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A Conformational Switch in Nuclear Hormone Receptors Is Involved in Coupling Hormone Binding to Corepressor Release

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Nuclear hormone receptors are ligand-regulated transcription factors that modulate gene expression in response to small, hydrophobic hormones, such as retinoic acid and thyroid hormone. The thyroid hormone and retinoic acid receptors typically repress transcription in the absence of hormone and activate it in the presence of hormone. Transcriptional repression is mediated, in part, through the ability of these receptors to physically associate with ancillary polypeptides called corepressors. We wished to understand the mechanism by which corepressors are recruited to unliganded nuclear hormone receptors and are released on the binding of hormone. We report here that an α-helical domain located at the thyroid hormone receptor C terminus appears to undergo a hormone-induced conformational change required for release of corepressor and that amino acid substitutions that abrogate this conformational change can impair or prevent corepressor release. In contrast, retinoid X receptors appear neither to undergo an equivalent conformational alteration in their C termini nor to release corepressor in response to cognate hormone, consistent with the distinct transcriptional regulatory properties displayed by this class of receptors.

Nuclear hormone receptors are a large family of hormone-regulated transcription factors that play critical roles in normal metazoan homeostasis, development, and differentiation (4, 9, 24, 29, 31, 32, 39, 47). Aberrant nuclear receptors are associated with a variety of human endocrine and neoplastic diseases (5, 15, 21, 23, 26, 33, 37). The nuclear hormone receptor family includes the steroid receptors, vitamin D_3 receptors, thyroid hormone receptors (T3Rs), two major classes of retinoid receptors (retinoic acid receptors [RARs] and retinoid X receptors [RXRs]), and a number of orphan receptors of unknown ligand specificity (4, 9, 24, 29, 31, 32, 39, 47). Many nuclear hormone receptors are expressed as multiple interrelated isoforms; for example, two different loci in vertebrates encode thyroid hormone receptors (designated T3Rα and T3Rβ) (28). In addition, different receptors can heterodimerize with one another, generating further combinatorial diversity (4, 9, 24, 29, 31, 32, 39, 47).

Nuclear hormone receptors function by binding to specific sites within the DNA genome and regulating the expression of adjacent target genes in response to cognate ligand (4, 9, 24, 29, 31, 32, 39, 47). Both negative and positive effects on transcription can occur (2–4, 8, 9, 14, 24, 29, 31, 32, 34, 39, 42, 47). These bimodal transcriptional properties of the nuclear hormone receptors are mediated, in part, by the ability of these receptors to associate with ancillary polypeptides called corepressors and coactivators (11, 12, 19, 20, 28, 41, 45, 46, 51–53). Corepressors associate with T3Rs and RARs in the absence of hormone, a context in which these receptors typically repress transcription (11, 12, 19, 28, 41, 53). Addition of hormone leads to a physical release of corepressors from the receptor and the physical association of coactivator proteins; in parallel, the receptor is converted from a repressor to an enhancer of transcription (reviewed in reference 20). The molecular mechanisms by which corepressors and coactivators mediate their effects on gene expression are not fully established but may include effects both on the general transcriptional machinery and on the chromatin template (references 36 and 50 and references therein).

Corepressors are encoded by at least two loci in mammals, designated SMRT/TRAC and N-CoR/RIP13 (11, 12, 19, 28, 41, 45, 46). Given that a critical component of the transcriptional response of T3Rs and RARs is regulated by the physical association and dissociation of corepressors, we wished to understand the mechanism by which corepressors are recruited to unliganded nuclear hormone receptors and are released upon the binding of hormone. We report here that an α-helical domain located at the very C termini of the T3Rs and RARs (Fig. 1) appears to undergo a specific hormone-induced conformational change that is required for displacement of SMRT; amino acid substitutions that abrogate this conformational change in T3Rs inhibit release of corepressor. In contrast to T3Rs and RARs, RXRs appear to fail to undergo this C-terminal conformational change and exhibit a hormone-resistant corepressor association that may be important in the role of RXRs as heterodimer partners for other nuclear receptors.

MATERIALS AND METHODS

Molecular clones. The construction of pSG5 vectors containing wild-type human T3Rβ, the P453A and P453H mutants of human T3Rβ, wild-type avian T3Rα, and wild-type human RXRα has been previously described, as has the creation of the pGEX vectors used for bacterial expression of the SMRT/TRAC corepressor (10, 41, 51). Mutants M1 through M4 of avian T3Rα were also used as pSG5 vector constructs, whereas v-ErbA was engineered as a pGEM-4Z construct (40). The ΔC-terminal mutant of avian T3Rα was created by cleaving the pSG5-wtT3Rα clone with *Xho*I, filling in the overhangs with Klenow fragment of *Escherichia coli* DNA polymerase I, and inserting an oligonucleotide containing a universal translational terminator.

Receptor-corepressor binding assay. Nonrecombinant glutathione *S*-transferase (GST), or a GST fusion of SMRT(trac1) corepressor (representing amino acids 71 to 769 of TRAC-1), was synthesized in *E. coli*, bound to glutathione agarose, and purified as previously described (41, 51). ^{35}S-labeled nuclear hormone receptors were synthesized in vitro by use of the appropriate pSG5 or pGEM vector in a coupled transcription-translation system (TnT system; Promega) (41, 51). For the in vitro binding assays, identical amounts of ^{35}S-labeled proteins were incubated with a 50% slurry of either GST bound to glutathione-

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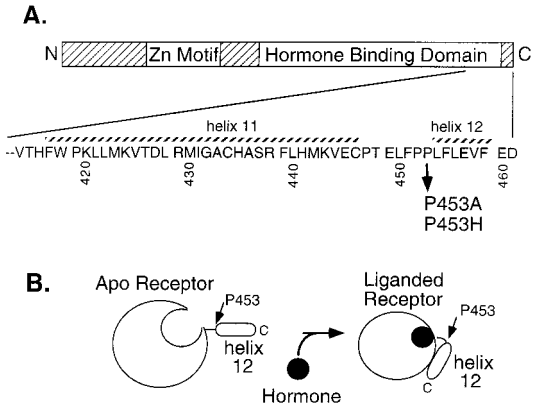

FIG. 1. (A) Schematic representation of the full-length human T3Rβ from N to C terminus, with the hormone binding domain and a zinc finger region (Zn motif) involved in DNA binding indicated. The amino acid sequence of the C-terminal domain is shown below, numbered from the receptor N terminus (48). The amino acids forming helixes 11 and 12, and the proline 453 mutations described in the text, are highlighted. (B) Schematic representation of the receptor conformational changes associated with binding of hormone. The proposed rearrangement of the C-terminal helix 12 and the overall condensation of the hormone binding domain are indicated.

agarose or GST-SMRT (trac1) bound to glutathione-agarose in 100 to 300 μl of HEMG binding buffer (40 mM HEPES [pH 7.8], 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol, 1.5 mM dithiothreitol, 1× Complete Protease Inhibitor [Boehringer Mannheim], 10 μg of bovine serum albumin per μl) for 1 h at 4°C with gentle rocking. The agarose beads were then washed four times with 1 ml of HEMG buffer in the absence of protease inhibitor and bovine serum albumin. Bound proteins were eluted in 30 μl of 50 mM Tris-Cl (pH 6.8) containing 100 mM glutathione, were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were visualized by autoradiography or phosphorimager analysis (41, 51).

