanced the interactions between this receptor and Gal4-SMRT (Fig. 8B, lower panel). Additionally, our previous data (Figure 3B) had showed that, in the presence of a RARE, RXR is important for the binding of RAR to SMRT. In addition, although R429Q TRβ1-VP16 interacted minimally with Gal4-SMRT in the absence of RXR, the interaction was enhanced more than 20-fold when RXR-VP16 was cotransfected. In fact, in the mammalian two-hybrid system, the interaction between TRβ1-VP16 and Gal4-SMRT was also enhanced by the cotransfection of RXR (6–fold). Although RXR-VP16 itself interacted minimally with Gal4-SMRT (data not shown), it did not interact to an extent where it could affect the synergistic level of interactions seen. Thus, SMRT appears to
Fig. 4. Specificity of TRβ and RARα for the Nuclear Corepressors Depends on the Proximal Corepressor Interacting Domain
A, Gel mobility shift assay was carried out using 4 μl IVT TRβ1; 2 μl RXR (or equal amount unprogrammed reticulocyte lysate); 20 ng of indicated GST-CoR construct; and DR+4 radiolabeled probe. B, Gel mobility shift assay was carried out using 4 μl in IVT RARα; 2 μl RXR (or equal amount UP reticulocyte lysate); 1 μg of indicated GST-CoR construct; and DR+5 radiolabeled probe. C, Gel mobility shift assay was carried out using 4 μl IVT TRβ1; 2 μl RXR (or UP reticulocyte lysate); 20 ng of indicated GST-CoR construct, and DR+4 radiolabeled probe. D, Gel mobility shift was carried out with 4 μl IVT RARα; 2 μl RXR (or UP lysate); 2 μg indicated GST-CoR construct; and DR+5 radiolabeled probe.

Fig. 5. The Binding of CoR-Interacting Domains to TR Is Blocked by Ligand
A, Gel mobility shift assay was carried out using 4 μl IVT TRβ1; 2 μl RXR (or an equivalent amount of unprogrammed reticulocyte lysate); 20 ng of indicated GST-CoR construct; DR+4 radiolabeled probe; and T₃, where indicated. The concentration of T₃ used was 100 nM. B, Gel mobility shift assay carried out as in panel A, using 20 ng of indicated GST-CoR construct.
particularly favor receptor heterodimers. Moreover, R429Q TRβ1, a mutant TR, appears to interact with corepressors mainly as a heterodimer with RXR, both in solution and on a thyroid hormone response element.

While RXR-VP16 was used to keep the total amount of VP16 moiety constant in each dimer pair, we next repeated these experiments in the presence of RXR-pKCR2. As shown in Fig. 8C, similar results are seen when RXR-pKCR2 is used instead (although the values are less pronounced). Again, the presence of RXR is more important in the context of receptor interactions with SMRT. In addition, cotransfection of RXR-pKCR2 increased interactions between R429Q TRβ1 and the Gal4-corepressor constructs 5- to 10-fold (data not shown).

While other studies have suggested that RXR can enhance the interaction between TRβ1 and NCoR, these studies used the LBD of the TR linked to VP16 (29). We therefore used the same paradigm as above in Fig. 8D, in the context of the nuclear receptor LBDs. In these experiments, RXR-VP16 had significant impact on the strength of the interaction of the R429Q LBD (117-fold), and, in contrast to the full-length TR (see above), also stimulated interactions when cotransfected with the wild-type LBD (although only 9-fold). In addition, the ability of Gal4-SMRT to recruit both wild-type and R429Q mutant LBDs was greatly enhanced by the addition of RXR. In contrast to the interactions seen with Gal4 NCoR, the interaction of the LBD of R429Q with Gal4 SMRT was enhanced by more than 300-fold by the addition of VP16-RXR, consistent with the preferences noted on the DR+4 element in EMSA. Both the absolute luciferase activity (with RXR) and the fold-enhancement by RXR were greater in these experiments (as opposed to experiments using full-length nuclear receptors), suggesting that the amino termini, and potentially the DNA-binding domains, of TR modulate interactions with corepressors.

To examine the specificity of the individual interacting domains in cells, constructs containing N2 or S2 were placed downstream of the Gal4 DNA binding domain, and used in a similar mammalian two-hybrid system. As shown in Fig. 8E (upper panel), transfection of TRβ1-VP16 with Gal4-N2 causes approximately 100-fold stimulation. In contrast, cotransfection with RAR-VP16 results in only about half that level of activity. While interactions with Gal4-S2 were weaker than with Gal4-N2, Fig. 8E (lower panel) shows that Gal4-S2 interacts strongly with RAR-VP16, but only minimally with TRβ1-VP16, consistent with the EMSA data (e.g. Fig. 4B).