Protease resistance assay. ^{35}S-labeled nuclear hormone receptors were synthesized in vitro by use of the coupled transcription-translation system. For each time point in the carboxypeptidase Y assay, 1 μl of in vitro translation product was diluted to 16 μl with 50 mM Tris-Cl (pH 6.8) containing either 1 μM cognate hormone or an equal volume of ethanol carrier. The samples were incubated on ice for 10 min, 4 μl of carboxypeptidase Y (1 mg/ml; Sigma) was added to each sample, and the samples were transferred to room temperature. After the indicated incubation period (0 to 80 min), the assay was terminated by addition of 20 μl of concentrated SDS-PAGE sample buffer, and the samples were rapidly frozen on dry ice. The samples were subsequently heated for 10 min at 98°C and resolved by SDS-PAGE (12% polyacrylamide gel); the radiolabeled proteolytic fragments were detected by autoradiography or by phosphorimager analysis. A similar procedure was used for the trypsin and elastase assays, except the dilution and incubation buffer was 50 mM Tris-Cl (pH 7.5) and different concentrations of protease (from 0 to 8 μg per sample) were used for a single time point (10 min). Elastase (type IV) and trypsin (tosyl-L-phenylalanine chloromethyl ketone treated) were obtained from Sigma.

RESULTS

Mutations in a putative hinge structure at the C terminus of T3Rβ inhibit the hormone-mediated release of SMRT. The wild-type T3Rβ binds to SMRT corepressor in the absence of hormone but dissociates from SMRT upon addition of hormone (Fig. 2). This binding and dissociation could be observed both for the full-length receptor (p55 in Fig. 2A) and for two smaller receptor derivatives that are the products of internal translational initiations by the in vitro transcription-translation system (p48 and p33 in Fig. 2A). These internal initiation products contain the same C terminus as the full-length receptor, as demonstrated by a shift of all three T3Rβ translation products to correspondingly faster-migrating species when a T3Rβ C-terminal deletion mutant was used in the transcription-translation reaction (data not shown). Similar T3R internal initiation products have been previously found both in vitro and in vivo (6).

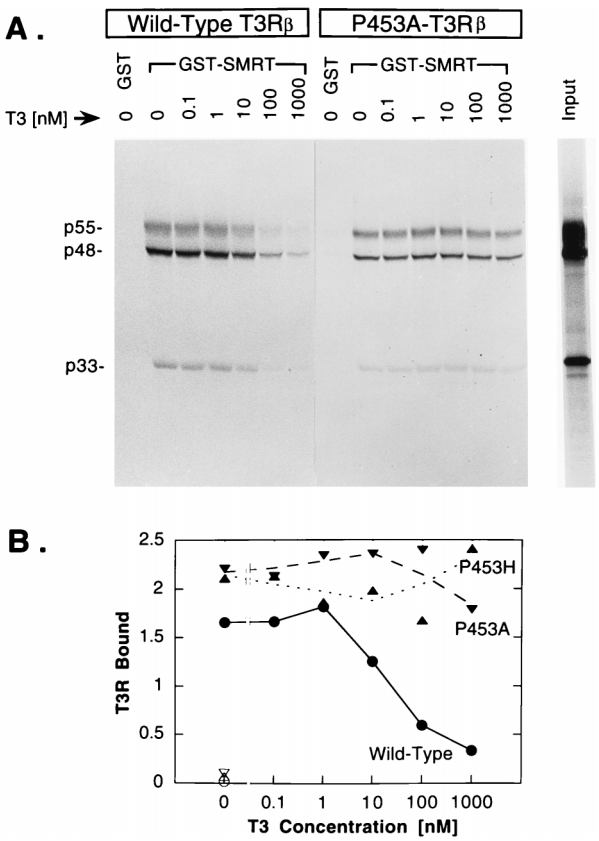

FIG. 2. Wild-type T3Rβ, but not the P453 mutants, releases from SMRT corepressor in the presence of hormone. (A) Radiolabeled wild-type or P453A T3Rβ proteins were incubated with immobilized GST or with an immobilized GST-SMRT derivative under different T3 hormone concentrations, as indicated above the lanes. Receptor bound to the GST or GST-SMRT matrix was subsequently eluted and analyzed by SDS-PAGE. The resulting autoradiogram is depicted, with the positions of the T3Rβ translation products indicated on the left. An aliquot of the wild-type T3Rβ in vitro translation mixture is shown in the input lane. (B) Abilities of wild-type and P453 mutant forms of T3Rβ to bind to GST (open symbols) or to the GST-SMRT derivative (closed symbols) were determined as for panel A and quantified by phosphorimager analysis.

The human endocrine disease resistance to thyroid hormone (RTH) syndrome is manifested as an impaired physiological response to thyroid hormone and is linked to mutations in T3Rβ (reviewed in references 5, 26, and 37). The RTH-T3Rβ mutants bind to SMRT corepressor but fail to dissociate from corepressor properly in the presence of hormone (51). As an apparent consequence, these RTH-T3R mutants can function as constitutive repressors and interfere in a dominant negative fashion with gene activation mediated by wild-type T3Rs (5, 13, 26, 37, 51). For some RTH-T3R mutants, the failure to release corepressor is due simply to an impaired ability of the receptor to bind hormone (13, 51). However, we identified a subclass of RTH-T3Rs that bound T3 hormone but nonetheless failed to release corepressor (P453A and P453H [Fig. 2] and references 13 and 51). The P453A and P453H mutants, therefore, appear to act as constitutive repressors because they are defective in the mechanism that couples hormone binding to corepressor dissociation (51).

Notably, both T3R mutants exhibiting this coupling defect were amino acid substitutions of a single proline, at codon 453 (Fig. 1A). The two mutants exhibited similar phenotypes, suggesting that loss of the proline, rather than the identity of the new amino acid, played the primary role in the coupling defect.

Proline 453 occupies an intriguing position in the T3R crystallographic structure, within a hinge-like domain that has been proposed to permit the C-terminal tail (helix 12) of the receptor to rotate from a relatively exposed position in the absence of hormone to a new position, more closely nested against the body of the receptor, in the presence of hormone (Fig. 1B and references 7, 38, and 48). The phenotype of the proline 453 substitutions suggested that this hinge-like domain might be important for corepressor release; we therefore investigated this question in more detail.

The C terminus of the wild-type T3R undergoes a conformational change that is abrogated by the proline 453 mutations. Carboxypeptidase Y is an exopeptidase that sequentially cleaves amino acids in a C- to N-terminal fashion (18). We hypothesized that if the conformational change proposed for the C terminus of the wild-type T3R actually occurs in solution, the receptor C terminus might be more exposed (and therefore more susceptible to carboxypeptidase Y degradation) in the absence of hormone but more sequestered (and resistant to carboxypeptidase Y) in the presence of hormone.

In the absence of hormone, carboxypeptidase Y produced a time-dependent degradation of the wild-type T3Rβ, whereas addition of T3 hormone rendered the wild-type receptor significantly more resistant to carboxypeptidase Y degradation (Fig. 3A; quantified in Fig. 3B). These results suggested that the C terminus of the wild-type receptor is indeed more accessible to exopeptidase in the absence than in the presence of hormone. In contrast to the wild-type T3Rβ, however, the P453A and the P453H mutant receptors were highly susceptible to carboxypeptidase Y degradation in both the absence and the presence of T3 hormone (quantified in Fig. 3B; also data not shown). The same hormone-induced resistance of the wild-type receptor, and the hormone-independent susceptibility of the P453 mutants, was also observed for T3Rβ derivatives representing only the C-terminal hormone binding domain (Fig. 3C). It should also be noted that in the absence of hormone, the patterns of carboxypeptidase Y susceptibility were nearly identical for wild-type and mutant receptors (e.g., Fig. 3C), indicating that in the unliganded receptor, a proline, an alanine, and a histidine at codon 453 were equally good substrates for the carboxypeptidase Y. We conclude that the C terminus of the wild-type T3Rβ is relatively accessible to exoprotease in the unliganded receptor and becomes more sequestered in the presence of T3 hormone, whereas the C terminus of the P453 mutant receptors is relatively accessible to protease in both the presence and the absence of hormone.

We next asked if the T3Rα isoform, which in common with T3Rβ exhibits a hormone-mediated dissociation from SMRT (Fig. 4A), displays a similar conformational change at its C terminus. Indeed, T3Rα was susceptible to carboxypeptidase Y degradation in the absence of hormone but became more resistant in the presence of hormone (Fig. 4C).

Both wild-type and P453 mutant receptors undergo hormone-induced conformational changes distinct from the sequestration of the C-terminal helix. In addition to the sequestration of the C-terminal tail described above, hormone binding also causes a more general, global structural condensation of the ligand binding domains of the wild-type T3Rs and RARs (7, 38, 48). This is manifested as an enhanced resistance of the liganded receptor to certain endopeptidases (22, 25, 30). We next tested if the P453 mutations affected these additional, more global conformational changes. As expected, the wild-type T3Rβ was sensitive to elastase and trypsin degradation in the absence of hormone but became more resistant in the presence of T3 (Fig. 5). Significantly, the P453A and P453H mutant T3Rβs were also sensitive to the endopeptidases in the absence of hormone and became more resistant in the presence of T3 (Fig. 5 and data not shown). These results confirm that both the wild-type and P453 mutant receptors bind hormone under the conditions used (13) and suggest that other than the very C-terminal helix domain, these receptor derivatives undergo generally similar, although not identical, conformational changes on binding hormone (see Discussion).

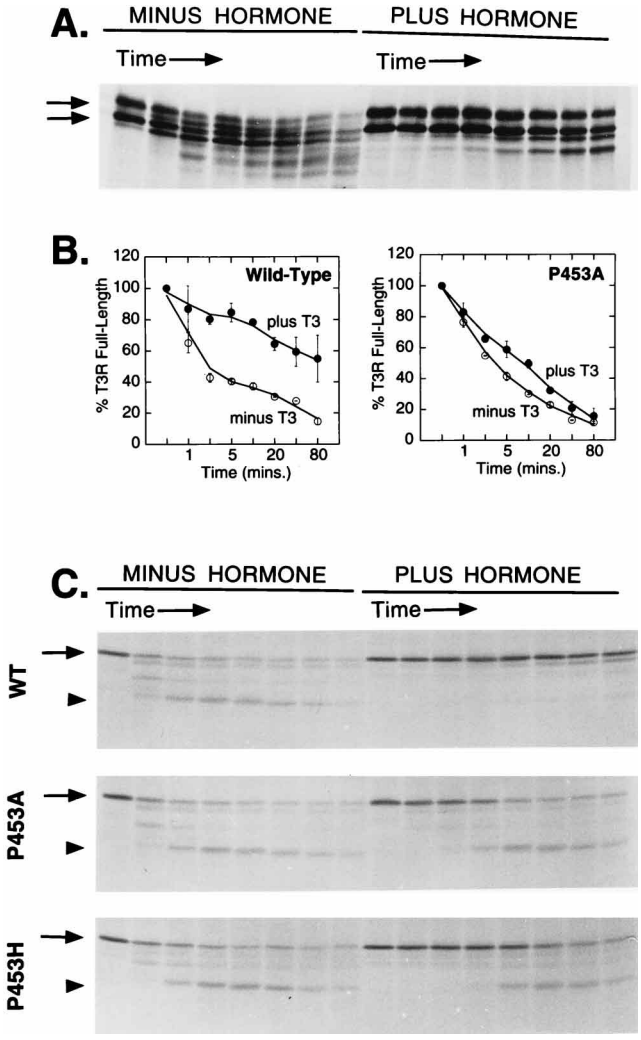

FIG. 3. Hormone confers resistance to carboxypeptidase Y for wild-type T3Rβ but not for the P453A or P453H mutants. (A) Radiolabeled wild-type T3Rβ was synthesized by in vitro transcription and translation and was exposed to carboxypeptidase Y for 0, 1, 2, 5, 10, 20, 40, or 80 min in the absence (left side) or presence (right side) of 1 μM T3 hormone. The resulting proteolytic products were analyzed by SDS-PAGE and visualized by autoradiography. Arrows depict the positions of the in vitro translation (p55 and p48) products prior to protease treatment. (B) The protocol used for panel A was repeated for the wild-type and the P453A mutant receptors, and the amount of radiolabel migrating as full-length receptor (p55) was quantified in the absence or presence of 1 μM T3 hormone. Error bars indicate the range obtained in duplicate experiments. (C) Analysis of the carboxypeptidase Y sensitivity of the C-terminal hormone binding domains of wild-type (WT), P453A, and P453H forms of T3Rβ. The protocol was identical to that described for panel A. Arrows indicate the translation products prior to protease treatment; arrowheads indicate a prominent proteolytic fragment generated by the carboxypeptidase Y degradation.