DISCUSSION

The identification and cloning of the nuclear corepressors have allowed for an understanding of the mechanisms by which members of the NR family regulate gene expression in the absence of ligand or in the presence of antagonists that recruit either NCoR or SMRT (35, 36). In addition to the NRs, NCoR and
Fig. 7. Certain TR Mutants Exhibit Altered Interactions with Corepressors

A. Gel mobility shift assay was carried out using 4 μl IVT R429Q TRβ1; 2 μl RXR (or unprogrammed reticulocyte lysate); and 20 ng or 1 μg of indicated GST-CoR construct (as indicated); and a DR14 radiolabeled probe. B. Gel mobility shift assay was carried out using 4 μl IVT Δ337T TRβ1, 2 μl RXR (or unprogrammed reticulocyte lysate); 20 ng or 1 μg of indicated GST-CoR construct (as indicated); and a DR14 radiolabeled probe.
SMRT can also be recruited by the oncoproteins PML-RAR, PZLF-RAR, and AML-ETO and thus may be linked to human disease by blocking myeloid differentiation through their ability to block transcription of target genes (26, 37, 38). The role of the corepressors in NR action in vivo has not been confirmed, although it is tempting to speculate that, in the context of thyroid hormone action, the corepressors are important in
the manifestations of hypothyroidism and in the presenta-
tion of the RTH syndromes (39–41). TR isoforms are able to widely regulate gene expression in a spec-
cific fashion based on their ability to bind to cognate response elements in the regulatory regions of target
genes. Once bound to the elements, the TRs recruit members of the corepressor family (NCoR/RIP13, SMRT/TRAC1) in the absence of ligand, which medi-
ates ligand-independent repression on positive TREs. The presence of T3 causes the corepressor complex to be released and the coactivator complex, which in-
cludes members of the p160 family, pCAF, CBP/p300, and possibly other coactivator molecules, to be re-
cruited. Specificity in the context of cofactor recruit-
ment and its ramifications for gene expression have not been ascertained. Recent work in the context of the p160 family suggests that although the LxxLL-
containing motifs (LXDs) mediate specific interactions with members of the NR family, the surrounding se-
quences and the spacing between LXDs are critical for nuclear receptor specificity (42–45).

Recent work has suggested that the L/I-x-x-I/V-I motifs in the corepressor IDs are required for interac-
tions with nuclear receptors (46–48). Both NCoR and SMRT contain two separate IDs, which have been independently identified by a number of separate groups. The more proximal ID2 of murine and human NCoR shares limited sequence homology with the ho-
logous region of SMRT (23%), whereas the distal ID 1 of murine and human NCoR share approximately 50% amino acid homology. Based on these differ-
ences, we and others have hypothesized that the two corepressors may differentially recruit nuclear recep-
tors. Initial studies using EMSA demonstrated that murine (m) NCoR and human (h) SMRT could interact with both the TR and RAR on their respective response elements (3, 4). However, these studies did not examine specificity in context of the interactions, nor did they examine the complexes with which the corepre-
sors may interact. Zamir et al. (49) demonstrated that both TR isoforms could interact with NCoR and SMRT on a thyroid hormone response element (TRE) but did not assess differences in binding, nor did they examine the specificity of the individual interaction domains. Wong and Privalsky examined interactions between individual interaction domains and a number of nu-
clear receptor isoforms. These data were generated in solution assays (GST pull-down and mammalian two-
hybrid assays) and did not incorporate the role of DNA binding into corepressor recruitment. However, these results showed that specificity in corepressor recruit-
ment exists, especially in the context of RAR isoforms and the amino acid sequences present in the hinge region (28). More recently, Hu and Lazar showed that the distal NCoR interacting domain interacts well with RXR, whereas the proximal domain vastly prefers TR (46). These data suggested that the corepressor inter-
acting domains might recognize receptor complexes, which we have examined in this report.

In the present study, we have examined the role of the TRb1 and RARz isoforms to recruit nuclear core-
pressors to TREs and RAREs, respectively. In addition, we have further evaluated the role of RXR in corepres-
sor recruitment to ascertain the role of homo- vs. heterodimerization in corepressor recruitment. Our data suggest that the TRb1 homodimer preferably re-
cruits NCoR while the RARz/RXR heterodimer prefer-
ably recruits SMRT on DNA response elements. This specificity appears to map the more proximal ID 2 region, as shown in Fig. 4. Furthermore, chimeric corepres-
sors require the SMRT ID 2 region (S2N1 or S2S1) to interact with the RAR. In contrast, the ID 2 of NCoR preferably binds the TRb1 homodimer. These data are consistent with the marked differences in the ID 2 regions between NCoR and SMRT and suggest that this region may exert specificity in the recognition of the TR and RAR.