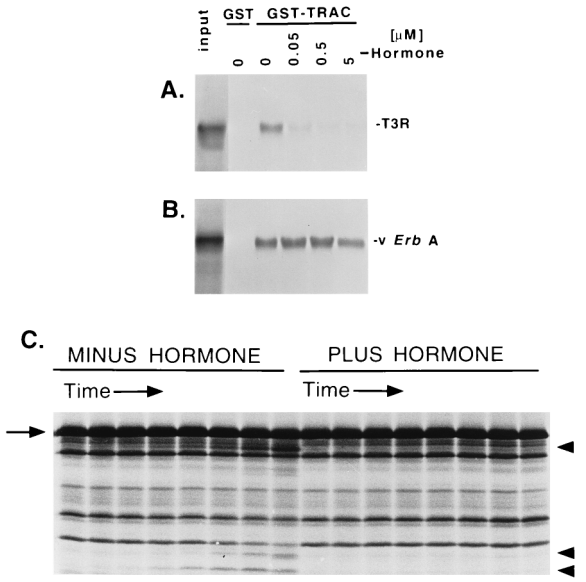

FIG. 4. T3Rα releases from corepressor, and gains resistance to carboxypeptidase Y, upon addition of T3 hormone. (A) Binding of avian T3Rα to GST, or to a GST-SMRT derivative, was determined by the protocol described for Fig. 2A. (B) Binding of the v-*erbA* oncogene product to GST, or to a GST-SMRT derivative, was determined by the protocol described for Fig. 2A. (C) Carboxypeptidase Y sensitivity of avian T3Rα in the absence or presence of 1 μM T3 hormone was determined by the protocol described for Fig. 3. Arrows indicate the full-length receptor prior to protease treatment; arrowheads indicate prominent proteolytic fragments generated by the carboxypeptidase Y degradation.

The C-terminal tail of T3R is not necessary for binding of SMRT corepressor in the absence of hormone but is essential for corepressor release in the presence of hormone. The v-*erbA* oncogene encodes a mutant form of T3Rα that lacks the C-terminal tail and behaves as a constitutive repressor in transfection experiments (14, 42). Paralleling these transcriptional properties, v-ErbA bound to SMRT in vitro in both the absence and the presence of hormone (Fig. 4A and B and references 11 and 41). These results suggested that the C-terminal receptor tail is not necessary for binding of the corepressor but might instead be required for corepressor release, an observation consistent with the repression properties of these receptors exhibited in transfection experiments (2, 3, 8, 43). However, the v-ErbA protein has multiple mutations relative to T3Rα, as well as a severely reduced affinity for hormone (14, 42). We therefore created a T3Rα derivative bearing only a single mutation, represented by a nonsense codon at position 401 (equivalent to 457 in T3Rβ). This ΔC-T3Rα mutant encodes only the first three codons of the C-terminal tail, and it exhibited a behavior similar to that of v-ErbA: the ΔC mutant bound to corepressor normally in the absence of hormone but failed to release corepressor properly upon hormone addition (Fig. 6). Endopeptidase experiments confirmed that the ΔC-T3Rα mutant nonetheless bound hormone under these conditions (data not shown). We conclude that the C-terminal α-helical tail in the wild-type T3R is not necessary for binding of corepressor in the absence of hormone but is required for correct corepressor release on addition of hormone.

We next tested the effects of mutating specific amino acids within the C-terminal α helix itself. Substitution of F403 (F459 in the T3Rβ numbering system) with proline (M4) or mutation of F403 together with additional flanking amino acids (M2 and M3) resulted in mutant receptors that bound corepressor normally but were impaired for corepressor release upon hormone addition (Fig. 7A and B). In contrast, a receptor bearing an E401K mutation (M1), although also in the C-terminal α helix, displayed normal corepressor release in response to hormone (Fig. 7). All four C-terminal mutant receptors bind hormone at nearly equal affinities (reference 40 and data not shown). We conclude that the integrity of the C-terminal α-helical domain is important for coupling hormone binding to corepressor release. Notably, these same C-terminal sequences have been previously implicated in transcriptional activation by nuclear hormone receptors (see Discussion).

RXRs lack the C-terminal hinge motif and fail to release from corepressor on binding hormone. Wild-type RXRs associate with SMRT corepressor in both the absence and the presence of the RXR ligand, 9-*cis* retinoic acid (Fig. 8A and references 1, 41, 45 and 46). The region of the T3R C terminus that we propose to be a hormone-operated hinge exhibits strong sequence conservation in all known isoforms of T3R and RAR; in contrast, the corresponding portion of the RXR C terminus lacks this conserved proline-rich motif (Fig. 8B). As a consequence, the RXR structure in this region has been proposed to form an extended C-terminal α helix that is longer and more stable than that seen for RAR and T3R (7, 38, 48). Does the more rigid C-terminal helix structure of RXRs prevent the necessary conformational change needed to displace corepressor? Consistent with this hypothesis, RXRα was equally susceptible to carboxypeptidase Y degradation in both the absence and the presence of its cognate hormone, 9-*cis* retinoic acid (Fig. 8C), contrasting sharply with the hormone-induced protection observed for T3Rs (see above). We suggest that this hormone-refractory association of RXRs with SMRT may contribute to the unique role RXRs play as heterodimer partners for a wide range of other nuclear receptors (see Discussion).

DISCUSSION

An α-helical domain at the receptor C terminus appears to undergo a conformational change on binding hormone. Evidence for this conformational switch was presaged by crystallographic analysis suggesting that this C-terminal α-helical domain is in an extended position in the unliganded RXR but is sequestered against the body of the receptor in the liganded T3R and RAR (7, 38, 48). Although obtained from structural studies of nonidentical receptors, these results have been interpreted as evidence for a reorientation of the C-terminal helix on hormone binding. Our current studies, using carboxypeptidase Y, provide new evidence that a C-terminal conformational change actually occurs in solution in the wild-type T3Rs and (unpublished data) RARs. For both T3Rs and RARs, the receptor C terminus was readily accessible to carboxypeptidase Y degradation in the absence of hormone but became significantly more resistant to exopeptidase in the presence of hormone. Several alternative interpretations need to be considered. First, the bound hormone itself might physically occlude the receptor C terminus from the exopeptidase; however, we note that many of our T3R mutants bind hormone without becoming protected from exopeptidase. Second, a portion of the receptor outside of the C-terminal domain may alter conformation on hormone binding so as to occlude the C-terminal helix from the exopeptidase; however, mutations in the C-terminal domain itself lead to loss of hormone-mediated exopeptidase protection, making this interpretation less likely. We favor a third explanation, consistent with the crystallographic data, that the C terminus itself moves from a relatively exposed to a relatively sequestered position in the presence of hormone.

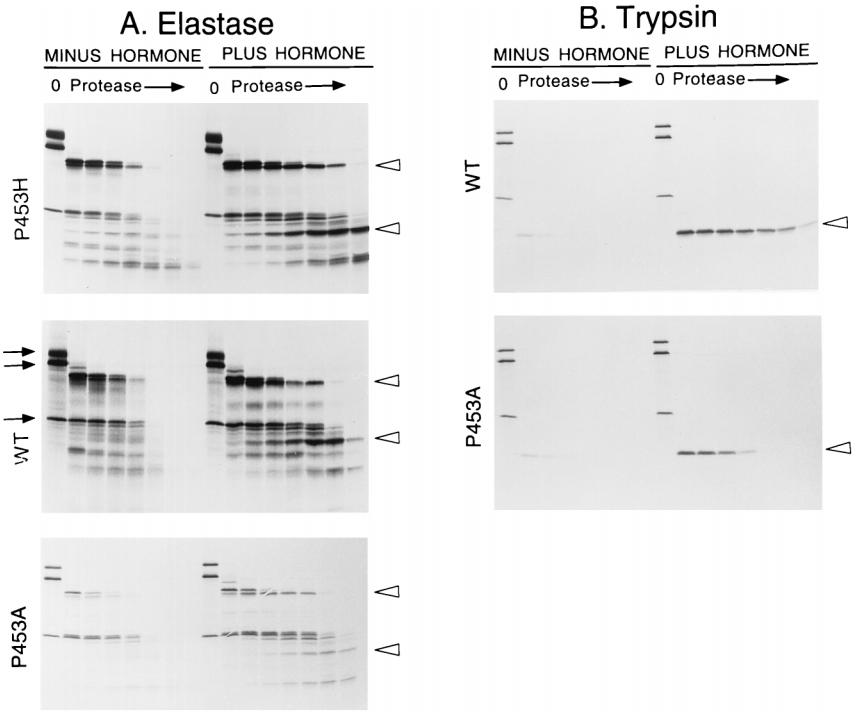

FIG. 5. Wild-type and P453A and P453H mutant receptors exhibit enhanced endopeptidase resistance in the presence of hormone. (A) Radiolabeled wild-type (WT) or mutant receptors were synthesized by in vitro translation and incubated with increasing amounts of elastase (0, 0.125, 0.25, 0.5, 1, 2, 4, or 8 μg per lane) in the absence or presence of hormone, as indicated. The resulting proteolytic products were resolved by SDS-PAGE and visualized by autoradiography. Translation products in the absence of proteolytic degradation are indicated by arrows; receptor fragments that exhibit enhanced resistance to protease in the presence of hormone are indicated by open triangles. (B) Experiments identical to those in panel A but using trypsin instead of elastase.

This conformational change in the receptor C terminus may serve as a switch regulating the association and dissociation of corepressors. T3Rs and RARs physically associate with corepressors in the absence of hormone but dissociate on hormone binding, a process paralleled by the conversion of these receptors from transcriptional repressors to transcriptional activators (reviewed in reference 20). What mechanism couples hormone binding to corepressor release? Our evidence suggests that the conformational change in the C-terminal α-helical domain plays a critical role in this hormone-mediated corepressor release. Our first line of evidence originates with the P453A and P453H RTH-T3Rβ mutants, which bind hormone but fail to release from corepressor. Both mutants represent substitutions of a critical proline at the base of the C-terminal α-helical domain that may impair the hinge function by which the C terminus reorients on hormone binding. Indeed, the P453A and P453H mutant receptors were readily degraded by carboxypeptidase Y in both the absence and the presence of hormone, suggesting that the C terminus of each of these mutants fails to sequester properly. Significantly, the P453 mutants nonetheless exhibited many of the more global hormone-mediated conformational changes observed for the wild-type receptor, manifested as an enhanced resistance to the endopeptidases trypsin and elastase. Thus, it appears that one specific effect of the P453 substitutions is to prevent the correct, hormone-induced reorientation of the C-terminal α helix.

We note, however, that although very similar, the endopeptidase patterns of wild-type and P453 mutant receptors were not identical. Therefore, we cannot fully exclude the possibility that, in addition to changes to the C-terminal helix, the P453 mutations may have secondary effects that extend to other portions of the hormone binding domain. The P453 mutants may exert these effects directly or by preventing subordinate conformational changes that occur in response to the reorientation of the C-terminal helix. However, our additional genetic analyses also strongly implicate the receptor C-terminal helix as playing a key role in hormone-mediated corepressor release (see below).

Deletion of the C-terminal α-helical domain yielded T3R derivatives that were constitutively associated with corepressor, indicating that an intact helix 12 is important for dissociation, but not association, of corepressor. Similarly, substitution mutations in helix 12 itself, especially in those amino acids that in the liganded receptor are oriented toward the body of the protein, can prevent corepressor release on hormone binding, perhaps by interfering with the proper sequestration of

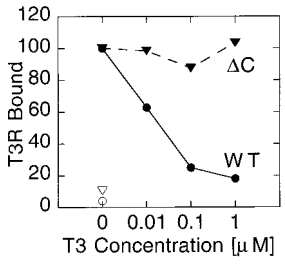

FIG. 6. A C-terminal deletion of T3Rα impairs release of corepressor in response to hormone. The ability of wild-type (WT) avian T3Rα, or a T3Rα mutant bearing a deletion of the C-terminal eight amino acids (ΔC), to bind to GST (open symbols) or to a GST-SMRT derivative (closed symbols) was quantified by the protocol described for Fig. 2B. Receptor binding in the absence of hormone is defined as 100%