Given the differences in the ability of the TR and RAR to recruit NCoR and SMRT, we next investigated the ability of TRb isoforms found in kindreds with RTH to recruit the corepressors to a TRE. Surprisingly, R429Q TRb1 exhibited altered corepressor specificity in the context of an underlying TRE. R429Q TRb1 preferred to interact with SMRT rather than NCoR on a DR4 element and did not interact with corepres-
sors well as a homodimer. Previous studies by others using the R429Q mutant demonstrated an inability to release SMRT as compared with the wild-type TRb1 isoform but did not examine the preference of the mutant TR for SMRT (40). Another RTH mutant Δ337T (30), which forms strong homodimers, preferably re-
cruits NCoR to the DR4 element.

The mammalian two-hybrid system also displayed strong differences between the R429Q mutant and wild-type TRb1. In this assay, the interactions between the nuclear receptors and corepressors occur in solu-
tion, so the effects of underlying DNA response ele-
ments can not be assessed. However, this assay was used to assess the role of heterodimerization in corepres-
sor recruitment in cells. Indeed, the addition of RXR enhanced the interaction of R429Q with NCoR and to a greater degree with SMRT, suggesting that its heterodimer form allows for the recruitment of SMRT. In contrast, RXR decreased the interaction of wild-
type TRb1 with NCoR, but increased interactions with SMRT in this context. Interactions between RAR and SMRT were also enhanced by the presence of RXR. Thus, SMRT appears to prefer to interact with nuclear receptor-RXR heterodimers. Although SMRT can in-
dependently interact with RXR in solution, the abso-
lute value of this interaction is weak when compared with the synergy imparted by the addition of RXR to TR or RAR. Thus, the heterodimer is favorably recognized by SMRT, while homodimer binding recognizes NCoR. Previous work by Zamir et al. (22) demonstrated that the orphan receptor RevErb, which binds DNA only as a homodimer, can recruit only NCoR but not SMRT; these findings are consistent with the data demon-
strated here, suggesting that the corepressor interact-
ing domains may recognize complexes as well as specific nuclear receptors.

In addition, the experiments performed with the LBD alone of the wild-type and mutant receptor fused to VP16 demonstrate that the A/B and DNA-binding domains influence corepressor recruitment in the presence or absence of RXR and help explain differences seen by a number of groups in the role of RXR in corepressor recruitment (28, 29). The A/B domain (33, 34) and DNA-binding domain (50) have been shown to influence receptor complex formation on TREs. Alterations in complex formation may explain their influence on TR-corepressor interactions. Alternatively, portions of the A/B and/or DNA-binding domains might conceivably interact directly with the nuclear corepressors.

The data presented here reinforce the need to examine cofactor interactions in the context of DNA binding, given the likely restrictions placed on the receptor once it is bound to DNA. Solution interactions performed by us and others have demonstrated equivalent interactions between NCoR and SMRT and the TR, whereas the introduction of a TRE brings out the differences in the isoforms in their ability to recruit corepressors. Indeed, Wong and Privalksky demonstrated, using solution interactions, that the TR bound equally well to both of the NCoR interaction domains, while on the DR + 4 response element it is clear that N2 is preferred over N1. In addition, the S1 domain appears to act to enhance binding to the heterodimer, suggesting that it plays a role in identifying complexes when bound to DNA, consistent with its ability to bind RXR (28, 46).

In summary, the work discussed here demonstrates that the complex formed by the nuclear receptor and the DNA response element present in the responsive promoter can dictate the preference for the corepressor that is recruited. In addition, the corepressor interacting domains appear to have preference for specific nuclear receptors, such that the RARx isoform prefers to interact with SMRT, which is mediated by the proximal S2 domain. It will be interesting to discern which portion of the interacting domains are important in the recognition of nuclear receptors and how complex formation furthers this specificity.

**MATERIALS AND METHODS**

**Plasmids**

All GST fusion plasmids were cloned into the vector PGEX-4T1 as EcoRI fragments or EcoRI-XhoI fragments. PCR was used to amplify the indicated amino acid sequences from either human NCoR or hSMRT (see Fig. 2), which were then placed in frame downstream of the GST moiety. GST-NCoR includes amino acids (aa) from human NCoR corresponding to mNCoR, aa 2063–2300; GST-N2 includes aa 2063–2142; and GST-N1 includes aa 2239–2300 (GST-N1’ includes aa 2239–2453). GST-SMRT includes hSMRTe (7), aa 2098–2507; GST-S2 includes aa 2098–2266; and GST-S1 includes aa 2267–2507. The chimeric N2S1 and S2N1 constructs were made by amplifying the separate domains with an artificial XhoI site introduced at the 3′-end of the proximal domain and 5′-end of the distal domain. The pieces were then ligated into PGEX-4T1 as EcoRI fragments. GST-N2S1 contains amino acids from hNCoR corresponding to mNCoR, aa 2063–2142, and hSMRTe aa 2267–2507; GST-S2N1 includes hSMRTe, aa 2098–2266, and NCoR, aa 2143–2300. All GST constructs were confirmed by dieoxy sequencing.