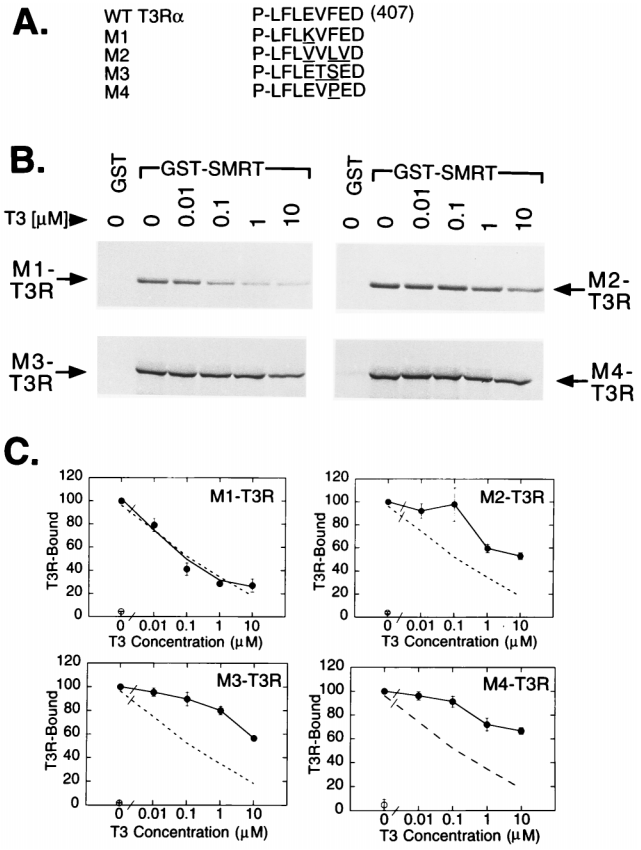

FIG. 7. Point mutations within the T3Rα C-terminal helix 12 can impair release of corepressor in response to hormone. (A) The wild-type (WT) and mutant T3Rα C-terminal sequences are depicted, with the amino acid substitutions underlined; codon positions are indicated in the T3Rα numbering system (42). (B) The effect of T3 hormone on binding of the mutant T3Rα proteins to corepressor was determined by the same form of assay as described for Fig. 2A; the resulting autoradiograms are presented. (C) Binding of radiolabeled mutant receptors to GST (open symbols) or to a GST-SMRT derivative (closed symbols) was determined as for panel B and quantified; error bars indicate the range obtained in duplicate experiments. Dashed lines indicate the results for wild-type T3Rα. Binding of receptor to GST-SMRT in the absence of hormone is defined as 100%.

this helix against the receptor. In contrast, a mutation in helix 12 oriented toward the solvent (E401K) had no detectable effect on corepressor release. Helix 12 includes the AF-2 domain, an amphipathic helix present in a variety of nuclear hormone receptors and previously implicated as important for binding coactivators and for transcriptional activation (20). Our own data, however, demonstrate that this same helix 12 domain is also important for the release of corepressor in response to hormone. Therefore, the effects of mutations in the AF-2 domain need to be carefully reevaluated, given that these lesions can potentially influence both the repression and activation properties of the receptor.

RXRs have a divergent C-terminal sequence and fail to release corepressor on hormone binding. RXRs associate with corepressors both in the absence and in the presence of the cognate hormone 9-*cis* retinoic acid (41, 45, 46). It is therefore provocative that the C-terminal hinge domain sequence found in all T3Rs and RARs is not shared by the RXRs. This lack of sequence conservation at the presumptive hinge region contrasts with the otherwise good sequence conservation observed for RARs, T3Rs, and RXRs within helix 12 itself. Therefore, the C terminus of RXR may not be able to reorient on hormone binding in the same fashion as that of T3R and RAR, and indeed, we could detect no evidence that 9-*cis* retinoic acid mediated a change in the accessibility of the RXR C terminus to carboxypeptidase Y. We suggest that the hormone-refractory corepressor association observed for RXRs may, at least in part, arise from the lack of a suitable hinge-like sequence in the RXR C terminus.

Why might RXRs lack this hinge function? Although RXRs can form heterodimers with a wide variety of other nuclear hormone receptors, such as T3Rs and RARs, the RXR moiety is often a silent partner in these relationships. That is, the heterodimer can activate gene expression in response to T3R- or RAR-specific ligands but not in response to RXR-specific ligands (27). In some cases, the RXR moiety in the heterodimer exhibits an impaired affinity for ligand; however, this alone cannot explain many of the features of the silent-partner phenomenon (16, 17, 27, 30, 35, 44, 49). We suggest that the hormone-refractory nature of the RXR-corepressor association contributes to this silent-partner phenotype by preventing activation of the heterodimer in response to RXR ligands. This proposal complements a recent observation that corepressors bind efficiently to receptor dimers but not to receptor monomers (53). Corepressor binding to an RXR-T3R heterodimer is therefore likely to require the dual interaction of corepressor

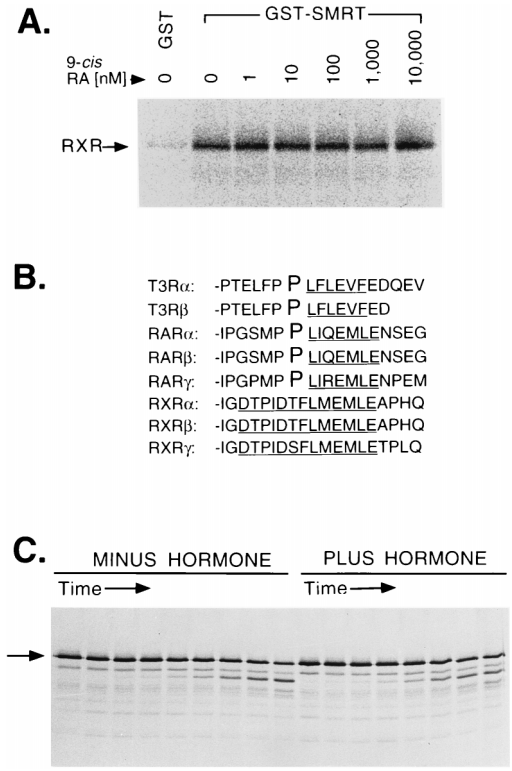

FIG. 8. RXR exhibits a hormone-resistant binding to corepressor. (A) The ability of RXRα to bind to GST, or to a GST-SMRT derivative, was determined over a range of 9-*cis* retinoic acid (RA) concentrations by the general protocol described for T3Rβ in the legend to Fig. 2A. (B) Comparison of the amino acid sequence of the C-terminal domains of different T3R, RAR, and RXR isoforms (38, 48). The T3Rα sequence is from rat; the remaining sequences are from human. Amino acids predicted to form the C-terminal α helix are underscored for each receptor (38, 48). (C) Carboxypeptidase Y sensitivity of RXRα was determined in the absence or presence of 1 μM 9-*cis* retinoic acid by the protocol described for T3Rβ in the legend to Fig. 3A. Arrow indicates the full-length in vitro translation product.

with both the T3R and RXR moieties. Addition of T3 hormone alone, by destabilizing the T3R-SMRT component of this dual interaction, would be sufficient to release SMRT from the heterodimer. In contrast, addition of 9-*cis* retinoic acid would neither destabilize the RXR-SMRT interaction nor release SMRT from the heterodimer, and the repressed state would be maintained. Intriguingly, the interaction between SMRT and RXRs is demonstrably weaker than that between SMRT and T3Rs or RARs (41, 51), suggesting that RXR homodimers may be less able to efficiently tether corepressors than are heterodimers. This prediction is consistent with the known transcriptional properties of RXR homodimers, which, unlike RXR heterodimers, are only weak repressors in the absence of hormone and do activate transcription in response to 9-*cis* retinoic acid.

The model presented above does not exclude the involvement of additional levels of regulation, such as conformational cross talk, in controlling the actions of the heterodimer partners (44, 49). In addition, given the close induced fit between ligand and receptor, structurally distinct hormone derivatives have the potential for manifesting distinct allosteric effects on receptor function (7, 22, 25, 30, 38, 48). For example, not all ligands that bind RARs appear to induce release of SMRT corepressor in vitro (18a). Conversely, there may be RXR ligands that, unlike 9-*cis* retinoic acid, are able to invoke the conformational changes necessary for corepressor release from RXRs. Further experiments will be necessary to understand the precise roles of these allosteric effects in regulating the association of nuclear hormone receptors both with corepressors and with coactivators.

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REFERENCES

1. **Allenby, G., M.-T. Bocquel, M. Saunders, S. Kazmer, J. Speck, M. Rosenberger, A. Lovey, P. Kastner, J. F. Grippo, P. Chambon, and A. A. Levin.** 1987. Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoic acids. Proc. Natl. Acad. Sci. USA **90:**30–34.
2. **Baniahmad, A., A. C. Kohne, and R. Renkawitz.** 1992. A transferable silencing domain is present in the thyroid hormone receptor, in the v-Erb A oncogene product, and in the retinoic acid receptor. EMBO J. **11:**1015–1023.
3. **Baniahmad, A., X. Leng, T. P. Burris, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 1995. The τ4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing. Mol. Cell. Biol. **15:**76–86.
4. **Beato, M., P. Herrlich, and G. Schutz.** 1995. Steroid hormone receptors: many actors in search of a plot. Cell **83:**851–858.
5. **Beck-Peccoz, P., and V. K. K. Chatterjee.** 1994. The variable clinical phenotype in thyroid hormone resistance syndrome. Thyroid **4:**225–232.
6. **Bigler, J., and R. N. Eisenman.** 1988. c-*erbA* encodes multiple proteins in chicken erythroid cells. Mol. Cell. Biol. **8:**4155–4161.
7. **Bourguet, W., M. Ruff, P. Chambon, H. Gronemeyer, and D. Moras.** 1995. Crystal structure of the ligand-binding domain of the human nuclear hormone receptor RXR alpha. Nature **375:**377–382.
8. **Casanova, J., E. Helmer, S. Selmi-Ruby, J. S. Qi, M. Au-Flieger, V. Desai-Yajnik, N. Koudinova, F. Yarm, B. M. Raaka, and H. H. Samuels.** 1994. Functional evidence for ligand-dependent dissociation of thyroid hormone and retinoid acid receptors from an inhibitory cellular factor. Mol. Cell. Biol. **14:**5756–5765.
9. **Chambon, P.** 1994. The retinoid signaling pathway. Semin. Cell Biol. **5:**115–125.
10. **Chen, H.-W., and M. L. Privalsky.** 1993. The *erbA* oncogene represses the actions of both retinoid X and retinoid A receptors, but does so by distinct mechanisms. Mol. Cell. Biol. **13:**5970–5980.
11. **Chen, J. D., and R. M. Evans.** 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature **377:**454–457.
12. **Chen, J. D., K. Umesono, and R. M. Evans.** 1996. SMRT isoforms mediate repression and anti-repression of nuclear receptor heterodimers. Proc. Natl. Acad. Sci. USA **93:**7567–7571.
13. **Collingwood, T. N., M. Adams, Y. Tone, and V. K. K. Chatterjee.** 1994. Spectrum of transcriptional, dimerization, and dominant negative properties of twenty different mutant thyroid hormone β receptors in thyroid hormone resistance syndrome. Mol. Endocrinol. **8:**1262–1277.
14. **Damm, K., C. C. Thompson, and R. M. Evans.** 1989. Protein encoded by v-Erb A functions as a thyroid hormone receptor antagonist. Nature **339:**593–597.
15. **de The, H., C. Lavau, A. Marchio, C. Chomienne, L. Degos, and A. Dejean.** 1991. The PML-RARα fusion mRNA encodes a functionally altered RAR. Cell **66:**675–684.
16. **Forman, B. M., K. Umesono, J. Chen, and R. M. Evans.** 1995. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell **81:**541–550.
17. **Hall, B. L., Z. Smit-McBride, and M. L. Privalsky.** 1993. Reconstitution of retinoid X receptor function and combinatorial regulation of other nuclear hormone receptors in the yeast, *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **90:**6929–6933.
18. **Hayashi, R.** 1977. Carboxypeptidase Y in sequence determination of peptides. Methods Enzymol. **47:**84–93.

18a. **Hong, S.-H., and M. L. Privalsky.** Unpublished data.