GAL4-NCoR and GAL4-SMRT were created by ligating the interacting domains of the respective corepressors downstream of the sequence encoding the GAL4 DNA-binding domain in the SV40-driven expression vector pECE. The VP-16 TRβ1 and RARα fusions were created by introducing an in-frame EcoRI site at the 5′-end of the receptor using PCR and ligating them downstream of the VP16 activation domain in AASV-PV16. They include aa 1–461 of hTRβ1, and aa 1–462 of RAR. The VP-16 R429Q mutant was made by introducing the mutant sequence as a PsiI-Asp718 fragment into the wild-type construct. The VP16-RXRα fusion was created by PCR and consists of aa 2–462 of the human isoform. IVT NCoR (aa 1579–2454) and IVT-N2 (aa 1579–2211) were cloned into pKCR2 and have been described previously (24, 51). Gal4-N2 was made by placing NCoR, aa 1579–2211, downstream of the Gal4 DNA-binding domain. Similarly, Gal4-S2 consists of SMRT, aa 2098–2266, downstream of GAL4 DBA binding domain. Construct integrity was confirmed by restriction endonuclease digestion and dieoxy sequencing.

**GST Fusion Proteins**

GST fusion proteins were expressed either in DH5α or BL21 Escherichia coli expressing thioredoxin by induction with 0.1 mM isopropyl-thio-galactosidase at 30 C (24). The proteins were isolated by lysis with lysozyme and purified on Sepharose beads. The bound proteins were eluted using a glutathione buffer. Verification of protein synthesis was obtained on SDS-PAGE. The amount of protein generated was quantified using the Bradford assay.

**EMSA**

EMSAs were carried out as previously described with either a 32P-radiolabeled DR + 4 or DR + 5 probe (52). GST-corepressor fusion proteins (GST-CoRs) were purified on Sepharose beads and eluted using a glutathione buffer. Nuclear receptors were in vitro translated in reticulocyte lysate (Promega Corp., Madison WI) using T7 polymerase. IVT NCoR or IVT N2 was translated similarly. For each EMSA, 4 μl of IVT TR or RAR were used. For experiments using RXR, 2 μl were used (or an equivalent amount of unprogrammed reticulocyte lysate as a control). The amount of GST protein used in each EMSA was identical and is indicated in each figure. Incubations were carried out for 20 min, and complexes were resolved on a 5% nondenaturing gel, followed by autoradiography.

**Cell Culture and Transfection**

All transient transfections were performed in CV-1 cells, which were maintained as described previously (24). Transient transfections were performed in six-well plates using the calcium phosphate technique, with each well receiving 1.7 μg of the upstream activity sequence (UAS-TK) luciferase reporter and 20 ng of a cytomegalovirus (CMV) β-galactosidase expression vector. Each well received 80 ng of Gal4-corepressor construct, along with 80 ng of VP16-TR or -RAR construct. The addition of 80 ng VP-16 RXR was controlled for by the presence of empty vector AASV-PV16 (EV-PV16). Similarly, the addition of 320 ng pKCR2-RXR was controlled
with empty vector pKCR2. Fifteen to 18 h after transfection, the cells were washed in PBS and refed with 10% steroid hormone-depleted FBS. To remove steroid and thyroid hormones, FBS was treated with 50 mg/ml activated charcoal (Sigma, St. Louis, MO) and 30 mg/ml anion exchange resin (type AGX-8, analytical grade, Bio-Rad Laboratories, Inc. Richmond, CA) as previously described (24). Forty to 44 h after transfection the cells were lysed and assayed for luciferase and \( \beta \)-galactosidase activity. Experiments were performed two to three times in triplicate. \( \beta \)-Galactosidase activity was used to control for transfection efficiency.

The data shown are the pooled results \( \pm SEM \) and are presented as relative or fold luciferase activity. In particular, the interactions between Gal4-NCoR (or Gal4-SMRT) and nuclear receptor-VP16 constructs are presented relative to luciferase activity in the presence of Gal4-NCoR alone. Fold luciferase activity was measured in the presence vs. absence of cotransfected RXR-VP16 to determine the effect of heterodimerization on interactions between Gal4-NCoR (or Gal4-SMRT) and nuclear receptor-VP16 constructs. For each experiment, the total amount of VP16 construct transfected was held constant with empty vector-VP16 (EV-VP16).

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