19. **Horlein, A. J., A. M. Naar, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamel, M. Soderstrom, C. K. Glass, and M. G. Rosenfeld.** 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature **377:**397–404.
20. **Horwitz, K. B., T. A. Jackson, D. L. Bain, J. K. Richer, G. S. Takimoto, and L. Tung.** 1996. Nuclear hormone receptor coactivators and corepressors. Mol. Endocrinol. **10:**1167–1177.
21. **Hughes, M. R., P. J. Malloy, D. G. Kieback, R. A. Kesterson, J. W. Pike, D. Feldman, and B. W. O'Malley.** 1993. Point mutations in the vitamin D receptor associated with hypocalcemic rickets. Science **242:**1702–1705.
22. **Ikeda, M., E. C. Wilcox, and W. W. Chin.** 1996. Different DNA elements can modulate the conformation of thyroid hormone receptor heterodimer and its transcriptional activity. J. Biol. Chem. **271:**23096–23104.
23. **Kakizuka, A., W. H. Miller, K. Umesono, R. P. Warrell, S. R. Frankel, V. V. S. Murty, E. Dmitrovsky, and R. M. Evans.** 1991. Chromosomal translocation t(15:17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor. Cell **66:**663–674.
24. **Kastner, P., M. Mark, and P. Chambon.** 1995. Nonsteroidal nuclear receptors: what are genetic studies telling us about their role in real life? Cell **83:**859–870.
25. **Keidel, S., P. LeMotte, and C. Apfel.** 1994. Different agonist- and antagonist-induced conformational changes in retinoic acid receptors analyzed by protease mapping. Mol. Cell. Biol. **14:**287–298.
26. **Kopp, P., K. Kitajima, and J. L. Jameson.** 1996. Syndrome of resistance to thyroid hormone: insights into thyroid hormone action. Proc. Soc. Exp. Biol. Med. **211:**49–61.
27. **Kurokawa, R., J. DiRenzo, M. Boehm, J. Sugarman, B. Gloss, M. G. Rosenfeld, R. A. Heyman, and C. K. Glass.** 1994. Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. Nature **371:**528–531.
28. **Kurokawa, R., M. Soderstrom, A. Horlein, S. Halachmi, M. Brown, M. G. Rosenfeld, and C. K. Glass.** 1995. Polarity-specific activities of retinoic acid receptors determined by a co-repressor. Nature **377:**451–454.
29. **Lazar, M. A.** 1993. Thyroid hormone receptors: multiple forms, multiple possibilities. Endocrinol. Rev. **14:**184–193.
30. **Leng, X., S. Y. Tsai, B. W. O'Malley, and M. J. Tsai.** 1993. Ligand-dependent conformational changes in thyroid hormone and retinoic acid receptors are potentially enhanced by heterodimerization with retinoic X receptor. J. Steroid Biochem. Mol. Biol. **46:**643–661.
31. **Mangelsdorf, D. J., and R. M. Evans.** 1995. The RXR heterodimers and orphan receptors. Cell **83:**841–850.
32. **Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans.** 1995. Overview: the nuclear receptor superfamily: the second decade. Cell **83:**835–840.
33. **McPhaul, M. J., M. Marcelli, S. Zoppi, J. E. Griffin, and J. D. Wilson.** 1993. Genetic basis of endocrine disease 4: the spectrum of mutations in the androgen receptor gene that cause androgen resistance. J. Clin. Endocrinol. Metab. **76:**17–23.
34. **Miner, J. N., M. I. Diamond, and K. R. Yamamoto.** 1991. Transcriptional factor interactions: selectors of positive or negative regulation from a single DNA element. Cell Growth Differ. **2:**525–530.
35. **Minucci, S., M. Leid, R. Toyama, J.-P. Saint-Jeannet, V. J. Peterson, V. Horn, J. E. Ishmael, N. Bhattacharyya, A. Dey, I. B. Dawid, and K. Ozato.** 1997. Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. Mol. Cell. Biol. **17:**644–655.

36. **Pazin, M. J., and J. T. Kadonaga.** 1997. What's up and down with histone deacetylation and transcription? Cell **89:**325–328.
37. **Refetoff, S., R. E. Weiss, and S. Usala.** 1993. The syndromes of resistance to thyroid hormone. Endocrine Rev. **14:**348–399.
38. **Renaud, J.-P., N. Rochel, M. Ruff, V. Vivat, P. Chambon, H. Gronemeyer, and D. Moras.** 1995. Crystal structure of the RAR-γ ligand-binding domain bound to all-*trans* retinoic acid. Nature **378:**681–689.
39. **Ribeiro, R. C., J. W. Apriletti, B. L. West, R. L. Wagner, R. J. Fletterick, F. Schaufele, and J. D. Baxter.** 1993. The molecular biology of thyroid hormone action. Ann. N. Y. Acad. Sci. **758:**366–389.
40. **Saatcioglu, F., P. Bartunek, T. Deng, M. Zenke, and M. Karin.** 1993. A conserved C-terminal sequence that is deleted in v-ErbA is essential for the biological activities of c-ErbA (the thyroid hormone receptor). Mol. Cell. Biol. **13:**3675–3685.
41. **Sande, S., and M. L. Privalsky.** 1996. Identification of TRACs (T3 receptor-associating cofactors), a family of cofactors that associate with, and modulate the activity of, nuclear hormone receptors. Mol. Endocrinol. **10:**813–825.
42. **Sap, J., A. Munoz, H. Schmitt, H. Stunnenberg, and B. Vennstrom.** 1989. Repression of transcription mediated by a thyroid hormone response element by the v-Erb A oncogene product. Nature **340:**242–244.
43. **Schulman, I. G., H. Juguilon, and R. M. Evans.** 1996. Activation and repression by nuclear hormone receptors: hormone modulates an equilibrium between active and repressive states. Mol. Cell. Biol. **16:**3807–3813.
44. **Schulman, I. G., C. Li, J. W. R. Schwabe, and R. M. Evans.** 1997. The phantom ligand effect: allosteric control of transcription by the retinoid X receptor. Genes Dev. **11:**299–308.
45. **Seol, W., H. S. Choi, and D. D. Moore.** 1995. Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. Mol. Endocrinol. **9:**72–85.
46. **Seol, W., M. J. Mahon, Y. K. Lee, and D. D. Moore.** 1996. Two receptor interacting domains in the nuclear hormone receptor corepressor RIP13/N-CoR. Mol. Endocrinol. **10:**1646–1655.
47. **Tsai, M. J., and B. W. O'Malley.** 1994. Molecular mechanisms of action of steroid/thyroid hormone receptor superfamily members. Annu. Rev. Biochem. **63:**451–483.
48. **Wagner, R. L., J. W. Apriletti, M. E. McGrath, B. L. West, J. D. Baxter, and R. J. Fletterick.** 1995. A structural role for hormone in the thyroid hormone receptor. Nature **378:**690–697.
49. **Willy, P. J., and D. J. Mangelsdorf.** 1997. Unique requirements for retinoid dependent transcriptional activation by the orphan receptor LXR. Genes Dev. **11:**289–298.
50. **Wolffe, A. P.** 1997. Transcriptional control—sinful repression. Nature **387:** 16–17.
51. **Yoh, S. M., V. K. K. Chatterjee, and M. L. Privalsky.** 1997. Thyroid hormone resistance syndrome manifests as an aberrant interaction between mutant T3 receptors and transcriptional corepressors. Mol. Endocrinol. **11:**470–480.
52. **Zamir, I., H. P. Harding, G. B. Atkins, A. Horlein, C. K. Glass, M. Rosenfeld, and M. A. Lazar.** 1996. A nuclear hormone receptor corepressor mediates transcriptional silencing by receptors with distinct repression domains. Mol. Cell. Biol. **16:**5458–5465.
53. **Zamir, I., J. Zhang, and M. A. Lazar.** 1997. Stoichiometric and steric principles governing repression by nuclear hormone receptors. Genes Dev. **11:** 835–846.